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Versatile and automated workflow for the analysis of oligodendroglial calcium signals in preclinical mouse models of myelin repair

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1 **Versatile and automated workflow for the analysis of oligodendroglial calcium signals in preclinical**
2 **mouse models of myelin repair**

3
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22 **Abstract**

23 Intracellular Ca^{2+} signals of oligodendroglia, the myelin-forming cells of the central nervous system,
24 regulate vital cellular processes including myelination. However, studies on oligodendroglia Ca^{2+} signal
25 dynamics are still scarce, especially during myelin repair, and there are no software solutions to properly
26 analyze the unique Ca^{2+} signal characteristics in these cells. Here, we provide a comprehensive
27 experimental and analytical workflow to acquire and analyze Ca^{2+} imaging data of oligodendroglia at the
28 population and single-cell levels in preclinical mouse models of myelin repair. We report diverse *ex vivo*
29 and *in vivo* experimental protocols to obtain reproducible Ca^{2+} imaging data from oligodendroglia in
30 demyelinated lesions. Importantly, we provide an analytical pipeline containing two free, open source and
31 cross-platform software programs, Occam and post-prOccam, that enable the fully automated analysis of
32 one- and two-photon Ca^{2+} imaging datasets from oligodendroglia obtained by either *ex vivo* or *in vivo* Ca^{2+}
33 imaging techniques. This versatile and accessible experimental and analytical framework, which revealed
34 significant but uncorrelated spontaneous Ca^{2+} activity in oligodendroglia inside demyelinated lesions,
35 should facilitate the elucidation of Ca^{2+} -mediated mechanisms underlying remyelination and therefore
36 help to accelerate the development of therapeutic strategies for the many myelin-related disorders, such
37 as multiple sclerosis.

38 Introduction

39 In demyelinating diseases such as multiple sclerosis (MS), myelin sheaths produced by
40 oligodendrocytes (OL) are destroyed by the immune system, resulting in OL death and axonal
41 degeneration and, ultimately, in both physical and neurological disabilities (Duncan & Radcliff, 2016).
42 Upon demyelination, new oligodendrocyte precursor cells (OPCs) and OLs can be generated and partial
43 myelin repair can take place (Franklin & Ffrench-Constant, 2017). However, since remyelination is often
44 incomplete, research tools to investigate oligodendroglial dynamics and roles during the processes of
45 demyelination and remyelination are of great importance for the development of therapeutic strategies.

46 It is now established that oligodendroglial Ca^{2+} signals translate environmental information into
47 cellular processes such as proliferation, differentiation and myelination but little is known about the
48 origin, dynamics or function of Ca^{2+} signals during myelin repair (Paez & Lyons, 2020; Pitman & Young,
49 2016; Maas et al., 2021). Recent *in vivo* studies in the zebrafish revealed that both OPCs and OLs are
50 capable of Ca^{2+} signaling (Baraban et al., 2018; Krasnow et al., 2018; Marisca et al., 2020; Li et al., 2022).
51 In mouse brain slices, spontaneous Ca^{2+} activity in OPCs (Balía et al., 2017) and OLs (Battefeld et al., 2019)
52 was shown to be high during postnatal development when the myelination process is still ongoing, and to
53 decrease in OLs as the brain matures (Battefeld et al., 2019). Then, Ca^{2+} signals of OLs are reactivated upon
54 demyelination in the adult mouse brain, suggesting a crucial role for oligodendroglial Ca^{2+} signals in
55 demyelinated lesions (Battefeld et al., 2019). To date, however, only one report has explored OL Ca^{2+}
56 signaling during demyelination in *ex vivo* mouse brain slices (Battefeld et al., 2019) and oligodendroglial
57 Ca^{2+} imaging studies in demyelinated mouse models *in vivo* are completely lacking. Moreover, in the
58 above-mentioned studies, not only were the regions of interest (ROIs) manually chosen, potentially
59 introducing bias in the ROI selection outcome, but also complex Ca^{2+} events with multiple peaks were
60 overlooked even though these events are a hallmark of oligodendroglia (see below).

61 Several pipelines for the analysis of Ca²⁺ imaging data from neurons and astrocytes in brain slices
62 and *in vivo* have been published. Unfortunately, problems arise when using neuron-oriented programs
63 such as CalmAn and EZCalcium for the analysis of oligodendroglial Ca²⁺ imaging data (Cantu et al., 2020;
64 Giovannucci et al., 2019). For instance, classifiers are used to recognize neurons as round somata of a
65 certain size (Giovannucci et al., 2019), while oligodendroglia possess less well-defined shapes and sizes
66 (Xu et al., 2021). Another important drawback of the available pipelines for neuronal Ca²⁺ imaging is the
67 difference in neuronal and oligodendroglial Ca²⁺ signal dynamics. Indeed, neuronal Ca²⁺ signals last around
68 100 ms, are characterized by fast kinetics and occur with a high frequency (Chua & Morrison, 2016). This
69 is in stark contrast with oligodendroglia that exhibit complex Ca²⁺ dynamics with signals that last anywhere
70 from several seconds to multiple minutes and that are characterized by slow rise and decay times (our
71 data and Battefeld et al., 2019). Another set of problems may arise when using available Ca²⁺ imaging
72 analysis for astrocytes in oligodendroglia: astrocytes are known to exhibit extensive signal propagation
73 within and between cells and Ca²⁺ signaling pipelines such as AQUA are therefore designed to trace Ca²⁺
74 events in time and space instead of identifying ROIs (Wang et al., 2019). Because we cannot assume that
75 intra- and inter-cellular signal propagation occurs extensively in oligodendroglia, these pipelines are not
76 immediately suitable for the analysis of oligodendroglial Ca²⁺ signaling. Older ROI-based astrocyte Ca²⁺
77 imaging analysis packages such as GECIquant and CaSCaDe rely on manual input which may introduce
78 bias, making them not fully desirable (Agarwal et al., 2017; Venugopal et al., 2019).

79 Here, we provide a comprehensive experimental and analytical workflow to investigate
80 oligodendroglial Ca²⁺ imaging data obtained either *ex vivo* or *in vivo* in two different preclinical mouse
81 models of myelin repair, the lysolecithin (LPC) and cuprizone (CPZ) models. We established reproducible
82 experimental protocols aiming to obtain Ca²⁺ imaging data of oligodendroglia both in brain slices and in
83 freely moving mice expressing genetically encoded fluorescent Ca²⁺ indicators. Next, we developed two
84 free, open source and cross-platform software programs, Occam and post-prOccam (Occam:

85 Oligodendroglial cells calcium activity monitoring), for the fully automated analysis of one- and two-
86 photon Ca^{2+} imaging data from oligodendroglia in demyelinated lesions (GNU GPLv3+ license; code
87 repository at: <https://gitlab.com/d5674/occam>). These highly versatile and accessible tools are suitable
88 for the analysis of Ca^{2+} imaging datasets obtained in diverse preparations, matching all the specific
89 requirements for the monitoring of the unique Ca^{2+} event characteristics of oligodendroglia. Our analytical
90 pipeline thus accelerates the elucidation of Ca^{2+} -mediated mechanisms underlying myelin repair and will
91 contribute to the development of therapeutic strategies in myelin-related disorders such as MS.

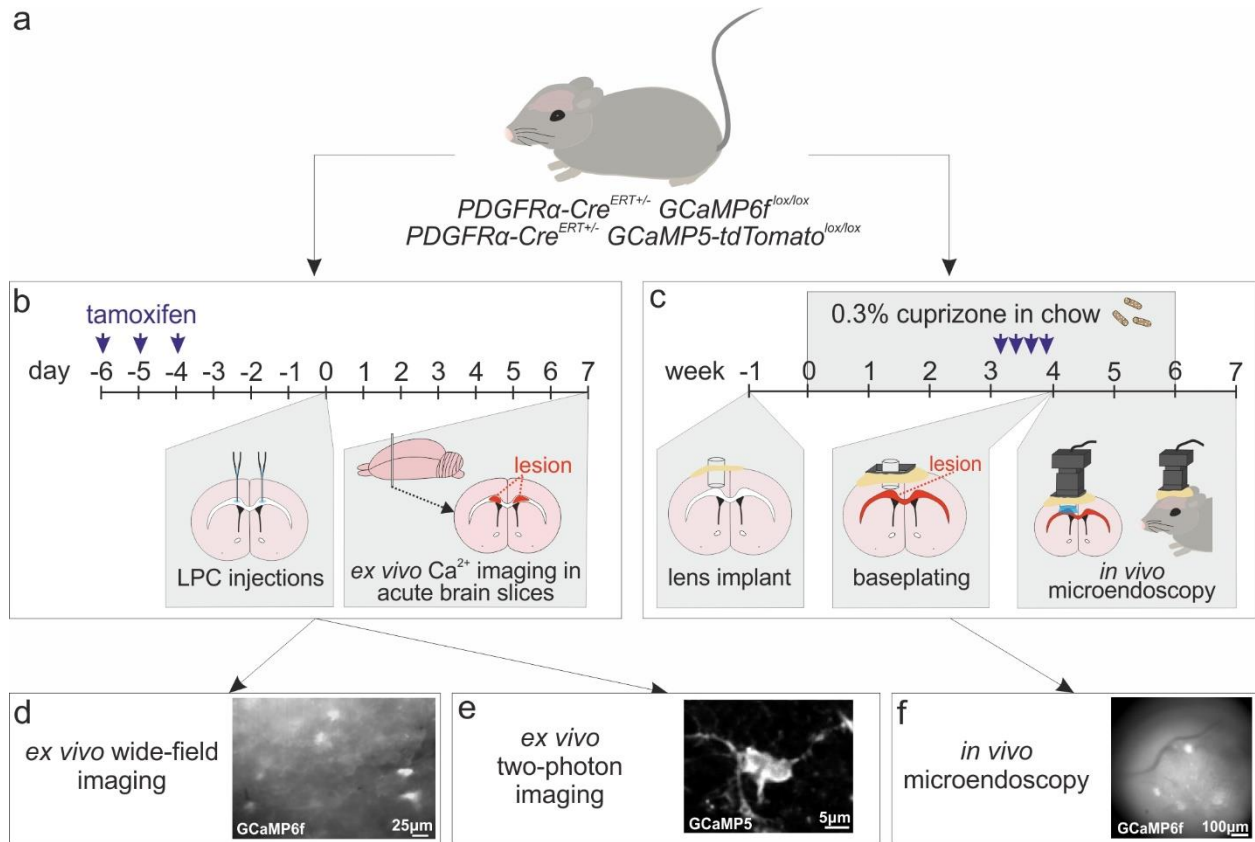
92 Results

93 Experimental paradigms to obtain *ex vivo* and *in vivo* oligodendroglial Ca²⁺ signals in demyelinated 94 lesions

95 The goal of this study was to develop both experimental paradigms and a fully automated
96 analytical workflow for the investigation of one- and two-photon intracellular Ca²⁺ imaging data from
97 oligodendroglia in demyelinated lesions of *ex vivo* and *in vivo* preparations (Fig. 1). We validated our
98 experimental and analytical workflow on data obtained in adult *Pdgfra*^{CreERT(+/-)};*Gcamp6*^{flox/Lox} and
99 *Pdgfra*^{CreERT(+/-)};*Gcamp5-tdTomato*^{Lox/Lox} transgenic mice (Fig. 1a). Ca²⁺ imaging in acute slices was
100 performed in LPC-induced lesions of corpus callosum as these lesions have several advantages: 1) since
101 these are focal lesions, they are localized in specific regions, 2) they can easily be identified by DIC video
102 microscopy at a low magnification as a brighter area than the surrounding non-lesioned dark-appearing
103 white matter (Sahel et al., 2015) and imaging can then be performed at a high magnification inside the
104 core of the lesion by either one- or two-photon Ca²⁺ imaging (Fig. 1b, 1d, 1e). In the LPC model, the
105 response of oligodendroglia to demyelination occurs in a stepwise manner (migration of OPCs to the
106 lesion site, OPC proliferation, OPC differentiation and remyelination), such that Ca²⁺ signals of
107 oligodendroglia at each step of the remyelination process can be analyzed separately (Sahel et al., 2015).
108 Tamoxifen injections to induce Cre expression a few days before the stereotaxic LPC injection are
109 sufficient to elicit a good level of GCaMP expression in oligodendroglia 7 days post-injection (dpi) of LPC
110 (Fig. 1b, 1d, 1e). This protocol led to a lesion-specific expression of GCaMP, as none was observed in intact
111 corpus callosum, probably because adult PDGFR α ⁺ oligodendrocyte progenitors revert to an immature
112 state and shorten their cell cycle upon demyelination (Supplementary Fig. 1; Moyon et al., 2015; Cayre et
113 al., 2021). In this work, we imaged oligodendroglia in *ex vivo* demyelinated brain slices by two different
114 techniques: one-photon microscopy used to study the whole oligodendroglial population's Ca²⁺ activity in

115 a wide field and conventional two-photon microscopy used to study the oligodendroglial Ca^{2+} signals at
 116 the single cell level (Fig. 1b, 1d, 1e).

a



117
 118

119 **Figure 1 – Experimental workflow for oligodendroglial Ca^{2+} imaging of *ex vivo* and *in vivo* preparations.**
 120 **(a)** Demyelinating lesions were performed in adult *Pdgfra*^{CreERT(+/-);Gcamp6^{flox/Lox}} and *Pdgfra*^{CreERT(+/-);Gcamp5-tdTomato^{lox/Lox}}
 121 *);Gcamp5-tdTomato^{lox/Lox} transgenic mice. **(b)** Four days after tamoxifen injection, demyelination was
 122 induced by LPC injection in the corpus callosum and *ex vivo* brain slices were performed 7 days after LPC
 123 injection. **(c)** Demyelination was induced by CPZ ingestion starting 1 week after GRIN lens implantation
 124 and tamoxifen injections were performed during the fourth week of CPZ ingestion. *In vivo*
 125 microendoscopy was performed from the fifth week of CPZ feeding and during the first week after CPZ
 126 withdrawal. **(d-f)** Ca^{2+} imaging in lesions was performed using wide-field microscopy (d) and two-photon
 127 microscopy (e) in *ex vivo* brain slices and wide-field microendoscopy (f) in freely moving mice.*

128

129 After successfully imaging the Ca^{2+} dynamics in demyelinated lesions in *ex vivo* brain slices, we
 130 extended our approach by including *in vivo* Ca^{2+} imaging in demyelinated lesions. The main difficulty
 131 encountered when imaging the mouse corpus callosum *in vivo* is related to the depth of this region,
 132 particularly at the level of the motor cortex where demyelinated lesions are often analyzed (Ortiz et al.,

133 2019). Although some lesions occur in the grey matter in MS, most of them actually occur in the white
134 matter; therefore, performing oligodendroglial Ca^{2+} imaging in corpus callosum lesions is particularly
135 relevant for this disease. For this purpose, we used *in vivo* one-photon microendoscopy which allowed us
136 to visualize Ca^{2+} signals of oligodendroglia in the corpus callosum, at 1.8 mm from the surface of the brain.
137 In order to monitor oligodendroglial Ca^{2+} signaling in callosal demyelinated lesions of freely moving mice
138 (Fig. 1c), we operated the open-source Miniscope V4 device, often used to follow neuronal Ca^{2+} activity
139 (Shuman et al., 2020; Cai et al., 2016). One interesting challenge in our approach was the fact that,
140 although the LPC-induced demyelination model is appropriate for brain slices, demyelination is already
141 obtained at 3 dpi (Sahel et al., 2015) and thus incompatible with *in vivo* microendoscopy which requires a
142 multi-step procedure extending over several weeks (Fig. 1c). We therefore used the CPZ demyelination
143 model in which intense demyelination occurs from the fourth week after CPZ ingestion without any
144 surgical intervention (Fig. 1c; Remaud et al., 2017). For these experiments, a GRIN lens was implanted
145 deep in the cortex above the target imaging region seven days before the start of the CPZ treatment. Five
146 weeks after the GRIN lens implantation, a baseplate was mounted onto the animal's skull providing
147 fixtures to attach the Miniscope and allowing us to set the working distance between the objective and
148 the lens (Fig. 1c). The tissue can thus recover for five weeks and any inflammation or blood accumulation
149 under the lens can be cleared before the beginning of Ca^{2+} imaging experiments (Zhang et al., 2019).
150 Tamoxifen was injected during the fourth week of CPZ treatment, when demyelination is advanced.
151 Finally, *in vivo* imaging was done during demyelination to record Ca^{2+} signals from oligodendroglia in the
152 demyelinated corpus callosum of freely moving mice (Fig. 1c, 1f).

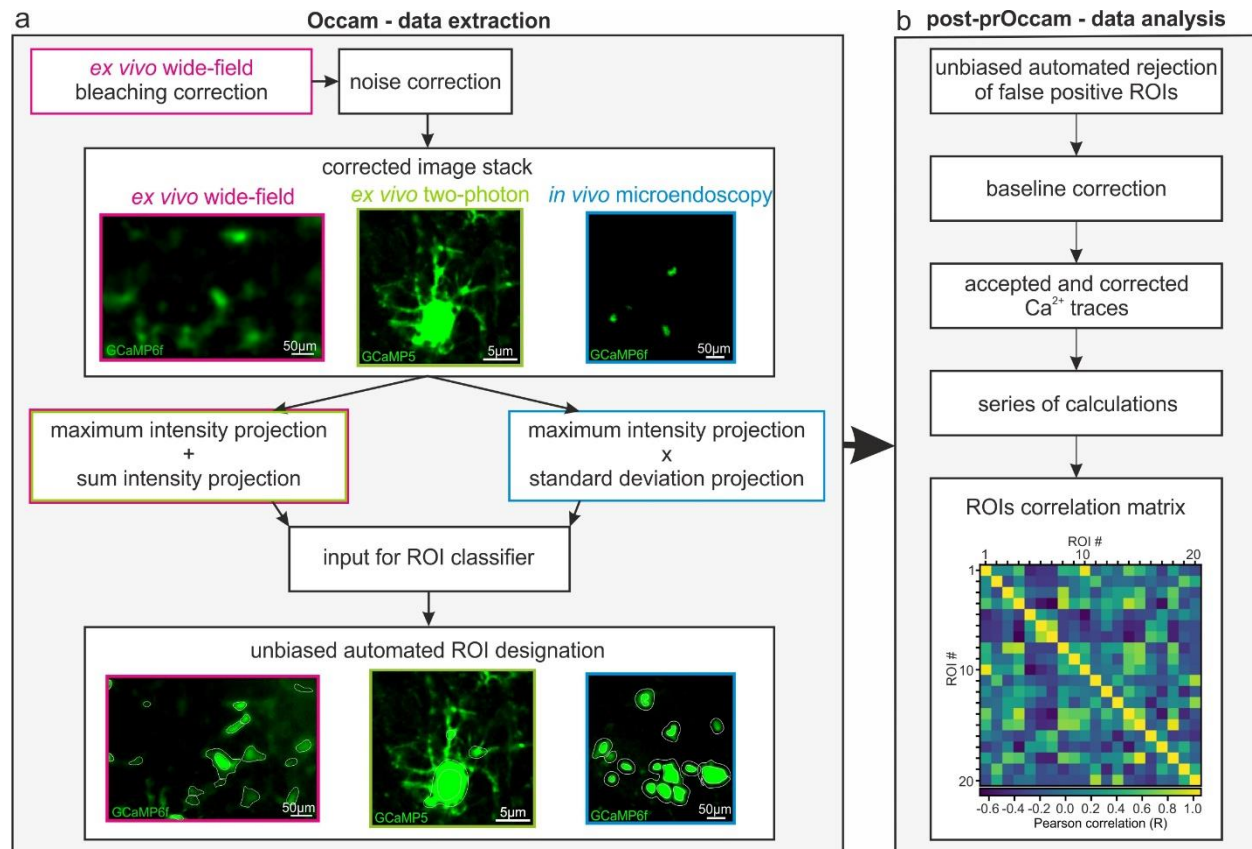
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156 **Occam and post-prOccam: Ca²⁺-signal analysis softwares for investigation of oligodendroglia in**
157 **demyelinated lesions**

158 To perform an automated and unbiased detection of active regions in different Ca²⁺ imaging
159 datasets from demyelinated preparations, we developed a configurable Fiji/ImageJ2 plugin named Occam
160 (Oligodendroglial cells calcium activity monitoring; Fig. 2a). We first validated our analytical workflow on
161 wide-field one-photon Ca²⁺ imaging data recorded in acute slices of corpus callosum which revealed high
162 levels of spontaneous Ca²⁺ activity in oligodendroglia of LPC-induced demyelinated lesions (Fig. 1b, 1d, 2a,
163 3a; Supplementary Video 1). Occam first performs noise and bleaching corrections of image stacks (Fig.
164 2a) and then uses the trainable machine learning-based WEKA Fiji/ImageJ2 segmentation plugin (Arganda-
165 Carreras et al., 2017) combined with a local maxima segmentation tool to define regions of interest (ROIs)
166 with fluctuating Ca²⁺ signals with high and medium pixel intensities (Supplementary Manual). Several
167 parameters of ROI detection can be configured by the user to optimize Occam's performance and to
168 obtain an automated and unbiased ROI designation (for instance, the minimum size of an active ROI or a
169 bleaching correction option). Once the ROI designation process is completed, Occam saves two files: one
170 file stores each ROI area size in pixels and the coordinates of the geometric center of the ROI, the other
171 file describes each ROI's fluorescence behavior over time as a vector listing the mean fluorescence pixel
172 intensity value in each frame contained in the image stack. The ROI fluorescence intensity vector is also
173 referred to as the ROI trace (Supplementary Fig. 2a, 2b; Supplementary Manual). These two files are then
174 read by the post-prOccam software to perform a systematic ROI analysis along with quantitative
175 calculations ultimately aimed at easing the detailed characterization of oligodendroglial Ca²⁺ activity (Fig.
176 2b). The post-prOccam software was implemented as a configurable program where users can set various
177 analysis parameters in a configuration file so as to adapt its operation to any specific dataset
178 (Supplementary Manual).



179

180 **Figure 2 – Occam and post-prOccam: an open source, fully automated and configurable analysis toolkit**
 181 **for oligodendroglial Ca²⁺ imaging of ex vivo and in vivo preparations. (a)** The Occam software is available
 182 as a Fiji (tested in version 1.53t and earlier) plugin and configurable for the analysis of wide-field, two-
 183 photon and in vivo microendoscopy Ca²⁺ imaging. Occam performs bleaching correction only on ex vivo
 184 wide-field image stacks and does noise correction according to imaging condition (Supplementary
 185 Manual). Then, it uses the maximum and sum intensity projections for ex vivo image stacks and the
 186 maximum and standard deviation projections for in vivo image stacks to build an input for the ROI
 187 classifier. The ROI classifier of Occam defines ROIs with significant Ca²⁺ fluctuations fully automated and
 188 is used by WEKA for ROI designation. **(b)** Output of Occam can be fed to post-prOccam which is a
 189 configurable Python-based software that first rejects any ROIs that do not show significant Ca²⁺
 190 fluctuations, then performs baseline corrections, subsequently performs a series of calculations for each
 191 ROI in the image stack and finally computes a ROIs correlation matrix of the Pearson correlation value of
 192 each ROI as compared to every other ROI. Occam and post-prOccam are available as free, multiplatform
 193 and open source softwares at: <https://gitlab.com/d5674/occam> and procedures are described in more detail
 194 in the Supplementary Manual.

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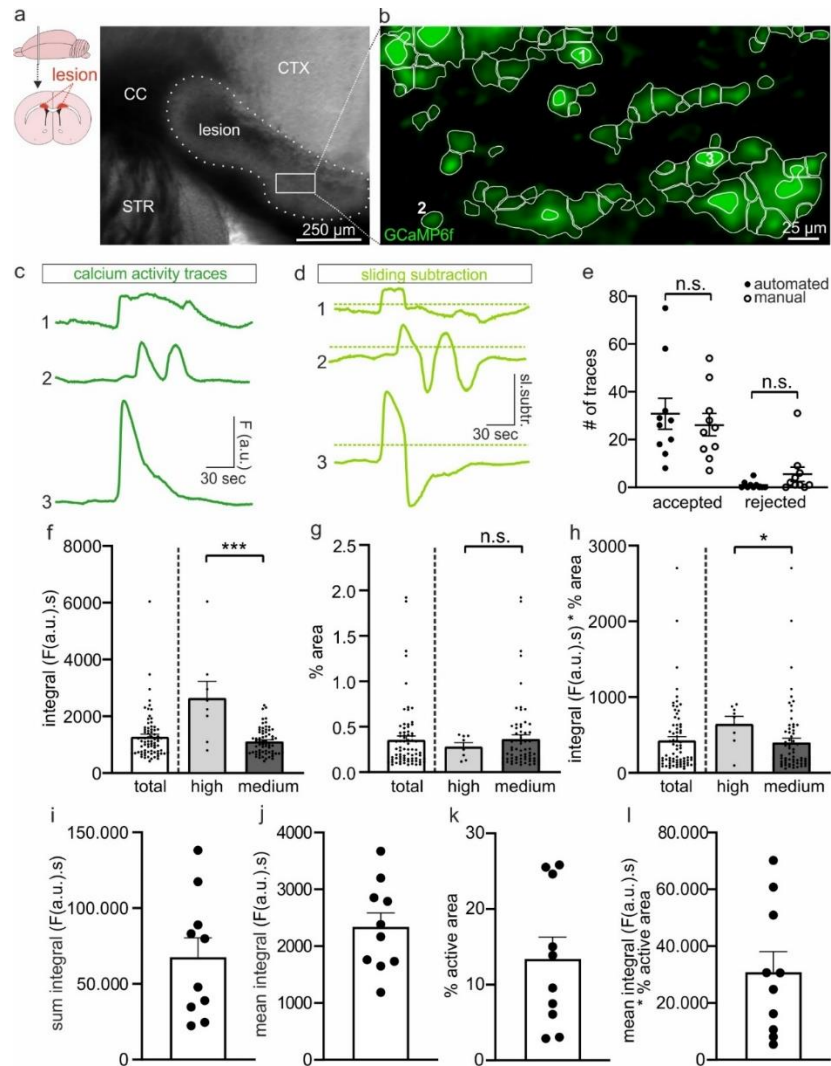
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197 Automated ROI detection algorithms cannot be error-free and almost always require an *a*
 198 *posteriori* rejection of false positive ROIs (Cantu et al., 2020). We developed the post-prOccam program
 199 that automatically fetches the data written by Occam and proceeds to an automatic rejection of ROIs
 200 lacking significant Ca²⁺ signal fluctuations. To reject false positive ROIs, we adapted an algorithm

200 previously used for the fluorescence-based tracking of exocytotic events (Yuan et al., 2015;
201 Supplementary Manual). Based on repeated intensity values subtraction in a sliding window over the
202 whole ROI vector of mean intensity values (Supplementary Fig. 2a-c), our method allowed us to single out
203 fluorescence intensity changes in an image stack that were greater than noise fluctuations (Fig. 2b, 3a-d).
204 Since Ca^{2+} signals of oligodendroglia in lesions were characterized by long-lasting kinetics (Fig. 3c; rise
205 time: 54.63 ± 9.67 frames, equivalent to 35.25 ± 5.36 s, $n=34$ events from $n=8$ fields), the sliding window
206 subtractions were computed between points distanced by 40 frames, as: $\text{intensity} = \text{intensity}(\text{ROI}[n+40])$
207 $- \text{intensity}(\text{ROI}[n])$, with n being the frame number in the stack (Supplementary Fig. 2b, 2c). Each initial
208 ROI trace is thus replaced by a new one, as computed from the sliding window subtractions, that is then
209 tested against user-defined threshold parameters set in the configuration file (Fig. 3d; Supplementary
210 Files 1-3). These threshold settings configure the rejection of ROI traces depending on both their Ca^{2+}
211 event kinetics and noise. To find the best settings to eliminate false positive traces, we empirically changed
212 the threshold parameters and compared the results with those obtained by a manual rejection of ROIs in
213 several imaging stacks (Fig. 3e). The threshold values that were found to be the most effective in rejecting
214 false positive ROIs were set as default values in the configuration file and provided in Supplementary File
215 1 (Fig. 3e; see detailed description of the threshold parameters in the Supplementary manual).

216 Once the accepted ROIs have been selected, post-prOccam performs a baseline subtraction for
217 each ROI trace by subtracting a mean minimum intensity value previously calculated from a configurable
218 number of trace points (Supplementary File 1; Supplementary Manual). We used a baseline subtraction
219 computation rather than the conventional $[(F(t)-F_0)/F_0]$ computation because we adapted the analysis to
220 the Ca^{2+} dynamics of oligodendroglia in lesions. Indeed, the oligodendroglial cells studied in this report
221 exhibited high overall levels of spontaneous Ca^{2+} activity, often right at the start of the recording, making
222 it difficult to determine with certainty the F_0 value of the resting fluorescence intensity. Finally, the post-
223 prOccam program performs a series of calculations to help characterize the Ca^{2+} activity of oligodendroglia

224 in lesions (Fig. 2b). Figure 3f-h illustrates part of the calculations performed for each ROI in a single image
225 stack, *i.e.* the mean integral, the percentage of active area and the mean integral multiplied by the
226 percentage of the active area. Since the complex features of oligodendroglial Ca²⁺ events preclude their
227 proper individual isolation (Supplementary Fig. 3), we found it more appropriate to calculate the mean
228 intensity integral of each ROI trace rather than to use a procedure for single event detection (see
229 discussion). As expected, high intensity ROIs exhibited significantly larger mean integrals than medium
230 intensity ROIs (Fig. 3f) and, despite similar mean percentage of active area between high and medium
231 intensity ROIs, the mean integral multiplied by the percentage of the active area remained larger for high
232 intensity ROIs (Fig. 3g-h). However, when considering all the analyzed stacks, the number of high intensity
233 ROIs was always largely smaller than the number of medium intensity ROIs (3.5±0.9 high intensity ROIs,
234 n=27.3±5.7 medium intensity ROIs and n=30.8±6.5 total ROIs for n=10 stacks). Moreover, the mean
235 integral of medium intensity ROIs and that of all pooled data (Total) remain similar (Fig. 3f). Therefore, we
236 hereinafter pooled the data from high and medium intensity ROIs for quantifications, but kept their
237 detection separate because the WEKA plugin used by Occam performed better when classifying ROIs in
238 these two categories. The average calculations for all ROIs in several image stacks show that Ca²⁺ activity
239 between demyelinated lesions at 7 dpi can be variable but sufficiently high to always allow us to detect
240 changes over only a few minutes of recording (Fig. 3i-l).



241

242 **Figure 3 – Using Occam and post-prOccam for analysis of ex vivo wide-field oligodendroglial Ca^{2+}**
 243 **imaging from a callosal LPC-induced demyelinated lesion. (a and b)** Representative image of a
 244 demyelinated lesion in 4x DIC microscopy (a) and of GCaMP6f expressing oligodendroglia in 40x wide-field
 245 fluorescence imaging displaying detected active ROIs (white) as obtained with Occam (b). **(c and d)**
 246 Representative corrected ROI traces (c) and sliding window subtraction traces (d) as obtained with post-
 247 prOccam. **(e)** Comparison of the manual rejection of false positive ROIs and the automatic rejection of
 248 false positive ROIs by post-prOccam revealed no differences in accepted and rejected ROIs, validating
 249 post-prOccam's performance. n.s.: not significant, two-way ANOVA followed by a Bonferroni multiple
 250 comparisons test. **(f-h)** Calculations performed by post-prOccam on each individual ROI of the image stack
 251 include amongst others the integral (f), percentage of active area (g) and integral multiplied by percentage
 252 of active area (h) and are separated for high and medium intensity ROIs. * $p < 0.05$ and *** $p < 0.001$; Mann-
 253 Whitney test. **(i-k)** Calculations performed by post-prOccam on all ROIs in an image stack include amongst
 254 others the sum integral (i), the mean integral (j), the percentage active area (k) and the mean integral
 255 multiplied by the percentage active area (l) ($n = 10$ stacks, $n = 10$ slices, $n = 7$ mice). Error bars represent
 256 standard error of the mean. Dot plots are presented as mean \pm s.e.m.

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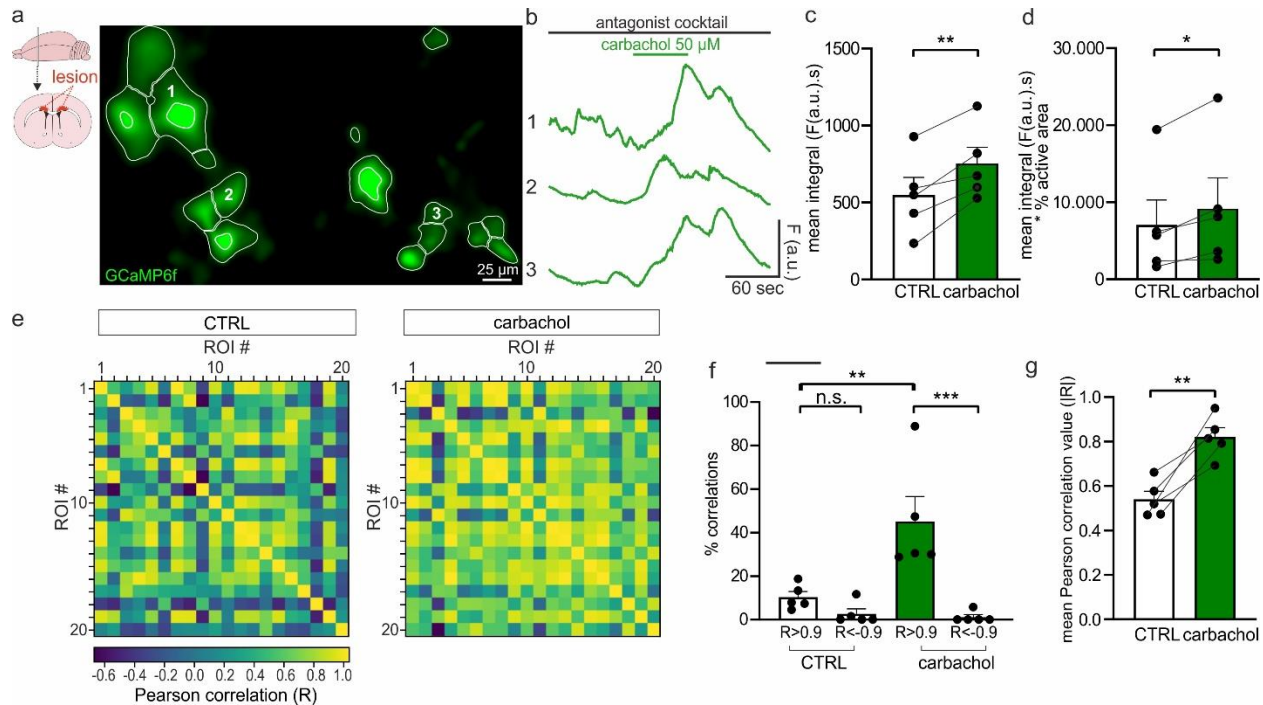
258 For informational and debugging purposes, a log file is produced by post-prOccam with a highly
259 detailed account of all the data processing steps and their outcome. The accepted ROIs and the
260 corresponding processed data of a stack are saved by post-prOccam in two separate files for further
261 statistical analysis. Of note is the pp-supervisor program that allows the scientist to run post-prOccam
262 automatically over a set of stacks located in a single directory using the configuration file that was
263 elaborated during the exploratory steps described above.

264

265 **Interval-based analysis and evaluation of synchronized Ca²⁺ signals of oligodendroglia in lesions**

266 One important feature of post-prOccam is its ability to compare ROIs only at specific time point
267 intervals of the recorded datasets. For instance, in the case of applications of pharmacological agents
268 during Ca²⁺ imaging recordings, a user might wish to compare Ca²⁺ signals in the absence or in the presence
269 of a drug in a single image stack. The default post-prOccam software behavior is to perform all the
270 calculations described in the previous sections on the whole ROI vector. However, in specific cases
271 mentioned above, it might be useful for the calculations to be performed only over selected ranges of the
272 ROI vector (that is, intervals of that ROI's acquisition time points). The configuration file provides a section
273 in which the user might list any number of ROI vector intervals over which to perform the previously
274 described calculations. To validate this feature, we bath-applied the muscarinic receptor agonist
275 carbachol in the presence of a cocktail of antagonists to stimulate intracellular Ca²⁺ signals of
276 oligodendroglia during one-photon Ca²⁺ imaging recordings of 7 dpi callosal LPC-induced lesions in brain
277 slices (Fig. 4a, 4b). Oligodendroglia express muscarinic receptors M1, M3 and M4, which, when activated
278 by carbachol, increase intracellular Ca²⁺ signals (Abiraman et al., 2015; Cohen & Almazan, 1994; Welliver
279 et al., 2018). In order to determine the extent of the Ca²⁺ signal activity changes upon the bath application
280 of carbachol, we directed post-prOccam to compare the Ca²⁺ signals in each ROI only during two distinct
281 acquisition intervals: the control interval before the carbachol application and the interval during which

282 the carbachol was applied. As expected, we observed that a bath application of 50 μM carbachol in the
 283 presence of a cocktail of antagonists indeed induced an increase in intracellular Ca^{2+} signals in
 284 oligodendroglia, as revealed by an increase in both the mean integral and the mean integral multiplied by
 285 the percentage of the active area, compared to the control (Fig. 4a-d).
 286



287
 288 **Figure 4 – Using Occam and post-prOccam for interval analysis and evaluation of synchronized Ca^{2+}**
 289 **signals of oligodendroglia in LPC-induced demyelinated lesions. (a)** Representative image of Ca^{2+} imaging
 290 in callosal LPC-induced lesions in *ex vivo* brain slices that were exposed to 50 μM carbachol to induce
 291 increases in Ca^{2+} signals in oligodendroglia in the presence of an antagonist cocktail containing 10 μM
 292 NBQX, 50 μM AP5, 10 μM GABAazine, 1 μM TTX and 50 μM mecamylamine. The image displays detected
 293 active ROIs (white) as obtained with Occam. **(b)** Representative Ca^{2+} traces showing Ca^{2+} increases during
 294 carbachol application as obtained with post-prOccam. **(c and d)** Mean integral (c) and mean integral
 295 multiplied by the percentage of active area (d) of Ca^{2+} signals in control and after exposure to carbachol.
 296 * $p < 0.05$, ** $p < 0.01$; paired Student's t-test. **(e)** Example of correlation matrixes obtained with post-
 297 prOccam before and after carbachol exposure. Each square indicates the Pearson correlation value of one
 298 ROI with another. Yellow indicates high positive Pearson correlation, while dark blue indicates high
 299 negative correlations. Note that traces 1, 2, 3 in b correspond to ROIs 7, 12, 14 in the matrix. **(f)** The
 300 percentage of correlations in the correlation matrix that is significantly negative ($R < -0.9$) or significantly
 301 positive ($R > 0.9$) in both control and carbachol exposed conditions. n.s.: not significant, ** $p < 0.01$,
 302 *** $p < 0.001$, two-way ANOVA followed by a Tukey's multiple comparisons test. **(g)** Mean Pearson
 303 correlation values for control and carbachol conditions. CTRL: control before carbachol exposure ($n = 5$
 304 stacks, $n = 5$ slices, $n = 4$ mice). ** $p < 0.01$; paired Student's t-test. Dot plots are presented as mean \pm s.e.m.

305

306 While it is now established that a population of astrocytes may exhibit high levels of synchronized
307 Ca^{2+} activity *in vitro* and *in vivo* (Kolzumi et al., 2010; Ingiosi et al., 2020), nothing is known about the
308 potential of Ca^{2+} signals in oligodendroglia to be synchronized. We thus tested this possibility in
309 demyelinating lesions by implementing a correlation calculation which allows the user to establish
310 whether ROIs within a given image stack show synchronized Ca^{2+} signals. The post-prOccam program
311 computes an inter-ROI Pearson correlation coefficient matrix between each ROI trace and every other ROI
312 trace (Fig. 4e; see Materials and Methods; Ingiosi et al., 2020). In control conditions, we found that most
313 ROIs did not show any correlated Ca^{2+} activity, as evidenced by a low percentage of correlated ROIs ($|R|$
314 < 0.9) and a mean Pearson correlation coefficient R value of 0.54 ± 0.24 (Fig. 4f-g). The remaining few ROIs
315 that displayed correlated Ca^{2+} activity had mainly a positive rather than a negative correlation (Fig. 4f).
316 Interestingly, upon application of the muscarinic agonist carbachol in the presence of a cocktail of
317 antagonists, a significant overall increase of the positive inter-ROI Ca^{2+} activity correlation was observed
318 (Fig. 4e-g), with a mean Pearson correlation value of 0.82 ± 0.04 . These data showed that the majority of
319 ROIs did simultaneously respond to the agonist. The post-prOccam software thus successfully determined
320 the degree of Ca^{2+} activity correlation between ROIs and was able to detect simultaneous increases of
321 that activity as induced by pharmacological agents. In order to adapt to any specifics of biological
322 applications, the Pearson correlation R value threshold might be configured. The percentage of correlated
323 ROIs as well as the mean Pearson correlation coefficient are reported for each image stack in the
324 corresponding output file generated by the program. Taken together, our results show that the Occam
325 and the post-prOccam software programs make for a configurable and automatable solution for the
326 analysis of oligodendroglial Ca^{2+} signals.

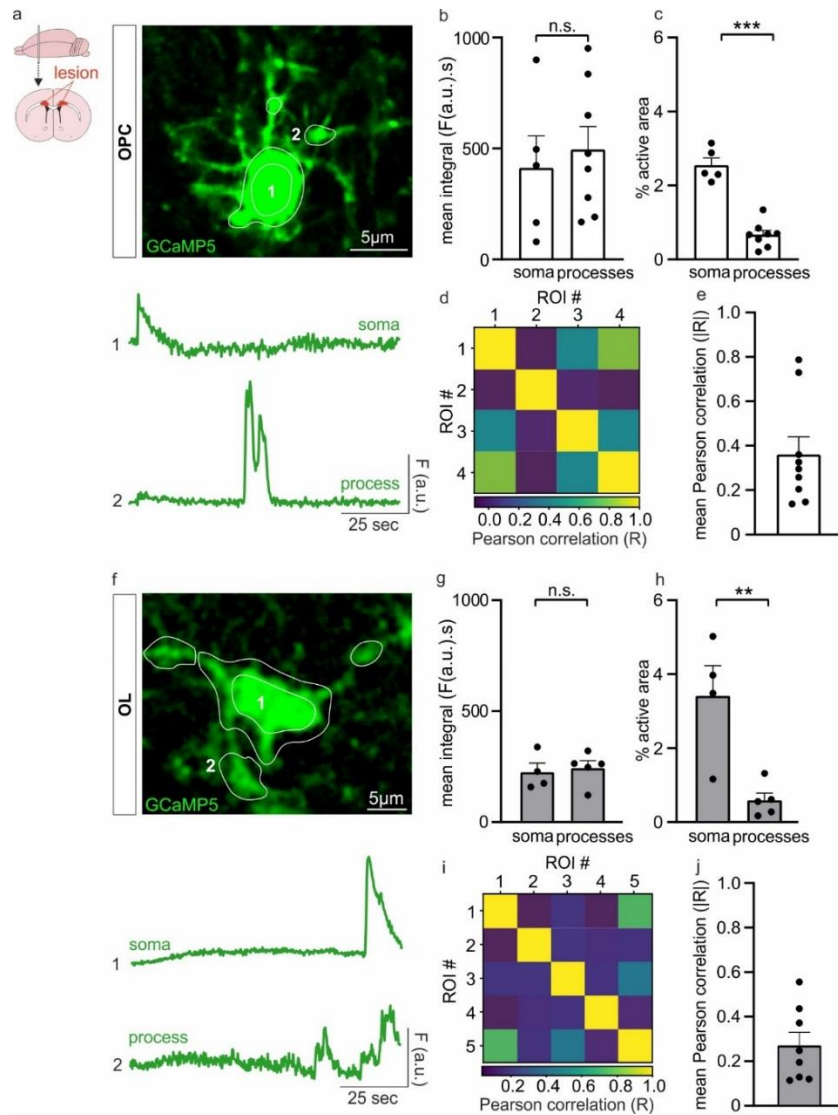
327

328

329 **Two-photon imaging of Ca²⁺ signals in single oligodendroglia in demyelinated lesions**

330 Although one-photon imaging allows one to determine the global Ca²⁺ signaling properties of
331 oligodendroglia in a large area of the LPC-induced lesion, the spatial resolution of this technique is not
332 sufficient to reveal local Ca²⁺ changes occurring specifically either in the soma or in the cell processes of
333 any individual cell. To test whether our workflow is suitable for the analysis of images obtained by two-
334 photon microscopy at the single cell resolution, we performed two-photon Ca²⁺ imaging of putative OPCs
335 and OLs in callosal LPC-induced lesions in *ex vivo* brain slices and analyzed the data using Occam and post-
336 prOccam (Fig. 5; Supplementary Video 2 and Supplementary File 2). We used morphological criteria to
337 distinguish OPCs and OLs, and only recorded cells for which we could connect the processes to a particular
338 soma. In corpus callosum, OPCs were characterized by a relatively small round soma and a stellate
339 arborization with thin processes (Fig. 5a; Chittajallu et al., 2004) whereas OLs had a larger soma and
340 principal processes often aligned with axons (Fig. 5f; Bakiri et al., 2011). Morphological determination of
341 putative OPCs and OLs allowed us to reveal that, even though the area of ROIs in the processes is
342 significantly reduced as compared to that of ROIs in the soma, the mean intensity integral of their Ca²⁺
343 signals is similar to that of Ca²⁺ signals from the cell soma (Fig. 5a-c, 5f-h). Although the mean number of
344 ROIs in OPCs and OLs was similar (9.44±1.97 in OPCs vs 8.00±2.19 in OLs) as was the rise time of Ca²⁺
345 events (3.61±1.14 s n=10 events from 5 fields for OPCs vs 4.16±0.68 s; n=37 events from 6 fields for OLs
346 p=0.71 for soma and process data pooled together, unpaired Student's t-test), Ca²⁺ signals had a higher
347 integral in OPCs than in OLs (Fig. 5 a-c, 5f-h, p=0.0391 for soma and process data pooled together,
348 unpaired Student's t-test)). Altogether, these results not only show high levels of Ca²⁺ activity in processes
349 of OPCs and OLs, but also that this activity is greater in OPCs inside demyelinating lesions. Importantly,
350 we also observed that, whatever the cell type involved (OPCs or OLs), the Ca²⁺ signals were poorly
351 correlated, which suggests that they occurred independently in the soma and in the processes and at
352 different subcellular locations (Fig. 5 d-e, 5i-j). In summary, these results show that Occam and post-

353 prOccam can successfully determine the characteristics of oligodendroglial Ca^{2+} signals recorded by
 354 different imaging techniques and at different spatial scales.



355

356 **Figure 5 – Using Occam and post-prOccam for the analysis of two-photon oligodendroglial Ca^{2+}**
 357 **imaging in callosal LPC-induced demyelinated lesions in *ex vivo* brain slices. (a)** Representative image of
 358 a putative OPC (a) and a putative OL (f) expressing GCaMP5 with examples of corrected Ca^{2+} traces from
 359 the soma and a process (bottom) obtained with Occam and post-prOccam. (b-c, g-h) Mean integrals (b, g)
 360 and % active area (c, h) for the soma and the processes of OPCs (b-c) and OLs (g-h). n.s.: not significant.
 361 ** $p < 0.01$, *** $p < 0.001$, unpaired Student's t-test. (d, i) Examples of correlation matrices describing a lack
 362 of synchronization of Ca^{2+} signals in the soma and processes of OPCs (d) and OLs (i). Each square indicates
 363 the Pearson correlation value of one ROI with another ROI. Yellow indicates high positive Pearson
 364 correlation, while blue indicates no correlation. Note that traces 1 and 2 in a and f correspond to ROIs 1,
 365 and 2 in the matrices in d and i, respectively. (e and j) The mean correlation value of OPCs and OLs (n=9
 366 stacks, n=9 slices, n=7 mice for OPCs and n=8 stacks, n=7 slices, n=6 mice for OLs). Dot plots are presented
 367 as mean \pm s.e.m.

368 ***In vivo* microendoscopy Ca²⁺ imaging of oligodendroglia during the demyelination process**

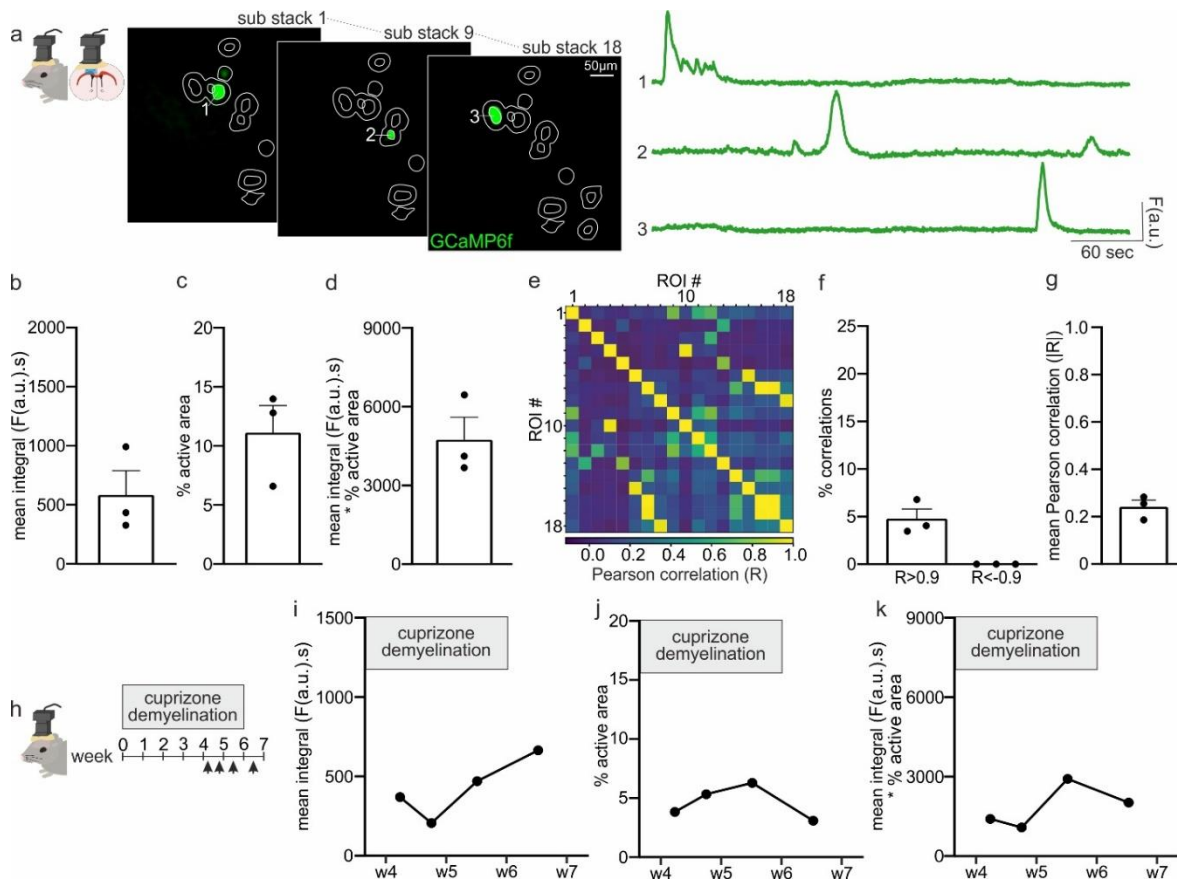
369 Although *in vivo* Ca²⁺ imaging has been extensively studied in neurons and astrocytes, only few
370 studies in the zebrafish have reported *in vivo* Ca²⁺ signals in oligodendroglia (Baraban et al., 2018; Krasnow
371 et al., 2018; Marisca et al., 2020; Li et al., 2022). Moreover, no current information exists on
372 oligodendroglial Ca²⁺ imaging in the demyelinated mouse brain *in vivo*. To fill this gap, we decided to adapt
373 our experimental and analytical workflow to the study of oligodendroglial Ca²⁺ signals during
374 demyelination in freely moving mice (Fig. 1c-f, Fig. 2a). As a proof-of-concept, we used the CPZ-induced
375 demyelination model and performed microendoscopic Ca²⁺ imaging recordings of oligodendroglia in the
376 corpus callosum of awake demyelinated mice (Fig. 6a-b; Supplementary Video 3). Four animals were
377 implanted according to the procedure described in Figure 1. Three of them were recorded during the fifth
378 postnatal week of CPZ feeding, when demyelination is advanced (Remaud et al., 2017), and sacrificed
379 afterwards in order to confirm the proper position of the GRIN lens above the corpus callosum. The fourth
380 mouse was maintained alive and imaged at four different timepoints to show that multiple recordings of
381 the same animal can be made over several weeks.

382 The image stacks we acquired contained 4200 frames collected over 7 minutes. To enhance the
383 precision of ROI detection by Occam in such large data files, the program first splits the image stack into
384 a configurable number of sub-stacks. Then, for each sub-stack, it performs the noise correction, uses the
385 maximum and standard deviation projections to build an input for the ROI classifier and proceeds to the
386 automated ROI designation using the WEKA Fiji/ImageJ2 segmentation plugin combined with a local
387 maxima segmentation tool (Fig. 2a; Supplementary Manual). This side-step from the *ex vivo* version of
388 Occam allows for accurate detection of ROIs in large image stacks collected over many minutes because
389 it also captures ROIs that are only briefly active and that would be overlooked when making a projection
390 image over the entire imaging duration. However, this procedure can lead to an overestimation in the
391 number of active ROIs if a specific region of the stack is active several times during a single experiment

392 and, thus, is detected as a ROI in multiple sub-stacks. To overcome this problem, Occam projects all ROIs
393 from all sub-stacks onto each other and allows the user to determine a degree of overlap for which
394 overlapping ROIs are merged into a single ROI (Supplementary Manual). Like for *ex vivo* preparations, ROIs
395 with high and medium pixel intensities were considered as displaying significant Ca²⁺ signal fluctuations.
396 As the number of sub-stacks can be configured by the user, the ROI detection process will be successful
397 even in recordings longer than 7 minutes, either in a single acquisition or in multiple acquisitions
398 performed on the same animal over weeks (Fig. 6). In sum, Occam features for *in vivo* experiments have
399 been adapted so that it can detect not only shortly occurring events, but also the repeated activation of a
400 single region over a long experiment. Once the ROI selection process has been performed, the
401 downstream processing by post-prOccam is identical to that described in the preceding sections
402 (Supplementary File 3).

403 Our analysis revealed that oligodendroglia *in vivo* show high levels of spontaneous Ca²⁺ activity in
404 the fifth week of CPZ-induced demyelination (Fig. 6a-d; mean number of ROIs of 15±4.58). We could
405 identify ROIs with multiple Ca²⁺ events with complex and long-lasting kinetics of several seconds (Fig. 6a
406 and Supplementary Fig. 3c). The mean rise time, however, was on average faster than in *ex vivo* wide-field
407 experiments (rise time: 31.48±5.54 frames, equivalent to 6.29±0.91 s, n=48 events from n=3 fields).
408 Furthermore, the low percentage of correlated ROIs and the small mean Pearson correlation coefficient
409 showed that most ROIs did not display any correlated Ca²⁺ activity (Fig. 6e-g), confirming the lack of
410 synchronized Ca²⁺ signals in oligodendroglia observed *ex vivo* in the LPC model. Finally, we performed *in*
411 *vivo* microendoscopy Ca²⁺ imaging in the same mouse during weeks 5 and 6 of CPZ-induced demyelination
412 and during week 7 after CPZ withdrawal (Fig. 6h). The mean pixel intensity integral, the percentage of
413 active area and the mean integral multiplied by the percentage of the active area remained relatively
414 stable during this period, indicating that the imaging conditions did not deteriorate over time (Fig. 6h-k).
415 The monitoring of oligodendroglial Ca²⁺ activity over weeks in the same animal may give insight into the

416 behaviour of this cell population during demyelination. To our knowledge, this is the first time that Ca^{2+}
 417 signals of oligodendroglia during demyelination have been recorded in real time and the successful
 418 validation of our workflow prompts future research on oligodendroglial Ca^{2+} signaling in the living injured
 419 mammalian brain.



420

421 **Figure 6 – Using Occam and post-prOccam for the analysis of *in vivo* microendoscopy Ca^{2+} signals of**
 422 **oligodendroglia in the demyelinated corpus callosum of freely moving mice. (a)** Representative images
 423 and corrected Ca^{2+} traces of microendoscopy Ca^{2+} imaging in freely moving mice during the fifth week of
 424 CPZ demyelination. The image displays detected active ROIs (white) in several sub-stacks as obtained with
 425 the *in vivo* analysis option of Occam. **(b-d)** Mean integral (b), % of active area (c) and mean integral
 426 multiplied by percentage of active area (d) during the fifth week of CPZ demyelination (n=3 mice). Data is
 427 presented as mean \pm s.e.m. **(e)** Correlation matrix of the *in vivo* experiment in a obtained with post-
 428 prOccam. Each square indicates the Pearson correlation value of one ROI with another ROI. Yellow
 429 indicates high positive Pearson correlation, while dark blue indicates no Pearson correlation. Note that
 430 trace 1, 2, 3 in a correspond to ROIs 1, 2, 3 in the matrix. **(f)** The percentage of correlations in the
 431 correlation matrix. **(g)** The mean Pearson correlation value. **(h)** *In vivo* microendoscopy Ca^{2+} imaging was
 432 performed in the same mouse at four timepoints, namely during weeks 5 and 6 of CPZ-induced
 433 demyelination and during week 7 after CPZ withdrawal. **(i)** Mean integral, **(j)** % of active area and **(k)** mean
 434 integral multiplied by percentage of active area during the fifth, sixth and seventh week in the same
 435 mouse.

436 Discussion

437 While it is suspected that oligodendroglial Ca^{2+} signaling plays a key role in myelin repair, the
438 characteristics of oligodendroglial Ca^{2+} signals in demyelinated lesions remain unknown. This is due to a
439 limited number of *ex vivo* or *in vivo* Ca^{2+} imaging studies as well as a lack of automated analytical tools
440 adapted to the monitoring of the specific characteristics of oligodendroglial Ca^{2+} activity. To fill this need,
441 we devised experimental conditions for *ex vivo* and *in vivo* Ca^{2+} imaging in mouse demyelinated lesions
442 and implemented an analytical workflow comprising two programs, Occam and post-prOccam, which
443 provide a reliable solution for the automated in-depth analysis of one- and two-photon oligodendroglial
444 Ca^{2+} imaging data. Tested in Windows 10 and Debian GNU/Linux, these cross-platform programs were
445 designed to be easily configurable to ensure an unbiased selection of active ROIs and to allow an efficient
446 analysis of large datasets. Licensed under the GNU GPLv3+ license, both programs can be freely used,
447 modified according to any specific need and question, and redistributed. Since the Ca^{2+} signal detection is
448 finely configurable in both programs, the use of our software solution might be extended to new use cases
449 such as other glial cell types.

450 By harnessing the features in these two programs, we could monitor the spontaneous
451 oligodendroglial Ca^{2+} activity in different toxic models of demyelination, both *ex vivo* and *in vivo*. In any of
452 these possible configurations, Ca^{2+} traces that were first obtained with Occam then undergo a post-
453 processing procedure with post-prOccam that processes them to reject ROIs with too small Ca^{2+}
454 fluctuations (false positives), which can be construed as a refinement of the ROI detection process. We
455 found that this automatic Ca^{2+} trace selection in our workflow performs equally well as a manual Ca^{2+}
456 trace selection, thus validating the procedure. Among other analysis options, the ability to restrict the
457 processing and quantification steps to specific acquisition intervals has proven useful to screen the effect
458 of drugs on oligodendroglial Ca^{2+} signaling. Furthermore, a number of features in post-prOccam make it
459 possible to spot eventual synchronized Ca^{2+} signals across different ROIs in a given frame stack. Here we

460 successfully applied these analysis modalities to detect, quantify and correlate Ca^{2+} activity increases upon
461 carbachol bath application to demyelinated brain slices in order to activate muscarinic receptors in
462 oligodendroglia, as previously described in cell culture (Cohen & Almazan, 1994; Welliver et al., 2018).
463 Interestingly, muscarinic receptor agonists can increase OPC proliferation, but block OPC differentiation
464 and remyelination (De Angelis et al., 2012; Abiraman et al., 2015; Welliver et al., 2018). Moreover, the
465 muscarinic receptor antagonist benztropine was found to be beneficial to induce myelin repair in an
466 animal model (Deshmukh et al., 2013) and the muscarinic receptor antagonist clemastine was shown to
467 improve myelin regeneration in a clinical trial with multiple sclerosis patients (Green et al., 2017). The
468 positive effect of these antagonists might thus be caused by the blockade of muscarinic receptor-
469 dependent Ca^{2+} signaling mechanisms, which is a subject for further research. These studies highlight the
470 importance of investigating the Ca^{2+} response of oligodendroglia to novel remyelinating drugs. The
471 workflow described in this report will facilitate these future studies.

472 Oligodendroglial Ca^{2+} events are unique in their variability and duration and are thus better
473 qualified using the ROI-based measurements number, size and mean pixel intensity integral instead of the
474 conventional measurements such as frequency, amplitude and duration of isolated events. Indeed, unlike
475 neurons, that display well-defined Ca^{2+} signals on a millisecond scale (Chua & Morrison, 2016),
476 oligodendroglia show complex Ca^{2+} events characterized by very slow and variable kinetics which make
477 their detection and isolation difficult, in particular because of their frequent convolution (Supplementary
478 Fig. 3a-c). This complexity of oligodendroglial Ca^{2+} events has previously been observed with two-photon
479 microscopy (Balía et al., 2017) and in recordings at single cell resolution (Baraban et al., 2018; Krasnow et
480 al., 2018; Battefeld et al., 2019; Marisca et al., 2020), indicating that complex Ca^{2+} dynamics are a hallmark
481 of oligodendroglia and it is therefore essential to evaluate them in an appropriate manner. Thresholding
482 techniques or event template detection methods commonly used on neuron and astrocyte Ca^{2+} imaging
483 datasets are not easily applicable to the unique and complex Ca^{2+} events observed in oligodendroglia. To

484 overcome this constraint, we evaluate activity levels by measuring the number and size of active ROIs as
485 well as the integral of the traces. Our measurements thus account for the activity throughout the whole
486 ROI trace or during configurable intervals without isolating Ca^{2+} activity events. In the eventuality that a
487 detailed description of Ca^{2+} events would be desirable, extra measurements may be performed either
488 manually or by other post-processing programs on the corrected active ROI traces as output by post-
489 prOccam. In our case, we manually inspected these corrected traces and could establish that the mean
490 rise time of Ca^{2+} events easily isolated by eye lasted from few seconds to minutes, independently of the
491 imaging condition (Supplementary Fig. 3). These slow kinetics are in line with the half-width duration of 9
492 s reported for myelin internodes in the neocortex (Battefeld et al., 2019), but our results also show that
493 oligodendroglial Ca^{2+} events in lesions are poorly correlated and may have long variable durations.

494 We could successfully and automatically detect oligodendroglial-related Ca^{2+} -active ROIs in wide
495 field as well as in single cell Ca^{2+} imaging frame stacks obtained in brain slices. These *ex vivo* recordings
496 revealed significant but uncorrelated spontaneous Ca^{2+} activity in oligodendroglia inside demyelinated
497 lesions, both at the population level and at the individual cell level. In particular, our two-photon Ca^{2+}
498 imaging experiments showed that Ca^{2+} signals in ROIs located in soma or processes are not correlated,
499 suggesting that they occur independently in subcellular compartments. In this configuration, our results
500 also indicate that, compared to OLS, OPCs displayed a higher Ca^{2+} activity whose significance remain to be
501 elucidated. The experimental and analytical tools described in this study will help to further explore the
502 characteristics of these signals at different states of cell maturation and at different time points of the
503 demyelination and remyelination process. Importantly, our workflow for acquiring and analyzing *in vivo*
504 oligodendroglial Ca^{2+} signals will also allow users to investigate the role of these signals in the mammalian
505 brain in real time. We were able to corroborate that the oligodendroglial Ca^{2+} activity observed *in vivo* is
506 compatible with that observed in brain slices because it was variable but significant, poorly correlated and
507 characterized by complex slow kinetics. We also showed that Ca^{2+} activity can be stable in the same mouse

508 from one week to another. This type of *in vivo* experiments opens up new perspectives for analyzing how
509 the oligodendroglial Ca²⁺ activity changes over time during myelin repair and when the mouse is
510 performing a behavioral task.

511 In conclusion, our *ex vivo* and *in vivo* results suggest that oligodendroglial Ca²⁺ signaling plays an
512 important role during the demyelination process. Therefore, the presented experimental and analytical
513 framework will aid future investigations into oligodendroglial Ca²⁺ signaling during demyelination and
514 myelin repair. As such, it might contribute to the elucidation of Ca²⁺-related mechanisms implicated in the
515 success or failure of remyelination in demyelinating diseases such as MS.

516 **Materials and Methods**

517 **Experimental animals**

518 All experiments followed European Union and institutional guidelines for the care and use of laboratory
519 animals and were approved by both the French ethical committee for animal care of the University Paris
520 Cité (Paris, France) and the Ministry of National Education and Research (Authorization N° 13093-
521 2017081713462292). They were performed with male and female *Pdgfra*^{CreERT(+/-)};*Gcamp6f*^{Lox/Lox} or
522 *Pdgfra*^{CreERT(+/-)};*Gcamp5-tdTomato*^{Lox/Lox} transgenic adult mice (7 to 9 weeks old) obtained by crossing
523 *Pdgfra*^{CreERT} (stock 018280, The Jackson Laboratory) with *Ai95(Rcl-Gcamp6f)-D* (stock 028865, The Jackson
524 Laboratory, USA) or *Gcamp5-tdTomato*^{Lox/Lox} (stock 028865, The Jackson Laboratory, USA). Animals were
525 genotyped by PCR using specific primers for Cre. These mice express GCaMP6f or GCaMP5 in OPCs and
526 their progeny upon tamoxifen injection. Tamoxifen (1 mg in miglyol oil, Caesar & Loretz, Germany) was
527 administered intraperitoneally once a day for three consecutive days starting seven days before inducing
528 demyelination by LPC injection or during week 4 of CPZ administration (Fig. 1a-c). All animals had *ad*
529 *libitum* access to food and water and were exposed to a 12 hr light/dark cycle, a controlled average
530 temperature of 21 °C and 45% humidity.

531

532 **Intracranial injections of LPC to induce demyelinated lesions**

533 Mice were deeply anaesthetized using ketamine/xylazine (100/5 mg/kg, i.p.) or isoflurane (1.5 %) and
534 headfixed in a stereotaxic frame (Kopf Instruments, USA). Before starting the surgery, mice received a
535 subcutaneous injection of buprenorphine (0.1 mg/kg) as analgesic. Throughout surgical procedures mice
536 were placed on a 37 °C heating blanket and ophthalmic dexpanthenol gel (Chauvin Bausch & Lomb GmbH,
537 France) was applied to the eyes to prevent dehydration. The skin on the head was treated with betadine
538 and lidocaine and opened to expose the skull. The head was aligned using both lambda and bregma, and
539 two 0.50 mm diameter holes were bilaterally drilled in the skull at coordinates +1.40 mm anterior from

540 bregma and +0.95 and –0.95 mm lateral from the midline. The dura was removed and a glass pipette
541 connected to a Hamilton syringe containing LPC (1% in PBS; Merck, Germany) was lowered into the brain
542 until 1.80 mm depth from the brain surface reaching into corpus callosum. The pipette rested in the brain
543 for four minutes and subsequently 0.8 μ L of LPC was injected two times with 4 minutes between
544 injections. After another 4 minutes the pipette was slowly retracted from the brain and the LPC injection
545 procedure was repeated in the other hemisphere. Skin was sutured (Mersilene[®], EH7147H; Ethicon, USA)
546 and cleaned with betadine and mice were allowed to recover at 37 °C before returning to their home
547 cage.

548

549 **Acute brain slice preparation**

550 Acute coronal slices of 300 μ m containing demyelinated lesions were prepared as previously described
551 (Mozafari et al., 2020). Brain slices were cut using a vibratome (Microm HM 650V, Thermo Scientific, USA)
552 in a chilled cutting solution containing (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH₂PO₄, 30 NaHCO₃, 20 HEPES,
553 25 Glucose, 2 urea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 CaCl₂, 10 MgCl₂ (pH to 7.3-7.4; 95% O₂, 5% CO₂)
554 and kept in cutting solution for 6 to 8 min at 34°C. Slices were transferred to a standard extracellular
555 solution at 34°C for about 20 min. Extracellular solution contained (in mM): 126 NaCl, 2.5 KCl, 1.25
556 NaH₂PO₄, 26 NaHCO₃, 20 Glucose, 5 Na-pyruvate, 2 CaCl₂, 1 MgCl₂ (pH to 7.3-7.4; 95% O₂, 5% CO₂).

557

558 ***Ex vivo* wide-field calcium imaging**

559 Demyelinated lesions were recognized under the microscope at 4x as a brighter area in corpus callosum
560 as described previously (Sahel et al., 2015). After identifying the demyelinated lesion at 4x, cells expressing
561 GCaMP6f in the lesion were visualized with a 40x water immersion objective in a wide-field microscope
562 (Olympus BX51) using a LED system (CoolLED PE-2; Scientifica, UK) and a CCD camera (ImageQ, Optimos;
563 Scientifica, UK). Excitation and emission wavelengths were 470 nm and 525 nm, respectively. The CCD

564 camera and the LED system were controlled using a Digidata 1440A interface and Pclamp10.5 software
565 (Molecular Devices, USA). The image stacks were acquired at a frame rate of 1.75 Hz with 50 ms light
566 exposure for a total duration of 240 s using Micro-manager-1.4 plugin under Fiji (version 1.53k or later)
567 (Schindelin et al., 2012). Ca²⁺ imaging during bath applications of 50 μM carbachol were performed after
568 incubating the slices for five minutes with an antagonist cocktail containing 10 μM NBQX, 50 μM AP5, 10
569 μM GABA_Azine, 1 μM TTX and 50 μM mecamylamine.

570

571 ***Ex vivo* two-photon calcium imaging**

572 Two-Photon Ca²⁺ imaging was performed using a two-photon laser scanning microscope (Otsu et al.,
573 2014). A 40x water-immersion objective (Olympus40x LumPlanFL N 540x/0.8) in combination with a 900
574 nm excitation beam from a femtosecond Ti:Sapphire laser (10 W pump; Mira 900 Coherent, Santa Clara,
575 CA) was used to image GCaMP5 in individual putative OPCs and OLs identified by their morphology. Cells
576 with round soma and numerous processes extending towards various directions were designated OPCs,
577 while cells with elongated soma's containing thicker and more T-shaped processes were designated OLs.
578 GCaMP5 was detected with Hamamatsu photon counting PMTs through an emission filter (HQ500/40,
579 Chroma). Ca²⁺ signals of individual cells were imaged in high resolution at 3.60-6.27 frames per second
580 during 99 seconds.

581

582 **Cuprizone treatment and *in vivo* microendoscopy calcium imaging**

583 One week before starting the CPZ treatment, mice (P35-P42) were subjected to GRIN lens implantation
584 surgery (Fig. 1c). Animals were prepared for craniotomy in a stereotaxic apparatus as described above,
585 and a 1 mm diameter circular cranial window was drilled around a midpoint at coordinates +1.40 mm
586 anterior from bregma and +0.95 mm lateral from the midline. Using a blunt 23 G needle attached to a
587 vacuum pump, the cortex was aspirated in the middle of the cranial window from the top of the brain

588 surface until 1.40 mm depth. A 1.0 mm diameter GRIN lens (Inscopix, USA) was then implanted at 1.60
589 mm depth, just above the corpus callosum at the motor cortex level. This placement allows imaging inside
590 this white matter region at 1.80 mm depth as the working distance of the GRIN lenses used is $\pm 200 \mu\text{m}$.
591 The lens was held in place by super glue and dental cement (Unifast, Japan) and covered with kwik-sil
592 sealant (World Precision Instruments, USA) until the baseplate surgery. Animals were allowed to recover
593 at 37°C before being transferred to their home cage.

594 For the seven days following the lens implantation procedure, animals were fed cherry or bacon
595 flavored nutragel food (Bio-Serv, France). Subsequently, we supplemented the nutragel food with 0.3%
596 CPZ (Sigma) for the 6 following weeks to induce demyelination. During week 4 of the CPZ diet, 4 daily 1
597 mg tamoxifen injections were performed intraperitoneally to induce GCaMP6f expression in
598 demyelinated areas of *Pdgfra*^{CreERT(+/-);Gcamp6f^{fl}/Lox} mice. During week 5 of the CPZ diet, animals were
599 subjected to a baseplating surgical procedure (Fig. 1c). Animals were anaesthetized with
600 ketamine/xylazine (100/5 mg/kg, i.p.) and the kwik-sil seal was removed from the lens. Animals were then
601 headfixed in a stereotaxic apparatus, and a miniscope V4 (Open Ephys, Portugal) with a baseplate
602 connected to it was held over the lens until a clear image of the brain surface under the lens was obtained.
603 We then cemented the baseplate onto the head of the mouse. A Ca²⁺ imaging stack was obtained during
604 the baseplating procedure to ensure that the miniscope could efficiently detect Ca²⁺ signals from the
605 demyelinated corpus callosum. Mice were recovered at 37°C and returned to their homecage. As a proof
606 of concept, *in vivo* Ca²⁺ imaging was performed from week 5 of CPZ-induced demyelination (Fig. 1c). Image
607 stacks were obtained in freely moving mice with a sampling rate of 10 frames per second during 7 minutes.
608

609 **Occam and post-prOccam: an automated analytical workflow for oligodendroglial Ca²⁺ imaging data**

610 The analytical processing consists of two parts performed by two distinct software pieces thoroughly
611 described in the software user manual provided as supplementary information (Supplementary Manual).

612 Briefly, the analysis is composed of two main steps: first, the image stack is processed by Occam, a
613 Fiji/ImageJ2-based plugin written in Java (Fiji/Imagej2 version 1.53k or later); second, the data output by
614 Occam is processed by post-prOccam, a software written in Python, to both refine it and perform a series
615 of calculations on the refined data. Importantly, *ex vivo* one-photon, *ex vivo* two-photon and *in vivo*
616 microendoscopic Ca²⁺ imaging stacks are processed in slightly different ways to optimize the analysis of
617 Ca²⁺ signals acquired in these different preparations (Fig. 2a; Supplementary Manual). The softwares
618 described in this report are cross-platform Free and Open Source Software (FOSS) and licensed under the
619 GNU GPLv3+ license (available at: <https://gitlab.com/d5674/occam>).

620

621 *Preprocessing: noise correction and ROI definition.* The input for Occam is an oligodendroglial Ca²⁺ imaging
622 stack as recorded by the microscopy acquisition software. The Occam software initially performs noise
623 correction steps according to the imaging condition, and then proceeds to an automatic ROI designation
624 (Supplementary Manual). ROI designation is carried out using the machine learning-based WEKA
625 Fiji/ImageJ2 plugin that performs a trainable segmentation of frames (Arganda-Carreras et al., 2017)
626 combined with a local maxima segmentation tool. A ROI as designated by Occam is defined as a vector
627 containing the mean fluorescence intensity value of the corresponding region in the different frames of
628 the stack (that is, over the acquisition time points). Such ROI vectors are indifferently called ROI traces in
629 this report. ROIs are sorted in two different classes: 1) high and medium mean pixel intensity ROIs, that
630 correspond to Ca²⁺ active regions of the stack and 2) low mean pixel intensity ROIs, that are considered as
631 background. In this report, WEKA classifiers were trained on Ca²⁺ imaging stacks obtained from GCaMP-
632 expressing oligodendroglia in demyelinated lesions of the mouse corpus callosum in one-photon, two-
633 photon and *in vivo* imaging conditions, separately. Notably, training WEKA is straightforward using any
634 given set of image stacks. Occam's user interface allows one to configure various aspects of the
635 processing. For each processed image stack, Occam produces a set of comma-separated value (CSV) files

636 that contain the description of the mean fluorescence intensity of each ROI along the time-resolved
637 acquisition experiment. These files are then fed to the post-prOccam software.

638

639 *Post-processing: automated ROI refinement and quantifications.* The post-prOccam software is a
640 configurable software that processes the files produced by Occam. Before performing a baseline
641 subtraction, false positive ROI traces of a stack are rejected by using a sliding window-based subtraction
642 of intensities in each ROI trace and applying a detection threshold defined by the mean absolute deviation
643 (MAD) value on each subtracted trace (Supplementary Manual). Only ROIs that show significant Ca²⁺-
644 fluorescence fluctuations are accepted for further analyses. A number of parameters configuring the ROI
645 processing and the filtering stringency can be set in a configuration file (Supplementary Files 1-3). The
646 accepted ROIs of a stack are output to a file and calculations that are then computed on these ROIs are
647 output to another file (such as the surface area of each ROI in pixels, the ROI integral, the sum integral of
648 all ROIs, the total ROI surface area in pixels, the sum integral corrected by the surface area of all ROIs, the
649 Pearson correlation between each ROI and every other ROI; Supplementary Manual). Of note, this analysis
650 can be performed on specific acquisition time point intervals of ROI traces by listing desired intervals in
651 the configuration file. The post-prOccam software logs all the processing steps and their outcome to a file
652 so as to let the user scrutinize the inner workings of the program.

653

654 **Statistical analysis**

655 Data are expressed as mean \pm SEM. GraphPad Prism (version 9.3.0; GraphPad Software Inc., USA) was
656 used for statistical analysis. Each group of data was first subjected to Shapiro-Wilk normality test.
657 According to the data structure, two-group comparisons were performed using the two-tailed unpaired
658 Student's t-test or the non-parametric two-tailed unpaired Mann-Whitney U test for independent

659 samples; the two-tailed paired Student's t-test was used for paired samples. Multiple comparisons were
660 done with a two-way ANOVA test followed by a Bonferroni's multiple comparison test.

661

662 **Data availability**

663 The data generated and analyzed during this study are included in the manuscript and supplementary
664 files. Occam and post-prOccam programs as well as all the software documentation and a full example of
665 ex vivo widefield imaging data are hosted at <https://gitlab.com/d5674/occam> and published under a Free
666 Software GNU GPLv3+ license.

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680

681 **Competing interests:** The authors have declared that no competing interests exist.

682

683 **Author contributions**

684 D.A.M. and B. M-S. conducted one-photon Ca²⁺ imaging experiments. C.H. performed two-photon Ca²⁺
685 imaging experiments and D.A.M. performed *in vivo* microendoscopy Ca²⁺ imaging experiments. D.A.M., B.
686 M-S., C.H. and M.C.A. designed experiments and analysis. P.B., D.A.M., B. M-S wrote the ImageJ plugin.
687 M.C.A. and F.R. designed the Python software and F.R. wrote the code. D.A.M. and M.C.A. performed data
688 analyses and D.A.M., F.R. and M.C.A. wrote the manuscript. M.C.A supervised the project.

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