Supplemental information 1: Hyena endocast volume measurements

Note: The detailed instructions given here are intended for work in Dragonfly. Other programs have the same functions but these may have other names and work slightly differently.

Basic Terminology

Transverse plane – the plane seen from above/below in the direction of the scan (equivalent to dorsal/ventral view)
Coronal plane – the plane seen from front/back in the direction of the scan (equivalent to anterior/posterior view).
Sagittal plane – the plane seen from right/left in the direction of the scan (equivalent to left and right lateral view).
Horizontal plane – the maximum length of the cerebrum including OB. Similar to transverse plane but adjusted to the brain.
ROI – region of interest

How to create and rename a ROI

In top of the Segment panel, click "New…" and write the name of the ROI and click "OK". The ROI will appear at the top of the Properties panel. There you can change the colour (click the coloured square), rename it (double click its name) and make it visible/invisible in 2D or 3D view. In order to paint or do anything else with your ROI, you need to have it selected. The selected ROI (or other data/object) will be a lighter shade of grey in the properties panel.

2D and 3D brushes

The brushes are found in the Segment panel, quite far down. To select a brush, you must first have selected a ROI in the Properties panel, and you must have selected a 2D view (to use the 2D view brushes) or 3D view (to use the 3D view brushes) by clicking somewhere in that view. It does not matter which view you choose as long as it allows you to select the brush you want. Selecting the coronal view does not prevent you from drawing in the sagittal view etc. For this project you only need to use the round and square brushes. When selecting the 2D view brushes, you must also check if it is set to "Single slice" or "Multi-slice". The former draws only in 2D, on the slice you are drawing on (i.e. one click with the circular brush draws a disc, not a sphere). The latter draws in 3D, even if you are in the 2D view, and is essentially the same as the 3D view brush, you can even draw in the 3D view with it. As far as I can tell, the only difference between the 2D view multi-slice brush and the 3D view brush is which view you have selected when you pick the brush. In order to actually paint with your selected brush, you must hold down ctrl on your keyboard, and to erase you hold down shift instead. To change the size of the brush, simply hold down either ctrl or shift and scroll with your mouse. You cannot drag your view field with the brush selected, but you can zoom in or out by pressing the scroll button and moving the mouse. To make other adjustments to your view field, you must go back to the main panel and choose one of the "Manipulate" tools.

How to create and smooth a mesh

To generate a mesh from a ROI, right click the ROI and click "3D modelling". This will open a new panel called "Dataset tools". Check that "Object" is set to the ROI you want to make a mesh from. If not, you can click it and choose the correct ROI from the dropdown list. Change the sampling if needed (see "Sampling" below) and click "Export to a Mesh". If the mesh is large, Dragonfly will need to think for a bit and the spinning circle of death will appear. Just give it a moment. If the mesh is small, it will seem like nothing happened. Go back to the properties panel. The mesh should have appeared at the top of your list of data/objects. As a default, the mesh is not visible, you have to make it visible in 2D/3D view to see it. It will likely look a bit grainy to start. To smooth it, select the mesh, and click "Smooth mesh" further down in the properties panel. You cannot undo smoothing. If you smooth it too much you have to generate a new mesh.

Sampling – The sampling is essentially how many slices in each dimension Dragonfly will use to create the mesh. Every slice (sampling 1), every other slice (sampling 2), every third slice (sampling 3) etc. The lower the sampling (higher value), the blockier the mesh will look. If the scan is of high resolution and the ROI has a large surface (e.g. a whole skull), set the sampling to 3 in x, y and z. If the mesh is created very fast you can generate a new mesh with a sampling of 2 or 1. For endocasts, a sampling of 1 usually works fine, but if you are unsure, start with a lower sampling (i.e. a higher value). If you set the sampling too high and the ROI is too intricate, Dragonfly may freeze. If it does freeze, don't try to do anything, give it a few minutes. If it is still frozen, you will have to force quit and you will lose any unsaved progress. If it succeeds but takes a very long time to generate the mesh, the mesh will likely be too large to be viewed. If you try to view a mesh that is too large, Dragonfly will ask if you want to create a decimated mesh. Click no, it's easier to make a new mesh with lower sampling.

<u>Step by step:</u>

Part 0

1. Open the folder with the specimen and create a new subfolder with your name.
2. Open Dragonfly and load the session by clicking "File > Load session…" in Dragonfly or by dragging the session file into Dragonfly.
3. Create a mesh of the endocast and adjust colour (I like grey #949494) and smoothing so that the sulci are clearly visible.

Part 1: Separating the Olfactory bulb (OB)

4. Create a new ROI and name it "test1". Select a highly visible colour that differs from the endocast mesh and ROI.
5. Use the spherical brush in ROI "test1" to draw on the endocast in 3D view where the top and bottom of the circular fissure is that you will use to isolate the OB. The circular fissure is difficult to see in the 2D views.
6. In transverse view, check how well the skull is aligned with the direction of the scan. If it differs several degrees, you will need to adjust for it. The easiest way to do this is to scroll to a slice where you can clearly see the midline of the skull, then adjust the marker for sagittal view so that it is aligned with the midline. Note how much the yaw/pitch/roll changed and apply the corresponding change to the coronal view as well. Only one of the values will change. You can type in the exact value by right clicking the yaw/pitch/roll value you wish to change. E.g. if the pitch of the sagittal view increases by 3°, then the pitch of the coronal view needs to decrease by the same. Dragonfly does not allow you to type in values higher than 90, so if the pitch of the coronal view starts off at 180°, you need to type in -3° instead. Write down the corrected coronal yaw, pitch and roll in the excel sheet. You will need these again. Write down all three values even if only one has changed. The others will change later, and you will need to get back to the same values at a later step.
7. In sagittal view, use the markings you drew in step 5 to then adjust the marker for the coronal view so that it is aligned with the circular fissure. Scroll through the slices to find the best overall fit.
8. Create a new ROI and name it "test2". Select a colour that differs from the endocast, I like to use black for this step. Make this ROI visible in 3D view.
9. In "test2", in the adjusted view that is aligned with the circular fissure, draw a single large solid circle with the 2D brush that extends well beyond the edges of the endocast. This should be visible as a thin disc in 3D view, which allows you to see exactly where the cut will be. Erase and repeat this until you are happy with where the cut falls.
10. Once you are satisfied with the position of your cut for the OB, make a copy of the endocast ROI. In the copy, use the brush tool to erase the slice of the endocast where you previously drew your cut disc. Ensure that all endocast voxels in this slice are erased (Dragonfly usually draws a little stripy in adjusted views because the voxels aren't aligned with the view, but if you wiggle the brush around a little it will erase the stripes as well).

11. Then use the “Refine regions of interest - Process islands – Remove by largest (6-connected)…” tool to remove the major part of the endocast. This ROI should now contain only the OB. Rename it “OB_specimenID” and export it into the subfolder with your name.
12. If you have the ROI selected, you will find the volume under “Statistical properties” at “Volume (labelled voxels)” in the menu on the right. If this is not set to cm3, you can adjust your unit in preferences. Write down the volume in cm3 in the excel sheet provided. NOTE: Do not confuse the “Statistical properties - Volume (labelled voxels)” with the “Basic properties - Volume”. The latter is the volume of the entire bounding box, including non-labelled voxels or empty space surrounding the labelled voxels, in this case the OB.
13. Select the unedited “Endocast” ROI and write down its volume in the excel sheet as well.

Part 2: Separating the cerebrum from the cerebellum + brain stem

14. Reset all views to normal and make another copy of the endocast ROI. Rename it “CerebrumOB”. Zoom in on the region of the tentorium in sagittal view and scroll through it to give yourself an idea of how the shape of the tentorium changes as you move away from the midline to the left or right. The tentorium provides a clear boundary between the cerebellum and cerebrum but is not straight or flat and thus does not allow for a simple flat cut like for the olfactory bulbs. You will essentially need to manually segment away the cerebellum, one slice at a time. However, this will be done as straight and consistent as possible. Scroll back to roughly the middle slice in the sagittal view. In the “Main” menu on the left, use the rotate buttons to rotate the image until the line between the rostral point of the tentorium and the dorsal bump on the basisphenoid is vertical on the screen.
15. Select the “CerebrumOB” ROI and the square 2D (single slice) brush and enlarge the brush size so that it reaches between the tips of these two bones. Scroll through the images again and adjust the angle of the image if needed to keep the best overall alignment between the rostral edge of the tentorium and the basisphenoid bump. There is no perfect way to make this cut and you will maintain the same angle throughout the segmentation, so it’s worth spending some time ensuring you are happy with the angle before you start.
16. Once you are happy with the angle, scroll back to the middle slice, align the brush with the bony landmarks (careful not to cover any part of the cerebrum just dorsal to the tentorium, you only want to remove the cerebellum and brainstem) and erase. You do not need to erase all of the cerebellum and brain stem; we will do that later. Focus on getting the ideal line between the bony landmarks. Don’t move the brush more than needed, just align, erase with one click, scroll to the next slice, make minimal adjustments when needed to maintain alignment, erase, scroll, erase, scroll etc. This erase-scroll method means the segmentation is quite fast. Once you have reached the end (the side of the endocast) scroll back to the middle and repeat for the other side of the endocast (i.e. scrolling back instead of forward).
17. Once you are done, first duplicate the ROI just in case. Then use the “Refine region of interest – Process islands – Keep by largest (6-connected)…” tool to erase the cerebellum, which should now be a separate “island”. If this didn’t work, there is still a connection somewhere between the cerebrum and cerebellum and you need to scroll through the slices to look for and erase it. If you struggle to find the connection, erase all of the cerebellum+brainstem in a few slices distributed across the endocast and try the “Process Islands” step again – then only the sections of the cerebellum that are connected to the cerebrum will remain, helping you to pinpoint the connection. Once you have erased the cerebellum and brain stem, you are left with the cerebrum + olfactory bulbs.
18. To remove the olfactory bulbs from the cerebrum, in the list of ROIs on the right, select first the “CerebrumOB” ROI and then the “OB” ROI from before. An “A-B” button will appear below the list of ROIs in the Properties panel. Double check that “A” is the cerebrum and “B” the OB, then click the button and name the new ROI “Cerebrum”. View the new ROI to check that it looks right and export it as “Cerebrum_SpecimenID” to the subfolder with your name. Write down the volume in the excel sheet provided the same way you did for the OB.
19. The volume of the cerebellum+brainstem should equal the total volume minus the OB and cerebrum. However, to confirm that you have done everything correctly, it should be measured separately. Make

a new copy of the endocast and use the “A-B” function to remove first the OB and then the cerebrum. If the resulting volume does not match that automatically calculated in the excel sheet, you have done something wrong. If it does match, export the ROI as “CB+BS_SpecimenID” into your subfolder.

Part 3: Separating the anterior cortex

20. Use the 3D brush in ROI “test1” again to draw on the endocast in 3D view where the cruciate sulcus intersects the longitudinal fissure.
21. Imagine the line that represents the maximum length of the cerebrum+OB and identify the rostral-most point of this line on the OB and the caudal-most point on the cerebrum and highlight these points with the 3D brush in “test1”.
22. In the transverse view, find the highlighted rostral-most point on the OB. Use the square brush in 2D to highlight this point again in “test1” by drawing a big square with its caudal edge aligned with the rostral-most point of the OB. You may need to do this on two or more slices if the scan resolution is high, so that you can see the resulting line in sagittal view without zooming in too much.
23. Do the same for the caudal-most point of the cerebrum. Now you can see the two lines in sagittal view.
24. Adjust the coronal view to align it with the skull (rather than scan direction), using the coronal yaw, pitch and roll you wrote down earlier in the process.
25. In sagittal view, adjust the marker line for the transverse plane so that it is aligned with the points indicated by your drawn lines, this is the horizontal plane of the endocast. Write down the yaw, pitch and roll for the horizontal plane.
26. Adjust the marker for the coronal view so that it is 90° to the horizontal plane. You will need to do this by dragging the marker rather than changing the numbers for yaw, pitch and roll, or you will lose the alignment with the skull. It helps holding up a paper or something else with a right angle against the screen.
27. Move the coronal view marker to where you drew in the position of the cruciate sulcus. Study the position of the marker line in both the horizontal and sagittal view.
28. Create “test3” ROI and set colour to black. Like with the OB, draw one great circle where you wish to make the cut. Make the ROI visible in 3D view and check if you are happy with the placement. Adjust if needed. Be thorough here!
29. Make a copy of the “Cerebrum” ROI and in that, use the brush to erase the labelled voxels on the slice where you wish to make the cut. Then use the “Refine region of interest – Process islands – Remove by largest (6-connected)…” to remove the posterior cerebrum. Check that this step worked and that what remains is indeed the anterior cortex. Export this ROI as “AC_SpecimenID” and write down the volume.
30. Make another copy of the “Cerebrum” ROI and subtract the anterior cortex using the “A-B” function. Check that the volume of this ROI is the same as that calculated by the excel sheet to ensure nothing has gone wrong somewhere. Export the ROI as “PC_SpecimenID”.