1. Enter BoutonGui in the command line.
2. Input the mouse file name and mouse identifier (in case they aren’t explicit in the filename of the stack data used for analysis). Or, if you have a previous save file, type load to enter the ui to select that file and continue working on it.
3. Input the Stack Number to denote relative position of files loaded, then choose corresponding .mat file with the open file user interface. Click “Finish” in resulting window to load the filename (figData.stackfileName) and stackData (figData.stackData).
4. User Interface:
   1. The full stacks are shuffled relative to Stack Number to avoid formation/elimination bias. Z-stacks are not shuffled internally. shuffstack indicates your relative position within the shuffled stacks. Move up or down stacks using the ‘m’ and ‘,’ keys.
   2. Z-plane indicates your z position within the current stack. Move up or down in the z plane using the mouse scroll wheel.
   3. Axon indicates current axon under analysis. Change the current axon with ‘j’ and ‘k’ keys. Change to a new axon to avoid tracing from multiple axons being saved as a single axon.
   4. Bouton indicates the current bouton under analysis. Change the current bouton with the ‘u’ and ‘i’ keys. Some modes will automatically advance the current bouton tracker as you work. Return to a bouton id to override the data saved for that bouton.
   5. Status indicates the current input status for the “add boutons” mode. Change the status between alpha/beta/exclude/absent with q/w/e/r to enter large/small/ambiguous/absent boutons.
5. Use different modes to collect each type of data from the axons/boutons. The gui will warn you if a given axon/bouton is missing information from one of these modes.
6. Axons
   1. Use ‘A’ to enter axon tracing mode. Left click to add points to a line tracing the length of the axon. Right click to remove points from the axon trace. This data won’t be used to do backbone intensity of the axon, since it is too laborious to click all along the length in the center, but theoretically this could be used as a seed to regress to a local maximum to automatically center on the backbone. Instead, this will be used to calculate length of axon analyzed, as well as mark the axon so that it is not counted twice.
   2. Use ‘S’ to enter axon skipping mode. Every two left clicks will produce a line segment. Left clicking one of the two points will remove that line segment. Use this to indicate lengths of the axon that were not analyzed due to lack of clarity in the image in one of the time points. This will also be used to calculate the length of axon not analyzed, and subtract this from the length calculated from axon tracing mode.
7. Boutons
   1. Use ‘D’ to enter bouton center/status mode. Click on the center of the bouton to assign it a bouton number, as well as a status corresponding to the status indicator in the top right hand corner. Bouton numbers will persist, but bouton markers will clear from the image if you change plane/location/etc, in order to avoid clutter. Right clicking will remove a bouton center/status, but will leave a space in the numbering system so you can replace it. Both clicks will automatically advance the bouton number to the highest number (end of the stack).
   2. Use ‘F’ to enter bouton boundary mode. Use ‘F4’ to toggle ‘assisted’ versus ‘unassisted’ modes (but hit ‘F’ again to enter bouton boundary in the new assisted/unassisted mode.
      1. In assisted mode, the ui produces a trace from imsegfmm analysis of a filtered version of the current plane. Change the threshold using L/; while in ‘assisted’ mode in order to produce larger/smaller rois. Left clicking will overwrite any previous boundaries and replace it with the boundary shown on the screen. The raw and not the filtered image will be used for further analysis. Note that the trace produced runs through the middle of pixels immediately outside the region that will be committed for analysis.
      2. In unassisted mode, the ui produces a cursor with a width that can be increased/decreased using L/; while in unassisted mode. Left clicking will add to any previous boundaries (or create a new one, if no boundary is present). Right clicking will remove those pixels from any previous boundaries.
      3. The boundaries created in this mode will be used in conjunction with the bouton backbone trace and bouton cross trace in order to compute longitudinal intensity profile of the backbone and width of the backbone. Additionally, they can be used to calculate average intensity of the bouton region.
   3. Use ‘C’ to enter bouton backbone tracing mode. Left clicking will add a point to the trace, and right clicking on a point will remove that point from the trace. Use this to trace a representative portion of the axon on both sides of the bouton, as well as the straightest possible line along the longitudinal axis of the bouton. This will be used in conjunction with the bouton boundary to compute the intensity profile of the bouton relative to the axon.
   4. Use ‘V’ to enter bouton cross tracing mode. Left clicking will add a point to the trace, and right clicking on a point will remove that point from the trace. Use this to trace to include single straight line that crosses the bouton *and axon* at a maximum perpendicular axis, as well as trace through adjacent empty space for representative background fluorescence. This will be used in conjunction with the bouton boundary mode and adjacent axon boundary mode to compute the relative width change, and can also be used for a latitudinal intensity profile across either, as well as representative background intensity.
   5. Use ‘B’ to enter proximal axon boundary mode. This mode uses the same commands as bouton boundary mode, but use it to trace a region adjacent to the bouton representative of adjacent axon width at the widest point.
   6. **Note: All of the above data sets should be collected for Alpha, Beta, and Absent boutons at all timepoints. This will be used to confirm the elimination of that bouton.**
8. Use ‘X’ to commit the figure data to file in a short save, and ‘Z’ to perform a final save that will also generate figures for each axon and bouton.
9. Clear all data for the current axon (but not it’s boutons) using F1
10. Clear all data for the current bouton using F2

Terminaux boutons were not included in analysis (but were marked)

Increased axonal bouton dynamics in the aging mouse cortex

Grillo et al., 2013

Introduced EPB

Smallest serial section micro

Axonal Bouton Density Is Unaffected in the Ag Brain. We then measured the density and the dynamic properties of cortical EPBs. GFP-expressing EPB-rich axons in the Thy1-GFP-M line originate from cell bodies lying in L2/3 and L5 of the cortex or in the thalamus (26). We find that average bouton density is comparable in Ag and YA mice [YA: 0.061 ± 0.0047 EPB per micrometer; n = 27 axons, 17.8 mm, and 1,745 distinct EPBs (1,082 at day 4); Ag: 0.056 ± 0.0034 EPB per micrometer; n = 44 axons, 28.5 mm, and 3,034 distinct EPBs (1,588 at day 4); P = 0.33] (Fig. 3 A and B). Furthermore, bouton density remains stable over a 24-d period in both groups (Fig. S2A).

To measure EPB size and changes in size in the intact brain, we used animals that express cytosolic GFP in subsets of excitatory neurons (GFP-M) (27). We then modeled the axon as a cable filled with GFP molecules (Fig. 1B), and because more GFP molecules will accumulate in larger compartments (e.g., boutons), the intensity of the signal there will be higher. Because the size of 2P-imaged synaptic structures, especially in the z plane, is below the point spread function of the microscope (25), the intensity is directly proportional to the size of the structure. We developed a semiautomated software, termed “EPBscore” (Materials and Methods) that accurately and reproducibly measures axonal bouton intensity (i.e., size) in 3D from 2P image stacks (Fig. 1 C and D). To validate our analysis method, we correlated the in vivo intensity measurements with the volume determined from EM reconstructions of the same boutons (Fig. 1 E–H). Using serial section electron microscopy (SSEM), we reconstructed a total of nine boutons that previously had been imaged in vivo. All reconstructed boutons made synapses; the smallest of these fully equipped boutons had a volume of 0.236 μm3 and a relative intensity 1.92 times the intensity of the axonal backbone (Fig. 1 F–H). For each bouton we considered the total volume and the volume excluding eventual mitochondria. Both size measurements are highly correlated with the 2P intensity measurements: R2 including mitochondria = 0.74, P = 0.003; R2 excluding mitochondria, = 0.77; P = 0.002 (Fig. 1E). These results suggest that EPBscore is a powerful tool for detecting EPBs and for measuring and tracking their size in the living brain.

Briefly, 16-bit images are median filtered and saved. The resulting image is segmented with an adjustable threshold. Processes are traced in 3D, and an axon intensity profile is generated. The median intensity value of all pixels along the axon profile is set as the axonal backbone estimation. Peaks in the axon profile are scored as boutons and measured as backbone units of intensity. Fiducial points are chosen by the operator to align regions of interest over successive sessions to correlate boutons over time. The output is given in a Microsoft Excel format for further analysis. Axon length was confirmed using the NeuronJ plugin for ImageJ. Figures were prepared using Microsoft Office Suite and Adobe Illustrator.

EPBs were analyzed with EPBscore. To be included in the analysis, EPBs had to be at least two times brighter than the backbone in at least one session, based on SSEM reconstruction which showed that the smallest EPB that formed a structurally complete synapse was 1.92 times backbone intensity. EPBs had to be present in at least two consecutive imaging sessions at any point during 24 d to be included in the analysis. Using less conservative criteria, which may lead to an overestimation of axonal bouton dynamics, we find a similar difference in the fraction of EPB gain and loss between YA and Ag brains (Fig. S4). The same was true for other measurements of axonal bouton dynamics, suggesting that our conclusions are independent of EPB scoring criteria. The intensity values over time were exported to Excel (Microsoft) and were postprocessed using MATLAB scripts. We computed the intensity ratio over consecutive sessions as a measure of volume change (Fig. S6). The absolute intensity ratio was calculated as exp [abs(loga-logb)] (i.e., always the largest intensity value divided by the lowest, regardless of temporal sequence), where log is the natural logarithm of intensity a and b, which are the values of normalized intensity in consecutive sessions. If a = b, then the intensity ratio = 1. We assessed the noise level of this estimate of volume change by imaging EPBs in the brains fixed with 4% paraformaldehyde over a 4-d period and using the same analysis criteria used for the in vivo experiments (Fig. S7). We defined EPBs as “small” or “large” (Fig. 4 B–F) if their relative size fell in the bottom third or top third of the size distribution, respectively. Values for YA were 2.0–3.6 times backbone for small EPBs and >5.9 times backbone for large EPBs; values for Ag were 2.0–3.2 times backbone for small EPBs and >5.4 times backbone for large EPBs. The results in Fig. 4 did not change when the same size criterion was used for both age groups (i.e., large EPB size >5.6 times backbone). For large EPBs: YA TOR = 0.0017 ± 0.001, Ag TOR = 0.022 ± 0.005; P = 0.0002; YA ProbDest = 0.02 ± 0.05, Ag ProbDest = 0.17 ± 0.05; P = 0.003.

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