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Determining the thickness of convoluted cell layers in microtomography

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Abstract: Microtomography enables the three-dimensional imaging of cellular details of the entire mouse choroid plexus (ChP). This anatomical structure is vital for regulating the fluid balance in the brain. During aging, its epithelial cells flatten while its stroma expands. Quantification of the thickness of the ChP epithelial cell layer is hampered by the folded nature of the layers. We have developed a method to automatically quantify the thickness of the ChP epithelial cell layer even in the presence of folds and cell clusters. Visual inspection and tests with a wide range of parameter settings showed that the method is robust and provides realistic results.

Keywords: microtomography, mouse brain, choroid plexus, folded cell layers, thickness quantification

1 Introduction

Synchrotron radiation-based micro computed tomography (SRμCT) allows capturing cellular details of an entire mouse brain in three dimensions (3D) [1]. This imaging technique enables the complete analysis and quantification of thin and relatively large 3D structures, such as the choroid plexus (ChP). The ChP is an important tissue localized in all brain ventricles and produces the cerebrospinal fluid (CSF) in the central nervous system (CNS). To date, the ChP structure has mainly been studied using histology, magnetic resonance imaging (MRI) and electron microscopy. These imaging techniques lack either 3D coverage or sub-micrometre resolution. Quantification was limited to volume and surface area, without distinguishing between ChP epithelial cells forming the blood-cerebrospinal fluid barrier (BCSFB) and stroma. As these micro-anatomical substructures can behave differently, with epithelial cell flattening and stroma expanding during aging [2, 3], they should be quantified separately.

A convolutional deep neural network was trained to segment the ChP epithelial cell layer and the ChP stroma in SRμCT data of a mouse brain with sub-micrometre voxel lengths. Volume and surface area can be deduced from these segmentations. However, quantifying the thickness of the ChP epithelial cell layer directly from this segmentation, e.g., using skeletonization and a distance transform, is inaccurate due to the folded layer, with many regions of contact between adjacent folds, see Figure 1. To address this, we developed a method based on individual cell segmentation that can automatically determine the ChP epithelial cell layer thickness while accounting for these folds. We also evaluated the robustness of the method with respect to its parameters.

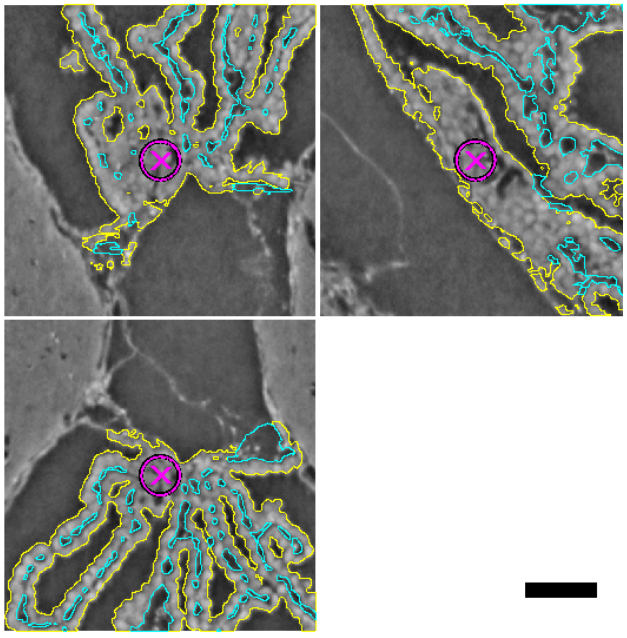

Fig. 1: Orthogonal slices centered at the largest ChP epithelial cell layer thickness (32.2 μm, magenta cross) as determined by the distance transform. The yellow and cyan contours show the deep neural network segmentation of the ChP ephithelial cell layer and the ChP stroma respectively. The scale bar is $60\,\mu\mathrm{m}$.

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2 Method

The imaged brain came from a wild-type, ten-week-old, female C57BL/6J mouse (Janvier Labs, France). The ethics committee of the Canton of Bern (licenses BE77/2018, BE73/2021) approved the tissue collection. The mouse was anesthetized with ketamine/xylazine. Thereafter, it was transcardially perfused with $20\,\mathrm{mL}$ phosphate-buffered saline (PBS) pH 7.4, followed by $100\,\mathrm{mL}$ of 4 % paraformaldehyde (PFA)/PBS. The brain was dissected and postfixed in 4 % PFA/PBS overnight. The brain was dehydrated by immersion in a $50\,\mathrm{mL}$ ascending ethanol series (50 %, 70 %, 80 %, 90 %, 100 %, 2 hours each) before storage in 100 % ethanol. All solutions were used at room temperature.

The entire mouse brain was imaged with isotropic $0.65\,\mu\mathrm{m}$-wide voxels using extended-field microtomography at the ANATOMIX beamline at Synchrotron SOLEIL (Saint-Aubin, France) as described previously [1].

The thickness of the ChP epithelial cell layer cannot be computed directly from the segmentation of the ChP epithelial cell layer, as the cell layers often touch each other, see Figures 1 and 2. Therefore, we segmented the bright ChP epithelial cells and determined the distance of the cell centroid to the border of the ChP epithelial cell layer segmentation. To avoid large distances due to cell clusters, cells closer to other cells than to the segmentation border were excluded. While this quantification is incomplete because some ChP epithelial cells are hardly visible, it enables realistic thickness measurements for touching ChP epithelial cell layers.

In detail, the image intensities within the ChP epithelial cell segmentation were rescaled from their minimum-maximum floating-point range to integer values ranging from 0 to 255. The cell segmentation consisted of thresholding this normalized masked image I_M using a sequence of thresholds from θ_1 to θ_N in intensity steps, i.e., of $\Delta\theta = 3$. The θ_1 and θ_N values were set, respectively, to the 25th and the 99th percentile intensity value in the segmentation I_M. Per threshold θ_n, the extracted connected regions were accepted if they met the following volume and shape constraints:

minimum volume	$4/3\pi(5.20/2)^3\ \mu\mathrm{m}^3$
maximum volume	$4/3\pi(20.15/2)^3\ \mu\mathrm{m}^3$
maximum elongation	4.50
maximum non-convexity	1.80

where elongation was determined by the ratio between the longest and shortest length of the principle axes of the ellipsoid with the same second central moments as the region. Non-convexity was calculated as the ratio between the volume of the region's convex hull to the volume of the region. Default parameter settings were based on visual inspections of initial tests.

All accepted regions were accumulated in a binary image and then checked again for volume and shape compliance to obtain the final binary mask M_C. Per cell segmentation, we calculated (i) the shortest distance r_B from the cell centroid to the ChP epithelial cell segmentation border using a distance transform, and (ii) half the distance to the nearest neighbouring cell centroids (r_N). Only cells where $r_B \leq r_\mathrm{N}$ were accepted for thickness measurements to avoid overestimating the thickness of the ChP epithelial cell layers in clusters of cells.

The method was evaluated by one of the authors by visually inspecting orthogonal slices of the three cells closest to the minimum, maximum, median and mean results. Furthermore, the influence of changing the default parameters on the extracted median and mean thickness values was tested for statistically significant differences at the 0.001 level using the Wilkson rank sum test resp. the two-sample t-test after randomly sampling 1000 values from each distribution to support independence of the samples.

3 Results

The proposed method was applied to the ChP epithelial cell layer in the third ventricle. Figure 2 shows orthogonal slices of the segmented ChP epithelial cell layer centred at a detected cell close to the median and maximum thickness (cell centroid marked by a magenta cross). It can be observed that the corresponding magenta sphere, which has a diameter equivalent to the extracted thickness, reaches to the border. Several folded layers are also visible. Visual inspection showed that the extracted cells stayed within a single layer as wanted.

The derived thickness values were consistent and realistic contrary to the measurements from the centre surface to the segmentation border, see Figure 1. Results were robust to changes of the geometrical constraints and the multi-level thresholds, see Tables 1 and 2 and Figure 3. Mean and median values were not statistically significantly different at the 0.001 level.

Fig. 4 shows the spatial distribution of all extracted ChP epithelial cells. The third ventricle is well covered. No particular spatial pattern in terms of thickness is recognisable.

4 Conclusion

We have developed a robust method for quantifying the thickness of the convoluted ChP epithelial cell layer, which has many touching layers. The proposed method will be applied to all ventricles and to a larger population of mice.

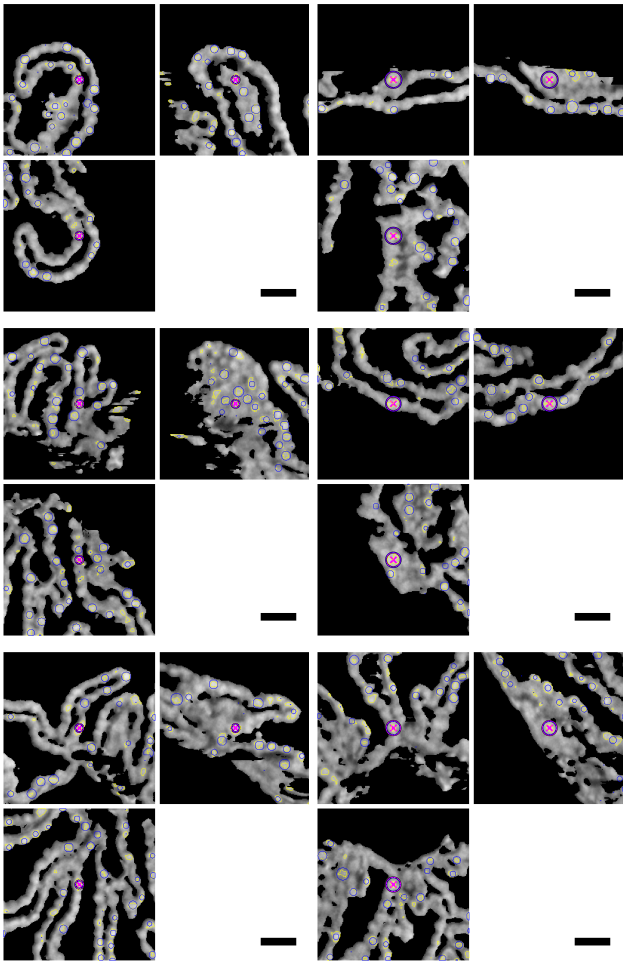

Fig. 2: Orthogonal slices of image within ChP epithelial cell layer segmentation centered (magenta cross) at results close to (left) the median and (right) the maximum thickness. Cell segmentations are shown as yellow contours. The associated sphere, with a diameter corresponding to the layer thickness is shown by blue contours. The scale bars are $30\,\mu\mathrm{m}$.

Tab. 1: Statistics of ChP epithelial cell layer thickness for the selected intensity threshold settings in the range of -40% to 40% from the default values, i.e. θ_1 = 25th percentile and $\Delta\theta$ = 3. #cells: number of extracted ChP epithelial cells.

Setting			Thickness [μm]				
θ_1	$\Delta\theta$	#cells	1st	Med	Mean	SD	99th
15th	2	25401	2.86	7.02	6.87	1.53	10.27
20th	2	25399	2.86	7.02	6.87	1.53	10.27
25th	3	24133	3.18	7.00	6.88	1.52	10.24
30th	4	22985	3.12	7.02	6.89	1.53	10.27
35th	4	22975	3.12	7.02	6.89	1.52	10.27

Tab. 2: Statistics of ChP epithelial cell layer thickness for the selected elongation and non-convexity parameter settings in the range of -30% to 30% from the default values, i.e. 4.50 for elongation (El) and 1.80 for non-convexity (nC). #cells: number of extracted ChP epithelial cells.

Setting			Thickness [μm]				
El	nC	#cells	1st	Med	Mean	SD	99th
3.15	1.26	8742	3.90	6.76	6.88	1.39	10.14
3.60	1.44	18820	3.64	7.02	6.91	1.44	10.14
4.50	1.80	24133	3.18	7.00	6.88	1.52	10.24
5.40	2.16	25087	2.86	6.76	6.78	1.61	10.27
5.85	2.34	25133	2.60	6.76	6.75	1.64	10.27

Author Statement

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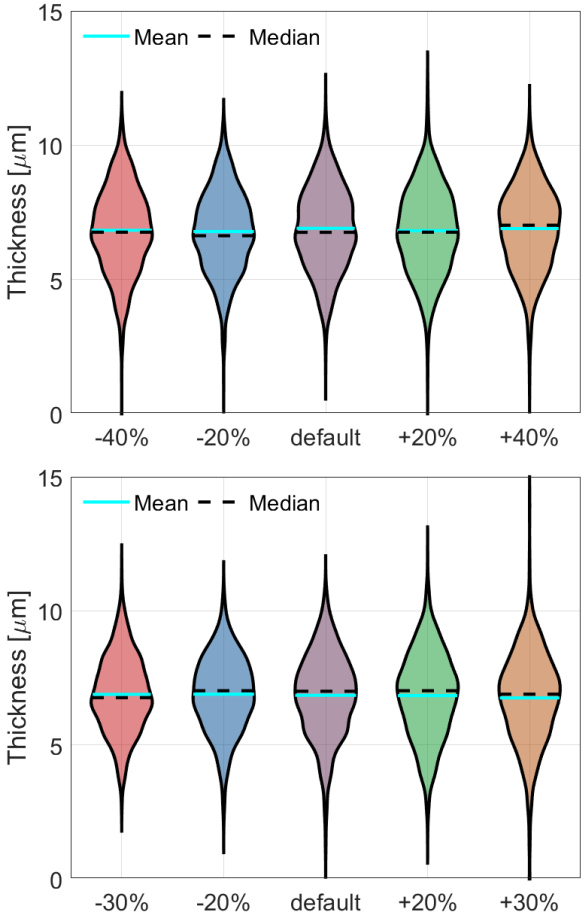

Fig. 3: Distributions of ChP epithelial cell layer thickness for 1000 random samples. (top) Multi-level intensity threshold parameters were changed in the range from -40% to 40% from the defaults, see Table 1. (bottom) Elongation and non-convexity parameters were changed in the range from -30% to 30% from the defaults, see Table 2.

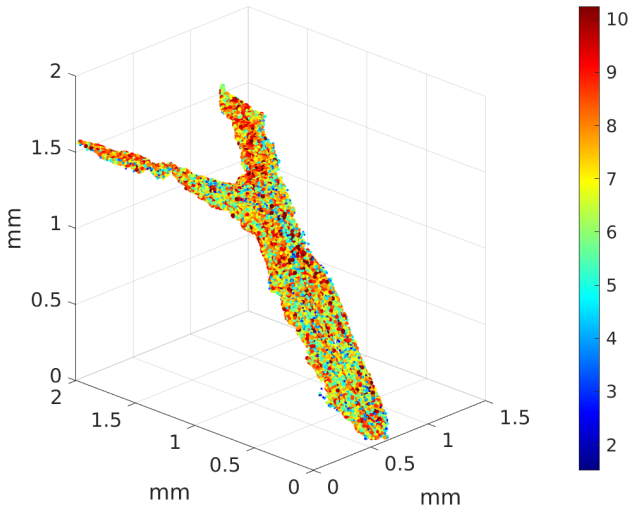

Fig. 4: Spatial distribution of detected ChP epithelial cells in third ventricle. Color encodes corresponding thickness in μm.