

Analysis of Landmark-Based Coordinates for Mouse Intracerebroventricular Surgery Using In Vivo X-Ray Microtomography



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Abstract Stereotaxic surgery on rodents is a delicate procedure essential for research on central nervous system physiology and pathology. Surgeons generally rely on post-mortem-based atlases that only represent the cerebrospinal fluid spaces in collapsed state. This synchrotron radiation-based in vivo tomography study with 12-week-old C57BL/6J mice enabled the extraction of the cerebrospinal fluid space and relevant stereotaxic landmarks to optimize the intracerebroventricular infusion. For this purpose, we injected a contrast agent into the right lateral ventricle. Its temporal distribution could be followed by a series of tomograms acquired at the BMIT-ID beamline of the Canadian Light

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Source. The segmented cerebrospinal fluid spaces and the semi-automatically determined stereotaxic landmarks were rigidly aligned to an anatomically oriented reference frame. For 14 mice, the 3D coordinates within the left lateral ventricle with the maximum distance to its surface were correlated to the animal's weight and sex. The stereotaxic coordinate frame showed an overlapping volume of 0.85 mm^3 of the left lateral ventricles. The optimized coordinates were $90 \mu\text{m}$ deeper for males with respect to females. Therefore, separate sets of stereotaxic coordinates for ventricular infusion should be considered for female and male mice with the aim to improve the success of the procedure.

Keywords Intracerebroventricular surgery · Cerebrospinal fluid · Synchrotron radiation · Microtomography · Brain coordinate systems · Image registration · Stereotaxy

1 Introduction

Intracerebroventricular (ICV) injection or infusion in rodents has been used for administration of, e.g., drugs [1], T-cells for cell therapy of neurological disorders [2], streptozocin or amyloid- β to create an Alzheimer's disease model [3, 4], and tracers to observe cerebrospinal fluid (CSF) clearance [5, 6]. Recently, *in vivo* synchrotron radiation-based X-ray microtomography employing ICV infusion of contrast agent through a radiotransparent cannula has been proposed for observing mouse CSF dynamics [7].

Common to these applications is the need for a targeted placement of a cannula or needle into the CSF space, based on externally accessible spatial reference points and knowledge of CSF space morphology. Common practice for rodents is to target one of the lateral ventricles and use skull landmarks. Once the orientation of the rodent's head has been fixed with respect to a reference coordinate system based on these landmarks, stereotaxic surgery allows precise navigation and placement of the cannula. The target coordinates for cannula placement have been reported to be established either from experience [1], or based on a reference atlas [2, 4]. The commonly referenced atlases [8, 9] were created using *ex vivo* imaging modalities, thus based on CSF spaces captured in a collapsed state. These might not, or only imprecisely, reflect the *in vivo* geometry.

To overcome this limitation, a map of the cerebral ventricles from *in vivo* imaging relative to the used skull landmarks is required. For targeting the lateral ventricle in rodents, the bregma and lambda landmarks generally serve as references [1]. This study employed *in vivo* synchrotron radiation-based microtomography (SR μ CT) and ICV infusion of X-ray contrast agent to image the mouse CSF spaces and the skull in a single acquisition at $8 \mu\text{m}$ isotropic voxel size. Intensity-based rigid registration was used to align tomography scans from 18 individuals along a common anatomically oriented reference frame. The bregma and lambda landmarks were determined with a semi-automatic approach [10, 11]

and formed the basis for a common stereotaxic coordinate system. Based on a segmentation of the left and right lateral ventricles, the point within the lateral ventricle with the largest distance to the ventricle surface was determined, both for each individual and on the intersection of segmentations for groups of either all individuals or individuals of the same sex. This is proposed as the ideal target location for ICV infusion, as it offers the largest margin of error.

2 Methods

2.1 *Surgery and Acquisition*

Eighteen 12-week-old C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). The mice underwent tracheotomy for artificial ventilation before stereotaxic surgery. After placing a 28G PEEK cannula (328OP/PK/Spc, Bilaney Consultants, Düsseldorf, Germany) into the right lateral ventricle, 5 μ L of ExiTron nano 12000 X-ray contrast agent (nanoPET Pharma, Berlin, Germany) was infused. Initially, the cannula was placed 0.95 mm distal, 0.22 mm caudal, and 2.30 mm ventral relative to the bregma. These coordinates were adopted from literature [12, 13], and denoted as Standard. Three datasets result from the use of Standard coordinates (CA004, CA005, CA009). The infusion location was adjusted during the experiments based on the microtomography data, with the new set of coordinates denoted as Modified. They were 1.20 mm distal, 0.03 mm rostral, and 2.30 mm ventral relative to the bregma. In one instance (CA023), the ventral coordinate was adjusted to 2.20 mm.

Tomographic imaging was performed at the BMIT-ID beamline of the Canadian Light Source in Saskatoon, Canada [14]. A monochromatic beam at 37.8 keV was used, with 0.6 m sample to detector distance. The detector system consisted of a 200 μ m LuAG scintillator, a tandem lens system, and a PCO Edge 4.2 sCMOS camera, producing an effective pixel size of $8 \times 8 \mu\text{m}^2$. 2000 projections were acquired over 180° , with 15 ms exposure time per projection. The tomograms were reconstructed using the Tofu toolkit [15].

A detailed description of the surgical procedures and the custom-designed setup for in vivo imaging are given in [7]. Protocols for animal experiments were approved by the University of Saskatchewan Animal Research Ethics Board (Animal Use Protocol no. 20230072) on September 26, 2023, in compliance with the Canadian Council on Animal Care guidelines.

2.2 *Landmark Detection and Alignment to Common Stereotaxic Reference Frame*

A tomogram acquired in the same fashion for an additional mouse not included in the further analysis steps was used as a template for anatomical skull

alignment. This dataset was manually aligned along anatomical directions. A dilated mask of the skull was created for image registration by applying a 3-voxel-radius median filter for reduction of noise, manual thresholding, extraction of the 20 largest connected components, manual exclusion of those components not belonging to the skull, and morphological dilation by a 15-voxel-radius sphere. To align a skull along the midsagittal plane, the image was aligned to its mirror image using intensity-based rigid registration with Elastix (version 5.0.1) [16, 17], optimizing the normalized correlation metric within the dilated skull mask to produce a transformation matrix T , and then transforming the volume with $\sqrt{T}$.

Subsequently, the tomograms of 18 mice were aligned first to the template and then along the midsagittal plane. The process is illustrated in Fig. 1. Alignment to the template employed rigid intensity-based registration in the neighborhood of the skull, using the dilated skull mask of the reference image. To further refine alignment of the skull to the anatomy-based coordinate system, the images were oriented along the midsagittal plane by registration to a mirrored copy, as

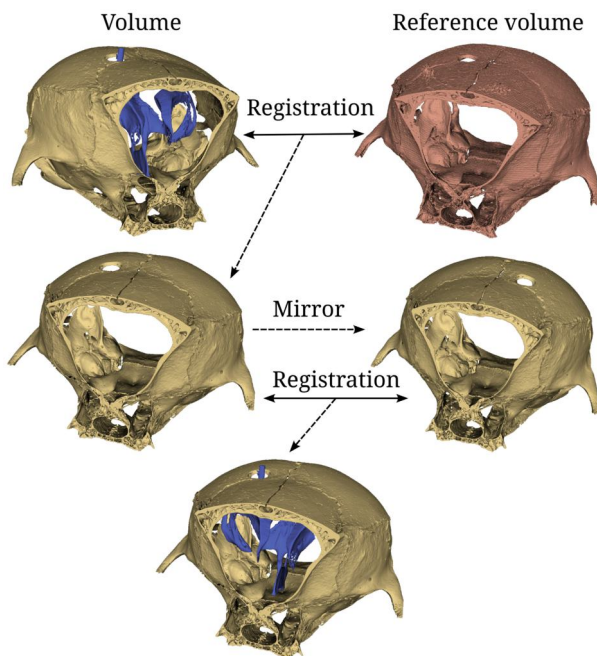


Fig. 1 Registration to a template and determination of the midsagittal plane to get the skulls anatomically aligned. The tomograms are rigidly aligned with the template, optimizing the normalized correlation metric within the skull region of the template. For alignment along the midsagittal plane, the volume is registered to its mirror image, again based on intensity values within the dilated skull mask. The contrast agent-filled ventricles are included in the initial and final state for illustration, the registration however was always based on intensities in the neighborhood of the skull

described above for the reference volume. The skull and the cerebrospinal fluid (CSF) spaces were segmented using manual thresholding. In few cases, the labels had to be amended manually to separate bone from CSF. The 20 largest connected components were identified and assigned to the skull, the CSF spaces, or were discarded.

To determine the location of the landmarks bregma and lambda on the skull, the height profile of the segmented skull in dorsal direction was computed after anatomical skull alignment, i.e. the distance of the first foreground element from the top of the volume, see Fig. 2b. Per individual, a rectangular region of the height map was manually defined per relevant skull suture, see Figs. 2c, e. The sutures were extracted by first removing the uneven background due to the surface curvature by applying a top-hat transform with a disk-shaped structure element with 50 pixel radius [18], and then thresholding using Otsu's method [19]. This gave the segmentations of the lambdoid and coronal sutures, as shown in Fig. 2d, f, respectively. The positions of the point landmarks bregma and lambda in the horizontal plane were determined as the intersections of a curve fit through the segmented sutures with the midsagittal line. A linear fit through the coronal suture was used to determine bregma, and a quadratic fit through the lambdoid suture for lambda. The dorsal-ventral position of the landmarks was determined manually, such that they lay on the skull's outer surface.

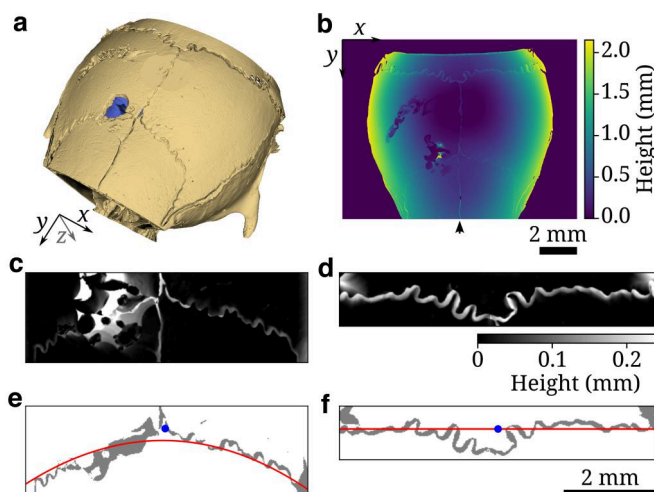


Fig. 2 Semi-automatic detection of the landmarks bregma and lambda on the anatomically oriented skull. (a) Volume rendering of the skull (beige) and contrast agent (blue). (b) Height map in dorsal-ventral direction measured from the top. 0 mm 2.16 mm The midsagittal line is marked by an arrow. (c) Region of the height map for fitting the lambdoid suture. (d) Linear fit (red) through a segmentation of the lambdoid suture to determine the lambda landmark (blue). (e) Region of the height map for fitting the coronal suture. (f) Quadratic fit through a segmentation of the coronal suture (red) to determine the bregma landmark (blue). 2 mm

Together with the midsagittal plane, the two landmarks are sufficient to define origin and orientation of the stereotaxic reference frame, as the plane fixes two of the rotational degrees of freedom, the first point determines the translation, and the second point the remaining rotation. The convention used here is to place the origin at the bregma, the x -axis orthogonal to the midsagittal plane pointing left, the negative y -axis passing through lambda in the caudal direction, and the z -axis in ventral direction, see Fig. 2a. All 18 volumes were transformed to this common stereotaxic coordinate system.

2.3 *Ideal Infusion Location*

The cannula for infusion of contrast agent was surgically implanted using a stereotaxic frame. The infusion generally is not always successful, despite targeting the same coordinates for cannula placement. Therefore, the in vivo ventricle morphology derived from microtomography was used to identify the ideal infusion location, promising the most reliable coordinates for successful infusion. The ideal infusion location here was specifically defined as the 3D target coordinates for cannula insertion with the highest chance of placement within the lateral ventricle assuming a uniform random deviation in any direction. This was determined based on the segmentation of the contrast agent-filled ventricles.

As the right lateral ventricle was subject to deformation caused by the cannula insertion, the analysis was performed on the left, contralateral side. Final results were then transformed to the right hemisphere, to allow a comparison with the actual cannula placement. This assumes that the left and right cerebral hemispheres are symmetrical, as done in the established atlases [9, 20]. The ventricles were filled with a sufficient amount of X-ray contrast agent to allow segmentation in 14 of 18 animals. For each of those 14 mice, the point within the left lateral ventricle with the maximum distance to the ventricle surface was determined by applying the distance transform to the segmentation and finding the maximum. These points were transformed to the right hemisphere by mirroring across the midsagittal plane, representing the ideal infusion locations per individual. To determine a globally ideal infusion location for the sample of 14 animals, the same method was applied to the intersection of all ventricle segmentations, and then again for two groups separated by sex, six female and eight male individuals. To test the generalizability of this approach, leave-one-out cross-validation was performed, i.e. the maximum distance location was computed on the intersection of all but one of the segmentations in a group, and the left-out dataset served to check whether this location was within the segmentation. The influence of weight and sex on skull size was examined using linear regression and statistical significance tests at the 0.05 level, employing Welch's t -test. Differences in the ideal 3D infusion location were tested for statistical significance at the 0.05 level using Hotelling's t -squared test. As Hotelling's assumes normality of the underlying distributions and this was difficult to verify given the sample size, it was complemented with a permutation

test [21]. Specifically, coordinates from all mice were pooled, and partitions into two groups of 6 and 8 individuals were drawn randomly 10 000 times. Hotelling's t^2 was determined for each partition, and the fraction of cases with t^2 greater than the value observed for the actual partition gave the p -value.

To quantify the benefit of utilizing a separate set of infusion target coordinates for female and male mice, the distance from the ventricle surface to each of the three intersection-derived ideal infusion locations (female, male, and all animals) was determined for all mice. A larger distance corresponds to a higher error margin for successful cannula placement. Additionally, the distance to the ventricle surface was determined for the coordinates adopted from literature (Standard).

2.4 Cannula Tip Location

The placement of the infusion cannula in the stereotaxic reference frame was determined by manually localizing the center of the cannula cross-section at the tip and at the entry point through the skull surface.

The inclination of the cannula with respect to the vertical was determined from the tip and entry point coordinates. This consisted of two angles, the rotation about the x -axis θ_x , for the projection of the cannula direction onto the plane $x = 0$, and the rotation about the y -axis θ_y , for the projection of the cannula direction onto the plane $y = 0$. The sign of $\theta_{x,y}$ was defined by the right-hand rule about the positive x and y axes.

3 Results

As an indication of skull size, the distance between the landmarks bregma and lambda was recorded and the correlation with weight and sex was examined, as shown in Fig. 3. Linear regression showed a weak positive correlation with weight, with Pearson correlation coefficient $R = 0.20$. In female mice, the mean bregma-lambda distance was 4.46 ± 0.20 mm and in male mice 4.52 ± 0.15 mm. The difference was not statistically significant, with $p = 0.48$ in Welch's t -test.

The three-dimensional distribution of the ideal infusion location among individuals, determined based on the ventricle segmentations, is shown in Fig. 4, and the mean values are listed in Table 1. The ideal mean infusion location from the individuals differed statistically significantly between female and male animals, with $p = 0.009$ in Hotelling's t -squared test and with $p = 0.017$ in a permutation test using the same statistical hypothesis test. Based on the individual results, the ideal infusion location for male mice was on average $90 \mu\text{m}$ more ventral, $55 \mu\text{m}$ more rostral, and $43 \mu\text{m}$ more medial than for female mice.

In addition to determining the point with the maximum distance to the ventricle surface in the lateral ventricle of each individual, this was also computed on the

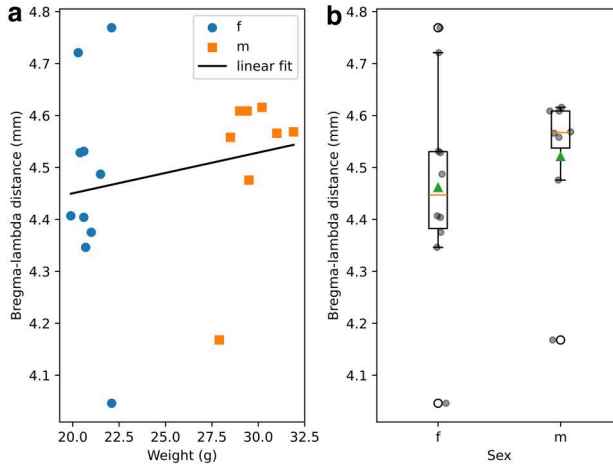


Fig. 3 Distance between the landmarks bregma and lambda relative to animal weight and sex. **(a)** The bregma-lambda distance showed a weak positive correlation with weight ($R = 0.20$). Within animals of the same sex, a moderate positive correlation ($R = 0.53$) was observed for male mice, but a weak negative correlation ($R = -0.17$) for female mice. **(b)** Data points (gray dots) with overlaid box plot of the bregma-lambda distance depending on sex. It shows the mean (green triangle), median (orange line), first to third quartile (box), minimum and maximum within $1.5 \times$ the interquartile range from the box (whiskers), and outliers. The mean difference of $60 \mu\text{m}$ between the groups is not statistically significant ($p = 0.48$ in Welch's t -test)

intersection of ventricle segmentations, once for all animals, and once grouped by sex. These locations are also reported in Table 1 and show larger differences, i.e. for males about 0.20 mm more ventral, 0.29 mm more rostral, 0.17 mm more medial compared to females. Additionally, the overlap of segmentations is shown in Fig. 5 in form of a consensus map, counting the number of coinciding ventricle segmentations in each voxel. The leave-one-out cross validation (LOOCV) had a 100% success rate when applied within each of the three groups, namely female, male, and all animals, meaning that the determined infusion location was within the ventricle segmentation of the left-out individual in all cases.

The distance from the ideal infusion location to the ventricle surface was determined for each of the three intersection-derived ideal locations (female, male, and all animals). In female animals, the distance to the ventricle surface from the female-specific location was 7.5% or $13 \mu\text{m}$ larger on average compared to the non-specific location. In male animals, however, the distance to the ventricle surface from the male-specific location was 6.1% or $11 \mu\text{m}$ smaller on average compared to the non-specific location. Applying the female ideal location to male mice and vice versa led to a 8.4% and 9.0% decrease relative to the non-specific location, respectively. The ideal infusion location derived from the collection of all animals had on average a $65 \mu\text{m}$ or 57% greater distance from the closest ventricle surface than the target location denoted as Standard.

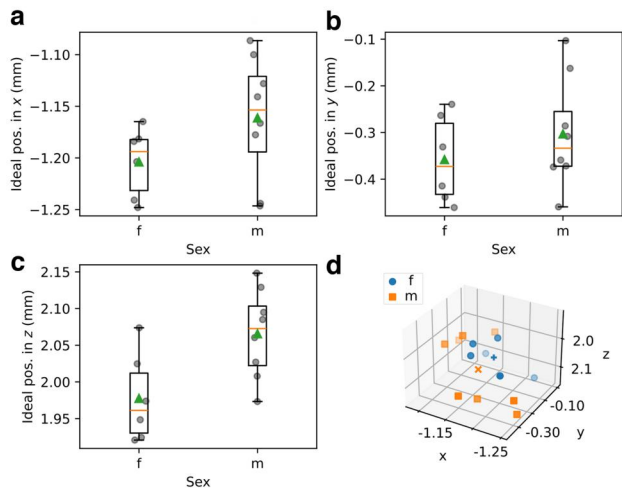


Fig. 4 Distribution of the ideal infusion location, defined as the point within the left lateral ventricle of each individual with the maximum distance to the ventricle surface. The points have been transformed to the right hemisphere, to be comparable with the coordinates used in surgery and actual cannula placement. (a–c) Data points (gray dots) with overlaid box plots of the ideal infusion coordinate grouped by sex along the three orthogonal directions. They show the mean (green triangle), median (orange line), first to third quartile (box), minimum and maximum (whiskers). $1.5 \times$ Coordinates are given in the stereotaxic reference frame, i.e. the origin is at the bregma, the x -axis points to the left, the y -axis runs rostrally, and the z -axis is in ventral direction. (d) Three-dimensional distribution of the ideal infusion location, with markers + and $\times$ indicating the mean location for female and male animals, respectively

For the surgery performed for this study, a systematic tilt of the infusion cannula was observed. While the rotation angle about the caudal-rostral axis θ_y varied around zero by a maximum of 5° , the angle about the right-left axis θ_x was consistently between 5 and 10° , resulting in the cannula tip landing 0.2 – 0.35 mm more caudal compared to the entry point, as shown in Fig. 6a. Figure 6b shows the distribution of the 18 cannula tip locations at the height of the infusion target, categorized by success of infusion. The infusion was categorized as a full hit, if no contrast agent was visible in the brain parenchyma. A partial hit signals some contrast agent in the parenchyma, but an adequate filling of the ventricle. The cannula was categorized as being out, if no or an insufficient amount of contrast agent perfused the ventricle.

4 Discussion

SR μ CT allowed capturing the in vivo morphology of the mouse cerebrospinal fluid spaces with one order of magnitude smaller voxels in each dimension compared to in vivo MRI [13, 22]. This made it possible to show differences in the mean

Table 1 Ideal infusion target locations, where x points left, y rostral, and z ventral, relative to the origin at the bregma landmark

Method	Group	x (mm)	y (mm)	z (mm)
Standard		-0.95	-0.22	2.30
Modified		-1.20	0.03	2.30
Individual	Female	-1.20 ± 0.03	-0.36 ± 0.09	1.98 ± 0.06
Individual	Male	-1.16 ± 0.06	-0.30 ± 0.12	2.07 ± 0.06
Individual	All	-1.18 ± 0.05	-0.33 ± 0.11	2.03 ± 0.07
Intersection	Female	-1.22	-0.39	1.95
Intersection	Male	-1.06	-0.10	2.15
Intersection	All	-1.09	-0.17	2.09
LOOCV	Female	-1.19 ± 0.06	-0.36 ± 0.06	1.97 ± 0.04
LOOCV	Male	-1.07 ± 0.05	-0.12 ± 0.09	2.14 ± 0.03
LOOCV	All	-1.09 ± 0.01	-0.18 ± 0.03	2.09 ± 0.01

The coordinates initially used for surgery, denoted as Standard, were adopted from the literature [12, 13]. They were adjusted during the experiments based on the microtomography data, with the new set denoted as Modified. These are listed alongside the results for the ideal infusion location determined from the individuals (mean $\pm$ standard deviation grouped for female, male and all), for the intersection of ventricle segmentations based on females, males and all. Generalization to unseen individuals was evaluated based on intersections from leave-one-out cross validation (LOOCV)

ventricle geometry, e.g., between female and male mice, on the order of $100 \mu\text{m}$, which would otherwise not be spatially resolved.

The distance between bregma and lambda in male C57BL/6J mice has been reported as $4.31 \pm 0.31 \text{ mm}$ for a sample of five 13-week-old mice [23] and as $4.80 \pm 0.15 \text{ mm}$ for a sample of twelve 10-week-old mice [11]. The value obtained here for eight 12-week-old male mice was $4.52 \pm 0.15 \text{ mm}$, lying in between the two cited results. A recent study in 26 female ≥ 8 -week-old C57BL/6 mice showed no statistically significant linear correlation between weight and bregma-lambda distance [24]. This suggests that weight is not a good indicator for skull size, and by extension for brain dimensions. From this standpoint, a scaling of stereotaxic infusion coordinates depending on the animal's weight has no empirical basis.

The landmarks bregma and lambda were defined as the intersection of the best fit line through the coronal and lambdoid suture with the cranial midline [9, 11]. Some works instead use the intersection with the sagittal suture [25, 26], which for the case of bregma has been observed to lead to larger stereotaxic errors [10].

Leave-one-out cross validation showed generalizability of the ideal infusion location approach. It demonstrated that the determination of the ideal infusion location on the intersection of ventricle segmentations was robust against the variations in ventricle geometry that were represented in the sample of 14 scans with successful ventricle infusion. It is possible that these do not capture all variations. On one hand, this is due to the limited sample size. On the other hand, it's possible that there is a variation in the geometry that makes infusion

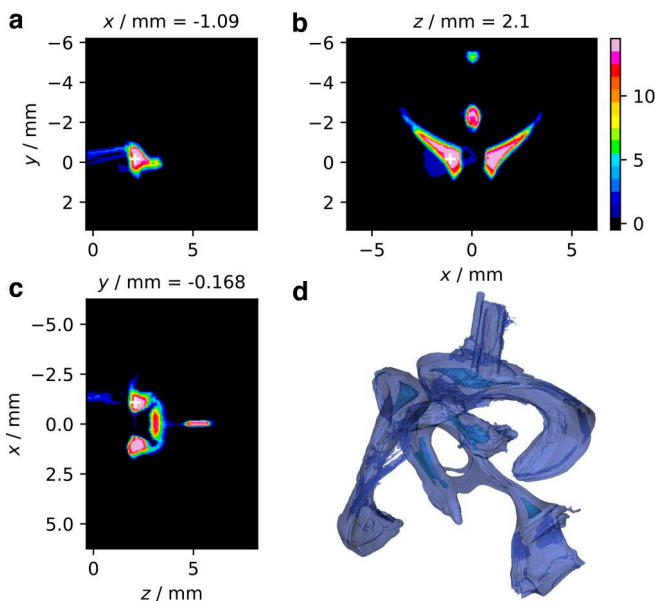


Fig. 5 The ideal infusion location was determined as the maximum of the distance transform on the intersection of all ventricle segmentations in the stereotaxic coordinate system (origin at bregma, x points left, y rostral, and z ventral). (**a–c**) Orthogonal slices through the consensus map of ventricle segmentations on 14 individuals, with the white cross marking the ideal infusion location. The colorbar indicates the number of coinciding ventricle segmentations at each point. (**d**) Volume rendering of the union (outer volume) and intersection (inner volume) of all ventricle segmentations

success at the locations chosen for the surgery less likely. This would mean that this variation is underrepresented in the 14 of 18 animals. Considering that the spread of cannula tip positions was twice the spread of the ideal infusion locations, it seems though that this is the more likely cause for failed infusions. To rule out a potential bias, future studies should devise a method for including also the cases with insufficient ventricle infusion.

The identified ideal infusion location based on the intersection of segmentations was 0.35 and 0.15 mm more dorsal compared to the coordinates adopted from literature (Standard) for female and male mice, respectively, and 0.27 and 0.11 mm more lateral. In female animals, it was additionally 0.17 mm more caudal, while in male animals it was 0.12 mm more rostral. Implanting the ICV cannula deeper than the center of the lateral ventricle may be necessary to apply enough pressure to ensure that the ventricle wall is penetrated, especially with polymeric cannulas with a blunter tip compared to metal needles. Therefore, selection of a more ventral target coordinate, e.g., the cited 2.30 mm, is warranted. After three surgeries, the target coordinates were changed based on experimental outcomes (Modified). The lateral change brought them closer on average to the ventricle center, and the rostral change negated the effect of the cannula tilt that was observed.

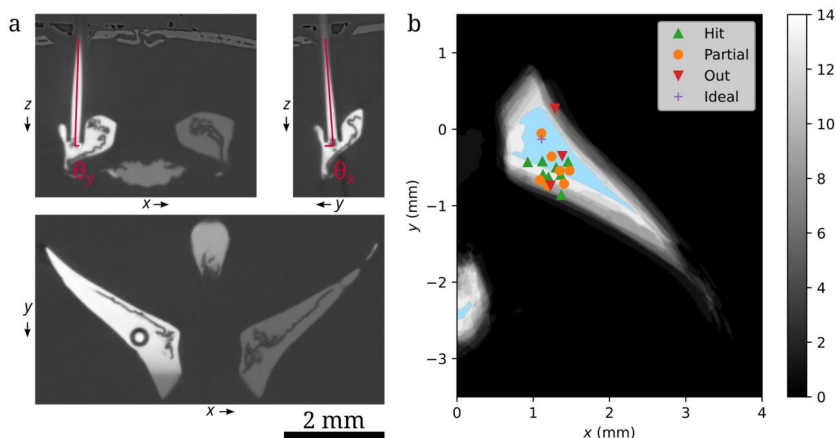


Fig. 6 Results of the surgery. **(a)** Orthogonal slices in stereotaxic coordinates (origin at bregma, x points left, y rostral, and z ventral) of the right lateral ventricle in an exemplary dataset (CA004), showing in red the projected cannula direction and the inclination with respect to the vertical. An inclination θ_x between 5 and 10° was observed consistently, while θ_y varied around zero by a maximum of 5° . This resulted in a deviation of 0.2 – 0.35 mm in caudal direction at the cannula tip relative to its entry point through the skull. **(b)** Horizontal slice through the consensus map of ventricle segmentations at the height of the ideal infusion location based on the intersection of all 14 segmentations. The colorbar indicates the number of coinciding ventricle segmentations at each point. The intersection is highlighted in light blue. The cannula tip locations of all 18 mice were projected onto this plane and categorized by success. The infusion was categorized as a full hit, if no contrast agent was visible in the brain parenchyma. A partial hit signals some contrast agent in the parenchyma, but an adequate filling of the ventricle. The cannula was categorized as being out, if no or an insufficient amount of contrast agent perfused the ventricle

The ideal infusion location derived from the intersection of ventricle segmentations changed by $291\ \mu\text{m}$ and $97\ \mu\text{m}$ when considering only female and only male animals, respectively, instead of all animals. On the other hand, the average distance to the ventricle surface increased only by $13\ \mu\text{m}$ or 7.5% for female animals and even decreased by $11\ \mu\text{m}$ or 6.1% for male animals for the sex-specific instead of the non-specific ideal infusion location. The evidence supporting separate ideal infusion locations for female and male mice is thus inconclusive.

5 Conclusion

The point within the intersection of the lateral ventricle segmentations with the largest distance to the ventricle surface, determined here on 6 female and 8 male mice, is proposed as target location for intracerebroventricular infusion. A precise placement of the infusion cannula to this location would have led to 100% success

in those 14 animals. It would increase the margin of error for cannula placement by 57% compared to the infusion location adopted from literature. Further studies with a larger cohort are necessary to assess the benefit of sex-specific infusion coordinates. The methodology for the detection of the bregma and lambda landmarks and establishment of the stereotaxic reference frame based on tomography images of the skull can be applied in studies focusing on other localized structures in the rodent brain. In vivo SR μ CT has proved highly suitable for the investigation of 3D CSF space morphology and when adding the temporal dimension lends itself to capturing CSF dynamics.

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Conflict of Interest All authors have no conflicts of interest.

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