StereoInvestigator-Cleared Tissue Edition Protocol for Brodmann's areas 44/45 (Broca's area)

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General information for Broca's area:

- We are using large specimens that include the entire Broca's area (approximately 4x4x2 cm). The protocol was adapted from a smaller (2x2x2 cm) block and therefore has larger image stacks (~40-70 GB each). For efficiency, the fused tiff files from LENS were converted to jpx using MBF Bioscience's MicroFile+ conversion software (MBF Bioscience, Williston, VT, USA; see Appendix A "Converting LSFM image stacks from tif to jpx"). Compression was lossy 20:1 and the pixel dimensions (3.6 µm isotropic) were embedded in the jpx files. The original specimen, half smaller, was 3.3-µm isotropic and the dimensions had to be entered into SI-CTE (StereoInvestigator Cleared Tissue Edition) every time a tif image stack was opened
- A total of about 48 400-µm slices can be expected in a large specimen from Broca's area. Counting is done in a 1-in-6 series, with adjacent slices replacing those that are damaged or have imaging/tiling artifacts. The final series contains a systematic-random number of slices such as 6, 13, 18, 24, 30, 35, 42, 48
- In I48, Broca's area has been imaged in four channels. Contour ALL cortical layers (essentially, I, II, III, V, VI), but only count cells in a selection of layers:

Stain/Immunolabel	Objective Used for LSFM	Fluorophore Wavelength (nm)	Layers counted
DAPI	**Left**	**405**	**not informative**
NeuN	**Left**	**638**	**3, 5, 6**
Calretinin (CR)	**Right**	**488**	**2, 3**
Somatostatin (Sst)	**Right**	**561**	**3, 5, 6**

- DAPI and calretinin were imaged together; NeuN and somatostatin were the other pair
- Contour all layers of NeuN first and make copies of the dat files to use for adjusting contours and counting CR and SST. Use the contour-only dat files to export the contour coordinates (see Appendix C: SI-CTE - Exporting Contour Coordinates
- As the image stacks of the different stains do not align, make a copy of the NeuN contour dat file and modify the contours of all layers to fit each CR and Sst and then repeat the counting steps. In the end, each slice will have three dat files: one for each of NeuN, CR and Sst.

Step-by-step instructions:

1. Open the program StereoInvestigator Clear Tissue Edition. A "welcome" message may pop up in the application. It is recommended to familiarize yourself with the software's concepts and

features prior to using it. Also, it is highly recommended to understand the basic principles of stereology. The StereoInvestigator software has help provided and documentation; further information can be found in Schmitz and Hof, 2005 and Slomianka, 2021. Click "Exit" on the bottom right corner of the pop-up window if you are a seasoned user

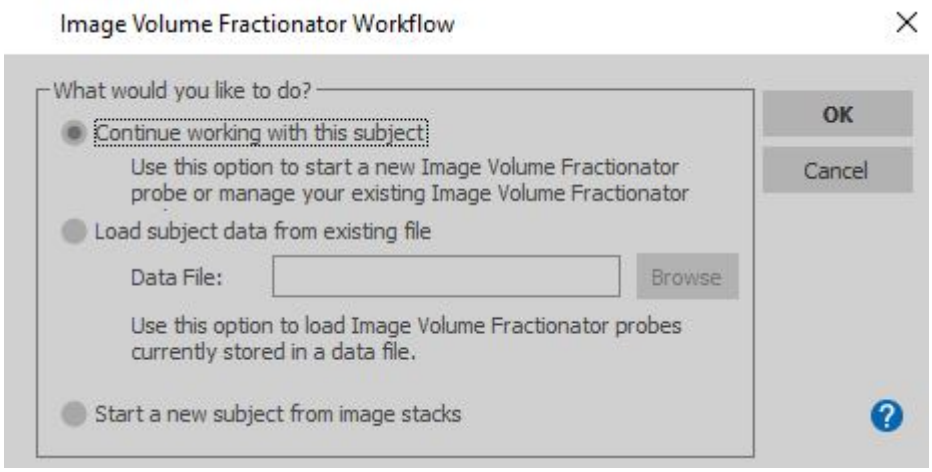
2. Open one NeuN image stack (only one jpx file: File > Open > Image Stack Open). When the first image stack is opened, the top of the image stack will have a z value of zero and z values for every slice in the stack will have a negative number. To achieve this for every subsequent image stack, the program must be closed and reopened before working with the next image stack.
3. Use the "PgDn" and "PgUp" buttons to move through the image planes, then move towards the middle of the image stack (e.g., around plane 100 of the 207 in specimen I48)
4. Select the "Zoom to Fit" icon (Image ribbon or in the Quick Access Toolbar at the very top left) to show the entire slice
5. In the Image Adjustment window, make the following adjustments to get good contrast between the cells and the background:
    a. Decrease the dynamic range by inputting the white value as needed (example range for one specimen: 2k-10k) until you can see the slice well, but not too overexposed
    b. Increase the contrast by increasing gamma to a value greater than 1.0 to 1.3 or 1.5. Be careful not to increase it so much that you lose the smaller and lighter-stained cells. A gamma of 1.3 has provided enough contrast on a few I48 NeuN-immunostained slices; some can stay at 1.0.
    c. Zoom in to 200-400% (on the Image ribbon our Quick Access toolbar at the upper left corner) to ensure these settings are ok for contouring and counting
    d. Record the white level and gamma values for this slice and stain
    e. Whenever you adjust the image, even if you reset the values to default, when you select the save button on the top left of the screen, there will be a pop-up asking you "Do you want to save this image stack?" ALWAYS select "No All." The data file will be saved, but the image stack will remain original. Then next time you use this file, check your files for the previously used white and gamma values to re-enter
6. Use the "PgDn" and "PgUp" buttons and note the values in each of the below columns in your records:

Total # of Image Planes	**Image Plane # Slice Begins Appearing**	**Layers I-VI first become visible (TOP of ROI Plane #)**	**Layers I-VI first become visible (TOP of ROI Z Depth)**	**Image Plane # Entire Slice is in View**	**Image Plane # Entire Slice is Last in View**	**Layers I-VI last visible (BOTTOM of ROI Plane #)**	**Layers I-VI last visible (BOTTOM of ROI Z Depth)**	**Image Plane # With Last Bit of Slice**

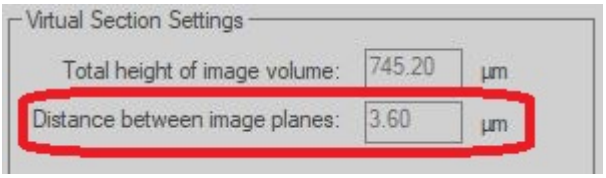
* The blue columns indicate where you can see countable cells in at least one area off the slice across all cortical layers without any gaps in the tissue slice–you will use these values in #12 of this protocol (step one of the upcoming workflow in SI-CTE).

**IMPORTANT! As you move through the workflow and enter different values, continue to record these values in your records (disector dimensions, grid size, etc.)

7. On the upper left-hand corner of the Probes ribbon, select "Image volume fractionator workflow" and the following window will open:

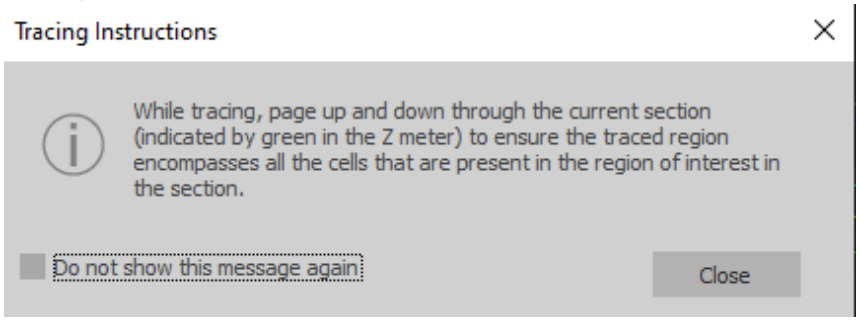


   a. If continuing with a slice that has already been started, select "Load subject data from existing file" and choose the corresponding dat file, then proceed to step #26; (If the message "Do you want the Marker Toolbar to use Marker names found in this file?" subsequently appears, click "Yes".)
   b. If starting a new slice, select "Continue working with this subject," then select "OK"
8. The image scale should be embedded in the jpx file. Using I48 as an example, the pixel dimensions are 3.6 µm isotropic. But if a window pops up for the pixel dimensions, enter 3.6 for the z on the left; then select the "Override X and Y" box and enter 3.6 into each of the x and y boxes. *NOTE: Verify image scaling of the slice images before starting with a new subject
9. In Step 1 of the IVF workflow ("Set up the subject"), fill in your name and the subject name (e.g., I48 Broca's area NeuN Slice 10).
10. Underneath "Subject Information" is "Virtual Section Settings" -- double check that the image scaling is correct as shown below:



11. Next set the Top and Bottom ROIs (regions of interest).
   a. Enter the value from the first/left blue column from step six above. This is where you can see countable cells in at least one area across all cortical layers at the top of the image stack. Then select "Set top of ROI"
   b. Enter the value from the second/right blue column from step six above. This is where you can see countable cells in at least one area across all cortical layers at the bottom of the image stack. Then select "Set bottom of ROI"

12. Set the “number of virtual sections” to 1. The virtual section thickness will populate automatically. Copy the virtual section thickness value into the corresponding column in your records ELNELN
13. Set the “disector height” to 50 µm.
14. Select “Next Step” to go to Step 2: “Trace the region of interest”. If the window below appears,
15. read it carefully, and click “close.”



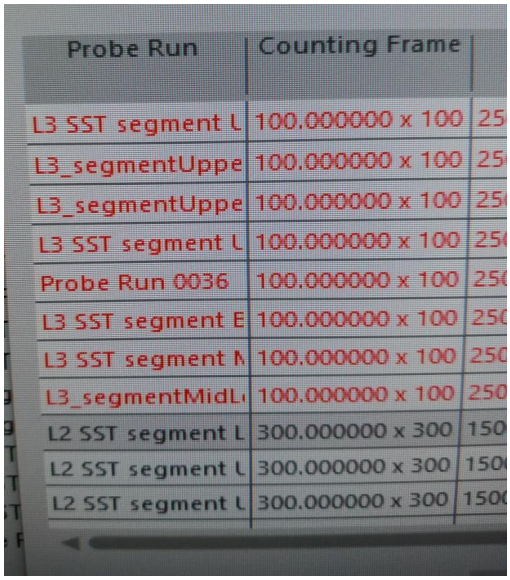
16. From the contour dropdown menu, select a contour type (the pial surface of Layer I is the easiest with which to start)
17. Tracing commands:
    a. Disable focusing with the mouse wheel to prevent accidentally changing image planes while drawing contours–you want all data points for the contours to be on one image plane:
        i. Click on “Move” tab
        ii. Deselect “Mouse Wheel Focus”
    b. Use “PgUp” and “PgDn” to scroll through z planes
    c. Use “Ctrl +” and “Ctrl –” to zoom in and out
    d. Use keyboard arrows to navigate the image stack in the x,y plane
    e. Use “Ctrl Z” to undo last point
18. Use the “PgDn” and “PgUp” to scroll down to the area with the most tissue in this section. Draw the contour here. Always draw the contour where the most amount of tissue is visible. MAKE SURE YOU DRAW ALL CONTOURS AT THE SAME Z DEPTH! (See Appendix B: “Determining Laminar Borders of Broca’s area”). If you want to move certain points that you drew, go to the “Trace” ribbon and click on “Select Objects.” Click anywhere on the contour and each point will become a small white square. Drag any points needing adjustment to their new location. When you are done, click “Select Objects” again to deselect it. This might be a time to consult a colleague with expertise in cortical neurohistology for any adjustments, as required.
19. Select “Next Step” to go to Step 3: “Set zoom level for counting”. Press Ctrl+ to zoom in to 400% for counting. The percentage is listed in the status bar at the bottom right of the tracing window.
20. Select “Next Step” to go to Step 4: “Define the counting frame size”. The counting frame size will vary depending on the immunolabel:

Immunolabel	**Counting Frame X,Y Dimensions (µm)**
NeuN	100 x 100
Calretinin	300 x 300
Somatostatin	300 x 300

21. Select “Next Step” to go to step 5: “Define SRS grid layout”. The grid size is different for each immunolabel.

Immunolabel	**Grid Size (µm)**
NeuN	2500 x 2500
Calretinin	2000 x 2000
Somatostatin	1500 x 1500

It is extremely important to ensure sampling consistency so that the same counting frame and grid dimensions are used for all the slices for a given immunolabel in a given region of interest across ALL specimens in a study (e.g., NeuN, in layer III, in Broca’s area of 10 brain specimens). For example, if the grid size for one probe run is 2500 x 2500 µm and another probe run for the same immunolabel has another grid size, clicking on the older probe run will revert the parameters and cause problems when exporting results later in step 8 of the workflow. Any errors will be highlighted in red as seen here:



22. Select “Next Step” to go to step 6: “Save sampling parameters”. Enter a name for the Sampling Parameter Set (e.g., I48 NeuN) and click “save your current settings.” Before counting, go to File -> Save As ->Data File, and save using the below file name structure:

    “sub-I48_sample-BrocaAreaS21_stain-NeuN_STER”

    “sub” = subject ID

    “S21” = slice 21 – this will change depending on the slice being worked on

    i. “NeuN” is the stain – change this to CR for calretinin, and SST for somatostatin.

23. Before clicking “Save” make sure you are in the correct folder on the computer. See Appendix B: “SI-CTE_Exporting Contour Coordinates” to export the contour coordinates for LENS

24. Select “Next Step” to go to step 7 “Count Objects.” On the left edge of the screen is the marker tool bar. Right click on a marker and rename it to correspond with the cortical layer and immunolabel, such as “Layer 3 NeuN ” or “Layer 5 Sst”. Select a different marker for each layer and each immunolabel, then use the same corresponding markers for each subsequent slice.

Immunolabel	Cortical Layer	SI-CTE Marker
NeuN	3	
	5	
	6	
Calretinin	2	
	3	
Somatostatin	3	
	5	
	6	

NOTE: the colors of the contours and markers match

25. In the workflow window, select the layer you want to count in section 1 and then select "Start Counting." Where there is more than one segment of a layer in that slice (as in the case of all slices counted in I48), multiple options will appear for that layer, so just choose the one at the top of the list
26. Click on "START COUNTING" and follow Appendix D "Counting Criteria." To summarize, count cells that are either within the counting frame or touching the green inclusion line. Do not count cells outside of the counting frame. If you imagine where the nucleus is in the very center of the cell and that is inside the counting frame, you count it. If the very center or the "nucleus" is split by the green line, you count it. If the very center or the "nucleus" is split by the red exclusion line, you do NOT count it. The z-meter is on the right side of the screen and displays a green area that indicates the dissector height (where you will count). Use the "PgUp" and "PgDn" keys to scroll through the image planes to find cells. To "count" a cell, click on the center of it to place a marker. If cells are brightest outside the dissector height, do not count them (the counting frame will change from red and green to all yellow). When cells are bright, then lose brightness, and then are bright again, that is a new cell and should be counted at its new brightest point. Once you count all the cells at the first site, click "Next" to continue to the next site and repeat the process. Do this until you have visited all sampling sites, then right-click on the probe run and rename it to

reflect the layer, immunolabel and grid dimensions. Select the next segment of that layer in the workflow window and continue marking cells with the same marker and using the same criteria. After counting all the segments of one layer (e.g., layer 3), change the marker and start counting in another layer (e.g., layer 5). Make sure to save the data file regularly as you count. After counting all the cells in all the layers of one slice's image stack, you may move on to the next step in the workflow.

27. Select "Next Step" to go to step 8 "View the sampling results" and click on "Display Probe Run List" at the bottom of the workflow window
28. Select all the probe runs for **one layer** (see note c.) by holding Ctrl while clicking each probe run -> View Results -> Export to Excel, then save the Excel file:
    a. The Excel files have the following naming structure: sub-**ID**_sample-BrocaArea**SliceNo.**_stain-**Type**_loc-**LayerNo.**_STER.xlsx
    b. Ex: sub-I48_sample-BrocaAreaS06_stain-NeuN_loc-Layer3_STER.xlsx
    c. *NOTE: when probe runs of segments from different layers are selected together, the number of sampling sites in the summary tab column F shows the total number of sampling sites for ALL layers combined and NOT the total number of sampling sites for each individual layer!
29. See Appendix E "Exporting Marker Coordinates"
30. See Appendix F "Exporting Counting Frame Coordinates"

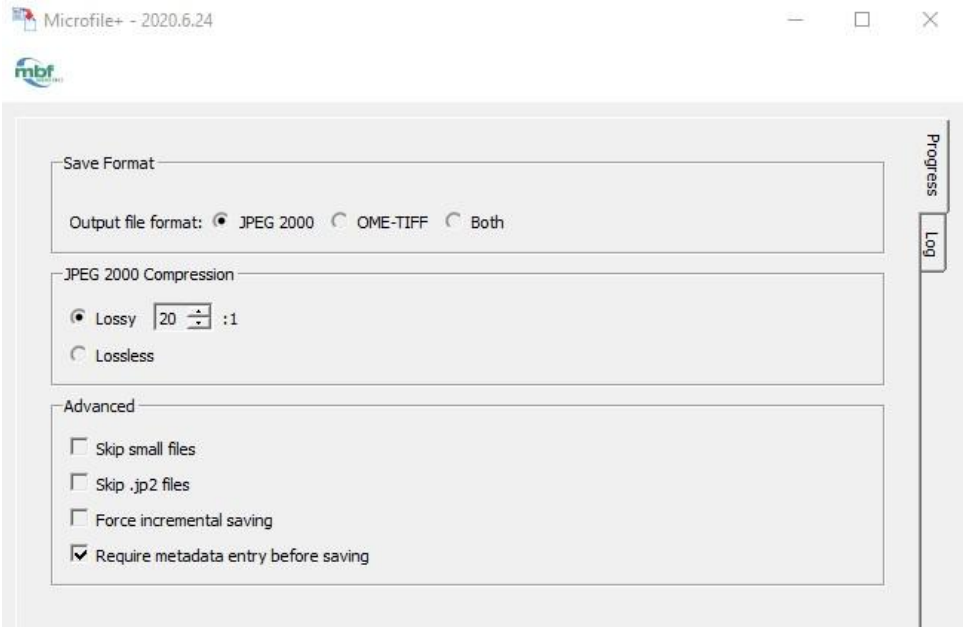
Appendix A:

Converting LSFM image stacks from tif to jpx

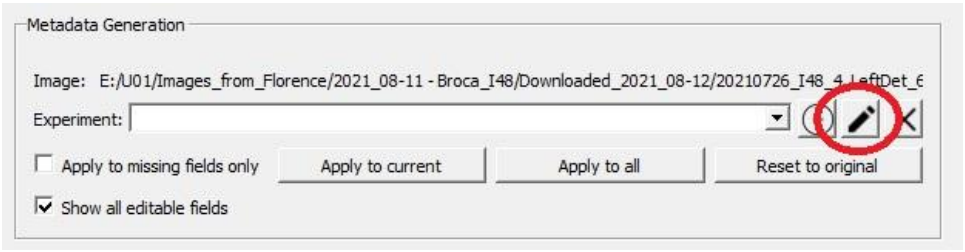
Purpose: to downsize large LSFM tif image stacks to smaller jpx format to increase efficiency in StereoInvestigator-Cleared Tissue Edition

Program: MicroFile+ (from MBF Bioscience)

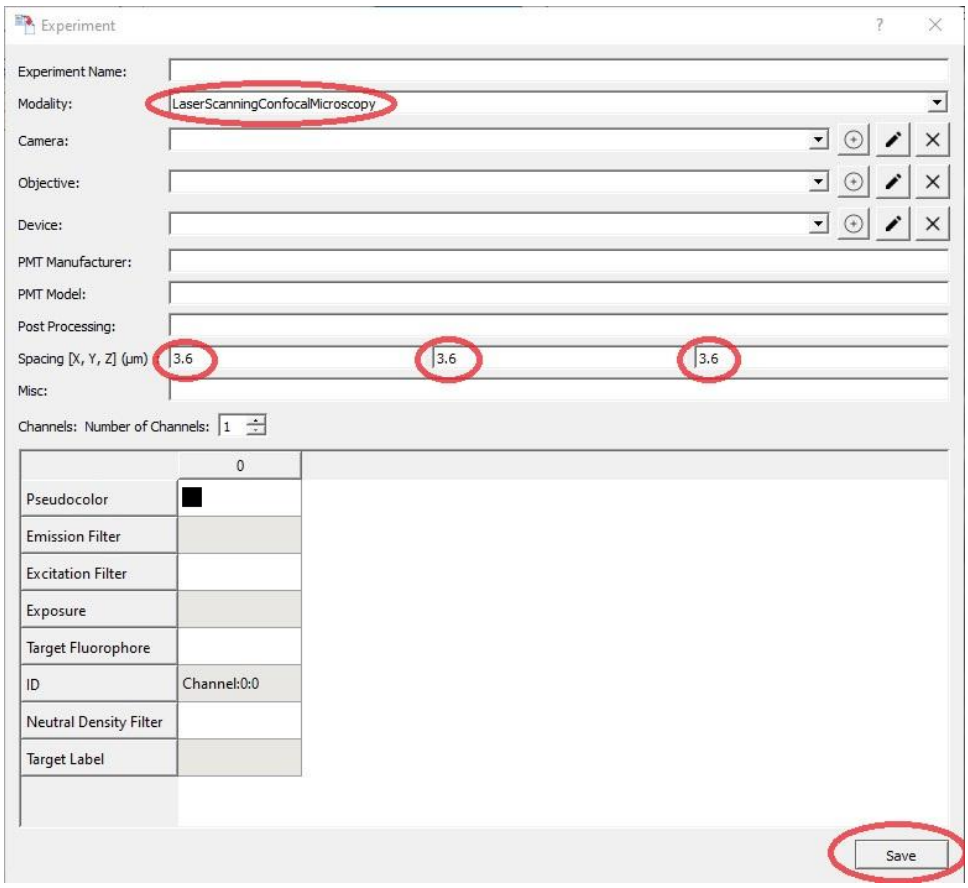
1. Open MicroFile+ from shortcut on desktop of U01 workstation (MBF system #5)
2. Click on the top option: "Convert Image(s)"
3. Make the selections as shown below, then click "Next" at the bottom right corner



4. Load files or drag and drop files into the Progress window
5. Click "Show Metadata" at the bottom left corner
6. In the Metadata Generation window, click on the "Edit Experiment" icon:



7. Select "LaserScanningConfocalMicroscopy" as the Modality and then enter the pixel dimensions in the Spacing fields (make sure these are correct!) and then click "Save": (If window below looks different on your screen, you may be using a different version of MicroFile+. See NOTES below.)

8. 

9. Click “Apply to all” in the Metadata Generation window. You will see the rows that are highlighted red fill with the scaling values. You should also see the red X at the end of the image name turn to a green checkmark (you may need to click on one of the image files for the red “x’s” to change to green checkmarks).
10. Click “Convert” and the status bar should appear
11. When completed, you will find the jpx image stack in the same folder as the tif image stack. Verify each image has been converted and that the file size is reduced. Enter date converted and jpx file size into your records

Appendix B:
Determining Laminar Borders of Broca's area

Layer descriptions:

- **Layer I:** plexiform molecular layer, a narrow band of myelinated fibers; low neuronal density
- **Layer II:** external granular layer, contains granule cells and pyramidal cells; thin and dense
- **Layer III:** external pyramidal layer with medium-sized pyramidal cells in the outer zone and larger pyramidal cells in the inner zone; large cells in packets at the bottom of the layer; upper layer has slightly larger cells than those of layer 2 and are more spaced out; answering my question about some large neurons that extend deeper than the large pyramidals of deep layer III: Some of these large rogue cells are actually from layer 5. They are large or very large and tend to be isolated or occur in small clusters. These are typical corticopontine and corticoreticular projection neurons. In a cortex in which layer 4 is absent or poorly defined these might be attributed as a very deep layer 3 or an upper layer 5 cell. However, their morphology defines them as layer 5.
- **Layer IV:** internal granular layer, contains stellate cells categorized as "granule" cells. It is conspicuous in most cortical regions except motor cortices, including Broca's area, and most visible in primary sensory areas; the absence of layer IV is due to the fact that it is very thin and very cell-sparse and in most planes of section it remains inconspicuous. It can appear as a thin, less intensely stained gap on Nissl preparations. NeuN does not show it well as the tiny modified pyramidal cells (spiny stellate cells, excitatory) intermix with deep layers III and upper V.
- **Layer V:** ganglionic (or internal pyramidal) layer, includes large pyramidal cells that tend to cluster in its upper portion.
- **Layer VI:** polymorphic (or multiform) layer, contains cells of many shapes but mainly spindle-shaped and small pyramidal cells; less cell-dense generally and has smaller neurons of variable shapes, and no large pyramid cells. Its cell density resembles that of the lower part of V, but V is characterized by more pyramids than VI that is more "polymorphic." NeuN can be reliably used to distinguish these borders.

Regarding magnification for drawing contours: A high magnification is useful to reveal a focal feature (e.g., a particular cell in III or in V) but not to establish the border that is better seen "from a distance" (think tabloid). It is highly recommended to change zoom levels. Also, when things become tangential you may have to go out of the plane to visualize in z how it looked before and after the plane you try to trace.

First use NeuN jpx files to draw laminar borders:

1. Draw along the pial surface as "layer I", the border between layers I and II as "layer II," etc. Make sure all contours are drawn on the image plane at the top of Section 1 and record the status of any drawn contours in your records
2. Save the dat file with the following format: "sub-I48_sample-BrocaAreaS30_stain-NeuN_STER" where "S" stands for "slice"
3. Take screenshots of questionable areas and send them to an expert neuroanatomist as required.
4. When ready to close contours for counting, follow the steps below

How to copy and close contours on adjacent layers:

Purpose: to run an image volume fractionator probe, the contour enclosing the layer in which you want to count must be closed. Colors are as used for I48, but the user has the ability to choose their own colors.

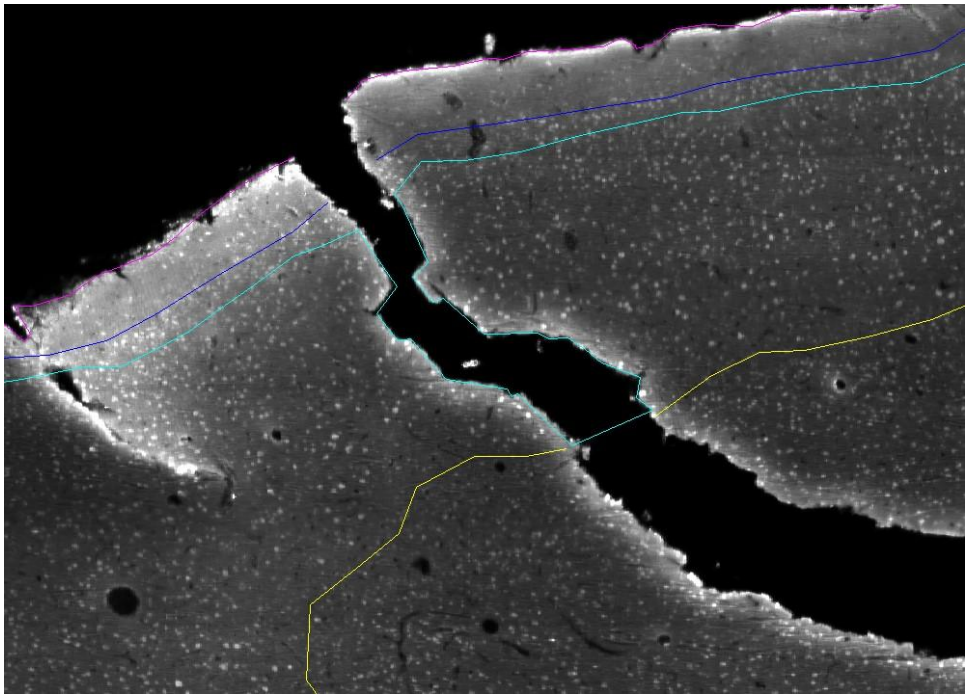
1. To enclose layer III within a contour, you will start by copying the border between layers III and V, then renaming the copy as "Layer III" and then closing one end by doing the following:
    a. In the Trace tab, click on "Select Objects"
    b. Click on the yellow border separating layers III and V so that the white data points appear, then right-click and select "Add to start of contour." Add one more point at a right angle to the contour, then Right click and "End open contour" -- this little "flag" will help differentiate between the two contours once they are overlaid and make them easier to close. Repeat with the other end of the yellow border (layer V), and on both ends of the teal border (layer III)
    c. Go to "Select Object" mode again, right click on the contour and select "Copy selected contour"
    d. Right click and select "Paste objects"
    e. Right click on the copy of the contour and select "Move selected contour." Move the copied contour further away from the original so it is easily distinguishable from the original contour (having them too close together may prevent you from being able to select one of them)
    f. Right click and select "Change contour to another type" then select "Layer III" -- it should turn from yellow to teal
    g. Change the angle of the flags so that when the contours are overlaid, the flags are extending in different directions
    h. Select the new layer III contour again, right click, and select "Move selected contour," then zoom in far and place it exactly on top of the yellow layer V contour by aligning the contour ends. This is best refined at a high zoom (>800%)
    i. Right click on it again and select "Add to start of contour" then follow along the edge of the tissue until you reach the teal border between layers II and III; then Right click and "end open contour."
    j. Right click on one of the white data points on either end, select "Splice contours" then click on the end of the other end point. You have closed one end of the layer III contour
2. To close the other end of the layer III contour:
    a. Navigate to the other end of the open layer
    b. Repeat steps b-i
    c. Right click on it again and select "Add to start of contour" then follow along the edge of the tissue until you reach the teal border between layers II and III; then Right click and "close contour

Notes:

- If there are multiple gaps in the contour due to tissue breakage, etc., connect the open-ended contour fragments where possible by adding to the start (or appending to the contour) and

splicing them together BEFORE closing the contour. Keep the number of contours for one layer to a minimum to reduce the number of probe runs needed.

- If there is a large break in the tissue, trace along the gap so as not to include it in the closed contour as shown in the teal layer III contour below:



Appendix C:

SI-CTE - Exporting Contour Coordinates

Purpose: to export the coordinates of the contours drawn in SI-CTE indicating the different cortical layers (segmentation)

1. Make a copy of the contour dat file in the File Explorer (right click on data file and select copy, then paste)
2. Open the copy in StereoInvestigator-Cleared Tissue Edition through the Image Volume Fractionator Workflow. Add in the subject Information, top and bottom ROIs (from your records) and dissector height (50um for I48)
3. Save this file: File -> Save As -> Data File -> use the same file name as in the dat file
4. Go to the drop-down menu below and select "MBF Ascii files (*asc)"
5. Click "Save"
6. Go to the documents and open this file by double-clicking on it – it will automatically open in Notepad
7. The file that appears gives you all the coordinates for every contour drawn. Select all, copy and paste into an excel sheet
8. Save the excel sheet with the same name as the dat file

Appendix D:

Counting Criteria

General rules:

- Rename each contour so it matches the layer (e.g., "Layer III")
- Rename each marker used so it matches the layer and immunolabel (e.g., "Layer III NeuN")
- Change the marker color so it matches the layer's contour color (e.g., for I48, layer 3 is teal, layer 5 is yellow, layer 6 is green)
- While some differences can be seen in cell size, there is no need to identify different cell types with different markers
- There are dark horizontal bands across some of the images--DO NOT adjust the image parameters (dynamic range and gamma) to brighten these regions while counting (you may need to replace with an adjacent slice if bands are too troublesome)
- Image adjustments (dynamic range and gamma) CAN be made when there are dark or light regions on a slice. Adjust such areas so they look similar in exposure/contrast to the rest of the sampling sites
- Use the approximate center of the cell body in the x, y, and z planes as the unique point when determining when to place a marker (imagine the nucleus is in the center of the cell)
- Place a marker on the image plane in which the cell body is the brightest and/or largest; it could also be the center plane in the z axis
- If having trouble determining the brightest and/or largest plane, determine the number of planes in which the cell can be seen:
    - If the cell appears across an odd number of planes, the middle plane is the plane to use when determining whether to count that cell
    - If the cell appears across and even number of planes, the first plane of the second half is the plane to use when determining whether to count that cell
- Treat the top plane of the optical disector as green and the bottom plane as red:
    - If the center of the cell appears in the top plane of the optical disector, count it
    - If the center of the cell appears in the bottom plane of the optical disector, do not count it
- Use the mouse wheel to scroll down and up through the section
    - Note: In specimen I48, the cells shift slightly to the right as you focus down through the slice. This is a small artifact after the corrections were made from the imaging being done at a 45-degree angle to the tissue surface
- Some neurons can be as big as 20um (so seen in 5.5 image planes of I48, which is 3.6 µm isotropic) and some as small as 5um (so seen in as few as one image plane in I48)
- If the disector touches a contour, zoom in on that area in the macro view window to determine which side of the disector is in the layer you are counting. CAUTION: it may just be a tiny corner that is in the layer being counted, and it's possible no cells will be visible within the disector AND within the layer being counted (i.e., place no markers at such a sampling site).
- If the disector falls on an image seam, do your best to identify neurons around it (do not place a marker on anything that looks like debris or an imaging artifact) Inclusion/Exclusion criteria for I48 NeuN, CR and Sst:DO place a marker if:
    - the center point of the cell body is:

        - inside the counting frame and NOT touching the red exclusion line
        - partially outside the counting frame but touching the green inclusion line
        - in the top plane of the optical disector
        - within the contour of the cortical layer in which you are counting (if the contour bisects the disector, zoom out or check the macro view window to determine the side on which to count)
    - the image plane where the cell appears brightest/largest is inside the disector (in the green zone on z meter). **Note: if the brightest and largest signals are not on the same plane, place the marker on the plane with the brightest signal**
    - the cell is faint and a solid spherical/oval shape (could be a small pyramidal or an interneuron)
- DO NOT place a marker if:
    - the center point of the cell body is:
        - outside the counting frame
        - touching the red exclusion line
        - in the bottom plane of the optical disector
        - outside the contour of the cortical layer in which you are counting (if the contour bisects the disector, zoom out or check the macro view window to determine the side on which to count)
    - the image plane where the cell appears brightest/largest is outside the disector (in the red zones on the z meter) **Note: if the brightest and largest signals are not on the same plane, place the marker on the plane with the brightest signal. If the center point of the cell touches the red exclusion line on the plane with the brightest signal, do NOT place a marker.**
    - the signal is not a solid spherical/oval shape, e.g., a strip of brightness near a blood vessel, a thin strip of brightness (likely a dendrite) or anything that looks like debris
    - pay extra attention if an object is near a darkened area (blood vessel) as there are often staining artifacts near them (i.e., non-specific staining)

<u>Additional notes and inclusion/exclusion criteria for I48 CR (counting zoom at 400%):</u>

- CR+ cells tend to be small in soma size
- DO place a marker if:
    - the object appears as a dash, a dot, then a dash (it is likely a cell)
- DO NOT place a marker if:
    - the object appears as only a dash (it is likely a dendrite or axon)

<u>Additional inclusion/exclusion criteria and info for I48 Sst (counting zoom at 400%):</u>

- Sst marks cell bodies as well as dendrites and axons
- Sst+ cells are a mixed population and will vary in size even within one layer
- Sst+ cells will not necessarily be clustered
- Sst will stain interneurons in subcortical tissue (white matter) so some Sst+ cells will appear deeper than layer VI
- DO place a marker if:
    - the object appears as a dash, a dot, then a dash (it is likely a cell)

- DO NOT place a marker if:
    - the object appears as only a dash (it is likely a dendrite or axon)
    - when scrolling up and down, the object is a bright spot that “snakes” through the image planes

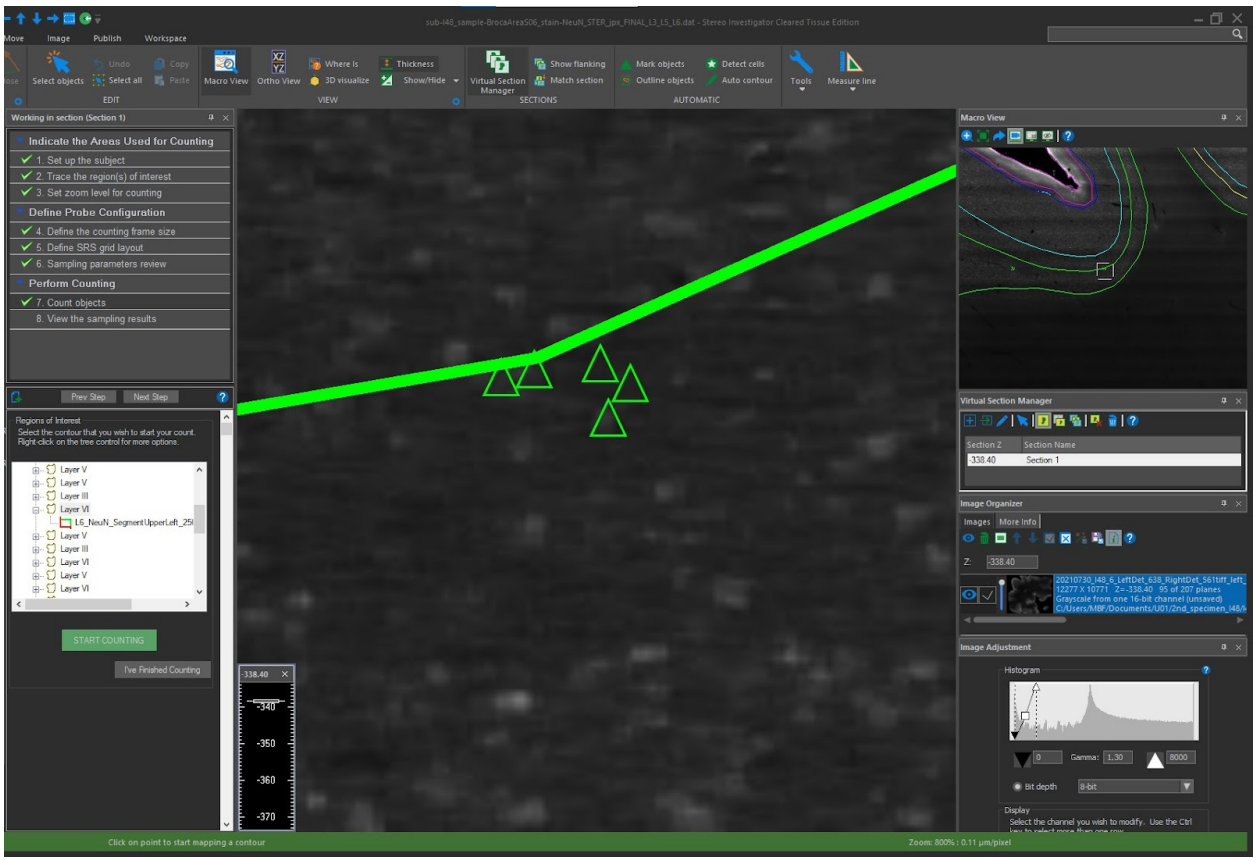
General observations of I48 Broca NeuN:

- Slice 30: when counting, it became obvious that signal intensity varies throughout different areas of the section as well as through the z (disector is in z planes 104-118), occasionally lighter in some areas on plane 104 than 118, and vice versa
- Slice 06, was counted at 800% zoom, then the zoom was changed to 400% ("Ctrl -") and the image planes within the 50-µm disector were scrolled through to verify marker placement and to ensure no countable neurons were missed

General stereology notes:

- On average, a counting frame will have around 5-10 countable cells, but it is ok if there is more, less, or even none
- Prior to taking a screenshot while counting, use macro view to show the location in the contour by zooming in on that segment (NOT in example below but can be helpful). Take the screenshot including the file name at the top, the workflow window on the left, the histogram and image organizer on the right and the zoom on the bottom right corner; then name the file following the format below:
- Subject_sampleregion_stain_slice##_layer#_layersegmentlocation_site##_zplane###
- Ex: “I48_Broca_NeuN_slice06_L3_segUL_site02_zplane095.jpg”
    - UL = upper left
    - UM = upper middle
    - UR = upper right
    - ML = middle left
    - MR = middle right
    - LL = lower left
    - LM = lower middle
    - LR = lower right

For slice 06, counted at 800% zoom, once all markers were placed in the counting frame, the zoom was changed to 400% zoom ("Ctrl -"), then scrolling up and down through the disector ensured marker placement and that no neurons were missed. Screenshot example:


Move
Image
Publish
Workspace
Select objects
Undo
Select all
Copy
Paste
EDIT
Macro View
Ortho View
Where is
3D visualize
Thickness
Show/Hide
VIEW
Virtual Section Manager
Show flanking
Match section
SECTIONS
Mark objects
Outline objects
Detect cells
Auto contour
AUTOMATIC
Tools
Measure line
Working in section (Section 1)
Indicate the Areas Used for Counting
1. Set up the subject
2. Trace the region(s) of interest
3. Set zoom level for counting
Define Probe Configuration
4. Define the counting frame size
5. Define SRS grid layout
6. Sampling parameters review
Perform Counting
7. Count objects
8. View the sampling results
Prev Step
Next Step
Regions of Interest
Select the contour that you wish to start your count.
Right-click on the tree control for more options.
Layer V
Layer V
Layer III
Layer VI
L6_NeuN_SegmentUpperLeft_25
Layer V
Layer III
Layer VI
Layer V
Layer VI
START COUNTING
I've Finished Counting
-338.40
-340
-350
-360
-370
Macro View
Virtual Section Manager
Section Z
Section Name
-338.40
Section 1
Image Organizer
Images
More Info
Z:
-338.40
Image Adjustment
Histogram
Gamma:
1.30
Bit depth
8-bit
Display
Click on point to start mapping a contour
Zoom: 800% : 0.11 µm/pixel

Appendix E:

Exporting Marker Coordinates

1. Open data file (.dat) in SI-CTE (images do not need to be loaded)
2. Publish tab > Marker Coordinates ("XYZ" icon)
3. Name text file to match the .dat file:
    a. Ex of dat file: sub-I48_sample-BrocaAreaS06_stain-NeuN_STER.dat
    b. Ex of txt file: sub-I48_sample-BrocaAreaS06_stain-NeuN_STER.txt
4. Save in a Marker Coordinates folder
5. Repeat for each slice
6. Once all coordinates have been exported to text files, open the first text file, add the below heading:
    a. `Marker  X(um)  Y(um)  Z(um)  Marker Diameter`
    b. Place this between the first line and the beginning of the coordinates list
    c. Save the .txt file
7. Repeat for each text file

Appendix F:

SI-CTE – Exporting Counting Frame Coordinates

1. Create a folder for all xml files for one stain of one case using "_CFC" for "Counting Frame Coordinates"
    a. e.g., "sub-I46_sample-BrocaArea_stain-NeuN_STER_CFC"
    b. e.g., "sub-I46_sample-BrocaArea_stain-Calretinin_STER_CFC"
2. Locate the stereology data file in Windows Explorer and create a copy
    a. e.g., original file: "sub-I46-sample_BrocaAreaS08_stain-NeuN_STER.dat "
    b. e.g., copy: "sub-I46-sample_BrocaAreaS08_stain-NeuN_STER - Copy.dat "
3. Cut and paste the copy file into the "CFC" folder and repeat with each slice .dat file
4. Open the first copy.dat file in SI-CTE
    a. images do not need to be loaded so uncheck "Load Images with Data File" at the bottom
5. In the Serial Section Manager, select section one. If the contours do not appear, click on the Image tab and "Zoom to fit"
6. Probes > Probe run list
7. Click on the Section Name column heading to list sections in ascending order
8. Select the top probe run from section 1
9. Click on "Draw Sites" button at the bottom of the window—the sampling sites will appear in the selected contour (sites can only be drawn for one probe run at a time)
10. Select the next probe run listed for section 1 and click "Draw Sites"
11. Repeat with each probe run in section 1. You should see counting frames in every contour that was counted
12. Close the Probe run list window
13. Repeat steps for all remaining sections (e.g., I46 has 10 sections counted per slice, I48 has one section counted per slice)
14. File > Save As > Data File Save As
    a. Delete "- Copy" and add "_CFC" for "Counting Frame Coordinates" at the end of the file. Be sure to select ".xml" from dropdown menu
    b. e.g., "sub-I46_sample-BrocaAreaS08_stain-NeuN_STER_CFC.xml")
15. If an image stack is open and you are prompted to save the image stack, select "No All"
16. Repeat the process (steps 1-15) for each slice of the case. In the end there should be one xml file for each counted slice of the specimen