Supplementary information

A combined clearing and quantitative analysis pipeline for human brain optical mapping

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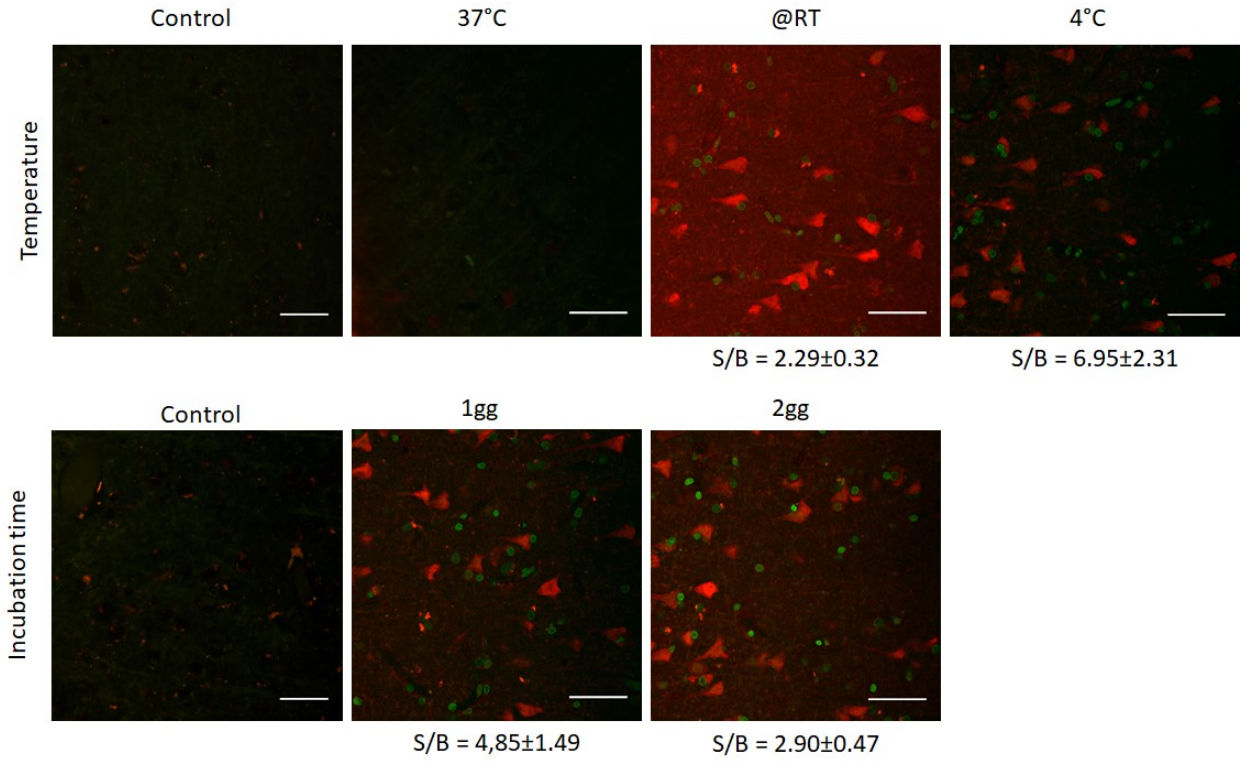
1. Immunostaining protocol optimization

Trials with different temperatures (37°C, @RT, and 4°C) and time of incubation (1 or 2 days) were performed in order to identify the best condition for optimal immunostaining. For each condition, images were acquired with the TPFM maintaining the same PMT gain and laser power. Signal to Background (S/B) analysis was performed with Fiji (http://fiji.sc/Fiji) to assess the best contrast: mean intensity of 10 square of 25px were analyzed for each condition, media and standard deviation were then calculated using OriginPro 9.0 (OriginLab Corporation). The protocol with the highest signal amplification corresponds to 4°C

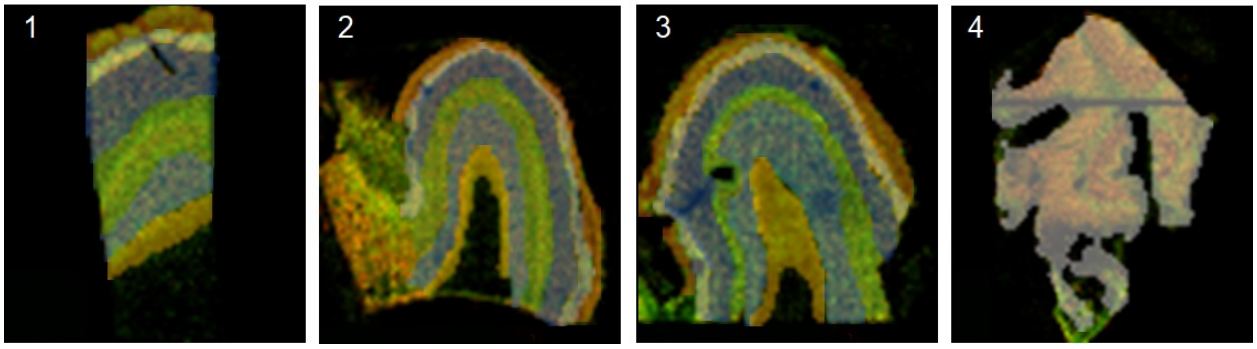
of incubation temperature and 24 hours of incubation time of the primary antibody.



Supplementary Figure 1: **Immunostaining optimization**. Representative two-photon images of tissue stained with anti-NeuN antibody (in red) and DAPI (in green) at different temperatures and incubation times. Scale bar = 50 µm.

2. Manual segmentation of the grey matter

In order to identify the different layers of the grey matter of the cortex, the maps obtained analyzing the mean volume distribution and the density distribution with a binning of 100 x 100 x 100 μm^3 were manually segmented using the software Fiji (Supplementary figure 2). Big blood vessels, tissue holes/breakages, and imaging artifacts were not reckoned drawing the masks. Sample 4 shows a disruption of the structural organization of the cortex, making layer classification impossible, only grey matter were segmented. The total volume for each mask was obtained performing this operation for all the z-plane of the sample.



Supplementary Figure 2: **Layers' assessment**. Layer masks (L1 red, L2 white, L3 blue, L4 green, L5 light blue, L6 yellow) of the middle plane of samples 1,2, and 3. Grey matter mask (in grey) is shown for sample 4.

3. Convolutional Neural Networks (CNN) parameters

The 2.5D CNN was implemented using the Aliquis® software ecosystem (https://www.bioretics.com/aliquis) version 2.4.3 with TensorFlow backend (https://www.tensorflow.org). Aliquis® is available online free of charge in capped mode.

The **network architecture** is as follows:

```
input 32x32x2 8-bit (promoted to float4 and remapped in range [0 1])
conv (relu) 32 5x5 + max pooling 2x2
conv (relu) 64 3x3 + max pooling 2x2
conv 64 3x3 (relu)
fc (relu) 128 + dropout (0.5)
fc (relu) 128 + dropout (0.5)
fc 2 (softmax)
```

The **network parameters** are as follows:

Layer name (type)	**Output**	**N. params**
conv2d_1 (Conv2D)	32	1632
max_pooling2d_1 (MaxPool 2)	32	0
conv2d_2 (Conv2D)	64	18496
max_pooling2d_2 (MaxPool 2)	64	0
conv2d_3 (Conv2D)	64	36928
conv2d_4 (Conv2D)	128	131200
dropout_1 (Dropout)	128	0
conv2d_5 (Conv2D)	128	16512
dropout_2 (Dropout)	128	0
output (Conv2D)	2	256

Total params: 205,024
Trainable params: 205,024
Non-trainable params: 0

Optimizer hyper-parameters are as follows:

```
type: SGD (mini-batch stochastic)
batch size: 256
epochs: 300
scale: 0.003921568627 (= 1/255)
learning rate: 0.01
weight decay: 0.00001
momentum: 0.9
loss: infogain_categorical_crossentropy
infogain weight matrix: 0.7; 0; 0 ;1
```

The **datasets** extracted from the raw images are as follows:

Summary **per-image dataset**:
training = 112 (80%); validation = 14 (10%); test = 14 (10%)

Summary **per-patch dataset** (number of 32x32x2 samples):
Total = 2'293'760; background 0 = 2'147'474 (94%); neuron 1 = 146'286 (6%)

Detailed **per-patch dataset** (number of 32x32x2 samples):
Training set: background 0 = 1'717'979; neuron 1 = 117'029
Validation set: background 0 = 214'747; neuron 1 = 14'629

Only the training dataset has been data-augmented.

4. CNN statistical assessment and grouping effect

4.1 CNN statistical assessment

The CNN validation detailed information is listed in the attached file (2.5D assessment.zip). Data are displayed for the four representative stacks of 100 x 100 x 100 μm^3, both for the manual segmentation (GT) and CNN automatic recognition. Performances are indicated for each neuron.

The manual segmentation has been performed on LAIRA® (https://laira.bioretics.com) and stored in the Ximage open-source format (https://github.com/bioretics/ximage). LAIRA® is available free of charge in trial mode.

4.2 Grouping effect

The 3D reconstruction of the meshes obtained through the 2.5D approach is sometimes characterized by a grouping effect: neurons that are too close to each other are reconstructed as single units, as shown in Supplementary Figure 3.

Supplementary Figure 3: Example of a group. 3D rendering of two neurons manually segmented (in blue), automatically reconstructed by the 2.5D approach (in red), and the superimposition of the two renderings.

5. Videos legend

Video1: Representative stack showing the neuronal segmentation obtained using the CNN. Imaging performed with TPFM, FOV of 450 x 450 μm^2.In red NeuN neuronal staining in green DAPI nuclei staining.

Video2: Navigation in the 3D rendering of the meshes of sample 1.