Overview This protocol describes a simple nonIHC-based approach that can be used by both the Deverman lab and the Baylor SATC to quantify the editing efficiency in the brains of Ai9 mice following Cas9 and gRNA delivery by the novel AAV-BI28 delivery vehicle.

Rationale The objective of this protocol is to quantity editing efficiency in CNS astrocytes. The fraction of astrocytes that is expressing tdTomato as a result of Cas9-based gene editing is closely estimated by quantifying the tdTomato+ area above background after thresholding. The rationale for this quantification approach is based on the fact that astrocyte processes are filled with tdTomato and astrocyte processes do not interdigitate (i.e., they occupy non-overlapping space in the brain parenchyma). Therefore, the area covered provides an approximate method for determining the fraction of astrocytes in which editing has occurred. The caveats of this method are that (1) off-target editing in neurons, which we do observe at a low rate, will cause an small overestimate of the signal, and (2) like IHC-based quantification methods, it is sensitive to inconsistencies in fixation and image quality.

Basic steps
(1) conversion to 8-bit grayscale images
(2) background subtraction
(3) Manual threshold setting
(4) measurement of % area above threshold in defined brain regions -cortex, thalamus, striatum

Detailed protocol

Preparing for measurements
1. Open Fiji.
2. Type command M to bring up the results window.
3. Right click to open options menu.
4. Open set measurements
5. Set as shown in the box to the right.
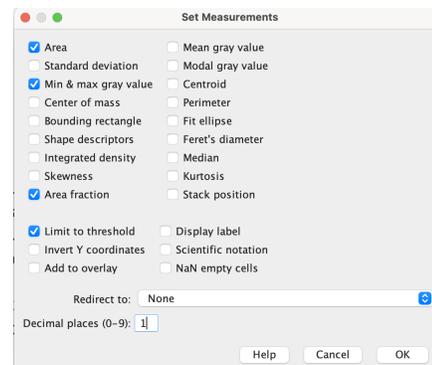
Preprocessing the images and making the measurements

1. Open ..._Cy3 file (or tdTomato channel) in Fiji.
2. Type>8-bit

3. Process>Subtract background → Rolling ball radius = 50.0, all other boxes unchecked

4. Image>Adjust>Manual Threshold = 10, 255 (the manual threshold setting should be set based on the guidelines outlined in Figure 1 below). We recommend checking several images with different manual threshold settings to identify a setting that can be used across all images.
5. Select brain regions using the polygon selection tool (See Figure 2).
6. Type command M.
7. Copy and paste the results window content into a csv/Excel/Google Sheets document
8. In the sheet, also provide the full image name and the brain region for each measurement
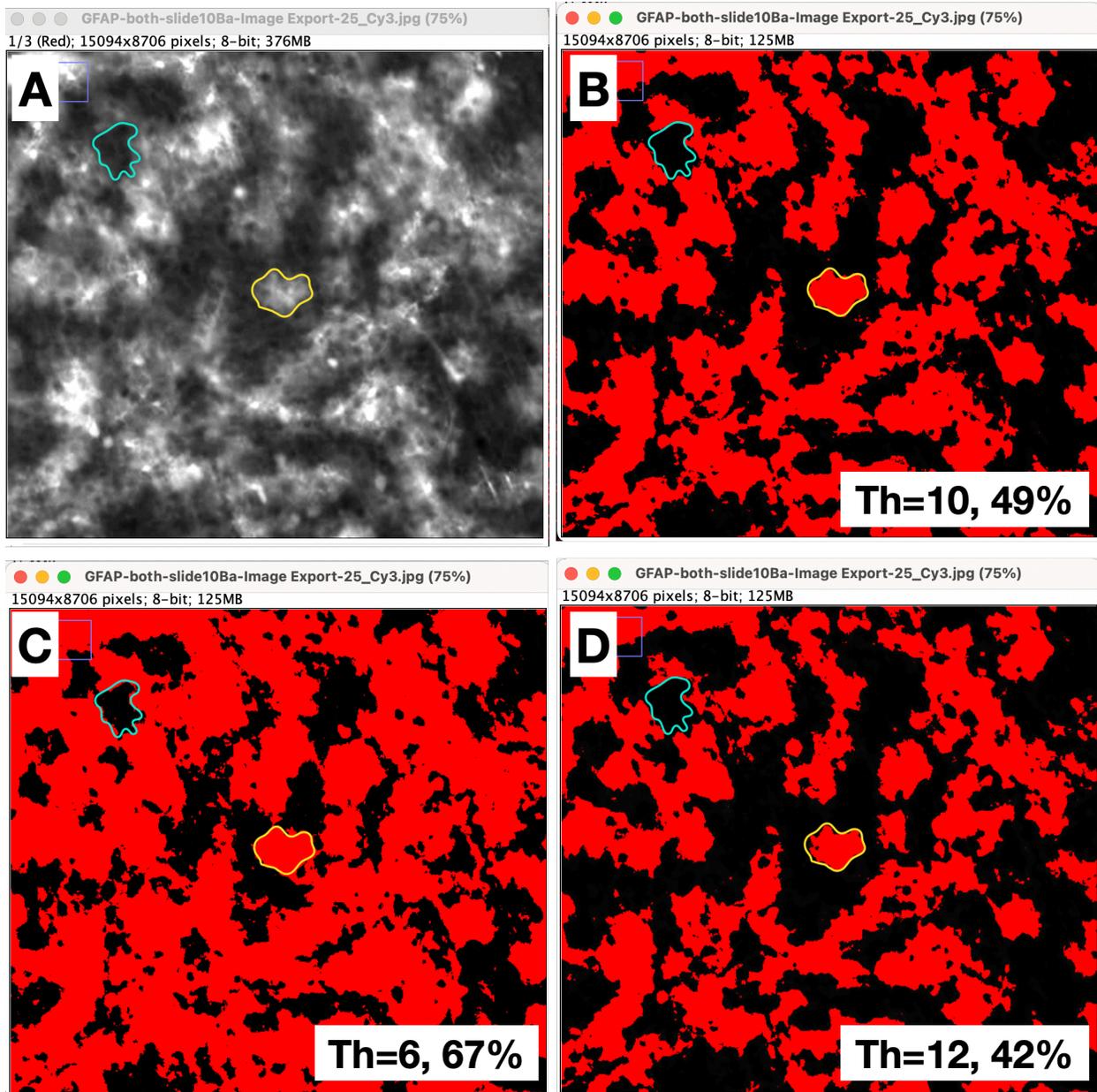
Figure 1. Settings for achieving an accurate estimate of the area covered by tdT+ astrocytes. Setting the threshold properly is critical to making this an accurate approximation of transduction. **A-D**. A zoomed in view of a representative image of tdTomato expression in an Ai9 mouse cortex following codelivery of AAV-BI28:GFAP-Cas9 and AAV-BI28-GFAP-NLS-GFP-gRNA-L1-R2. **A**. TdTomato expression in the cortex. **B**. The same image post background removal and thresholding at a level that closely matches the microscopy image. Note that the individual areas of below threshold signal (black) that are surrounded by signal above threshold (red, example negative cell with cyan outline) are similar in size to single cell areas above threshold that are surrounded by negative cells (yellow outline). This is an ideal setting to accurately quantify the area covered by tdTomato+ astrocytes. The same image where the threshold is set too low (**C**), which overestimates editing, or too high (**D**), which undercounts the fraction of tdTomato+ area.
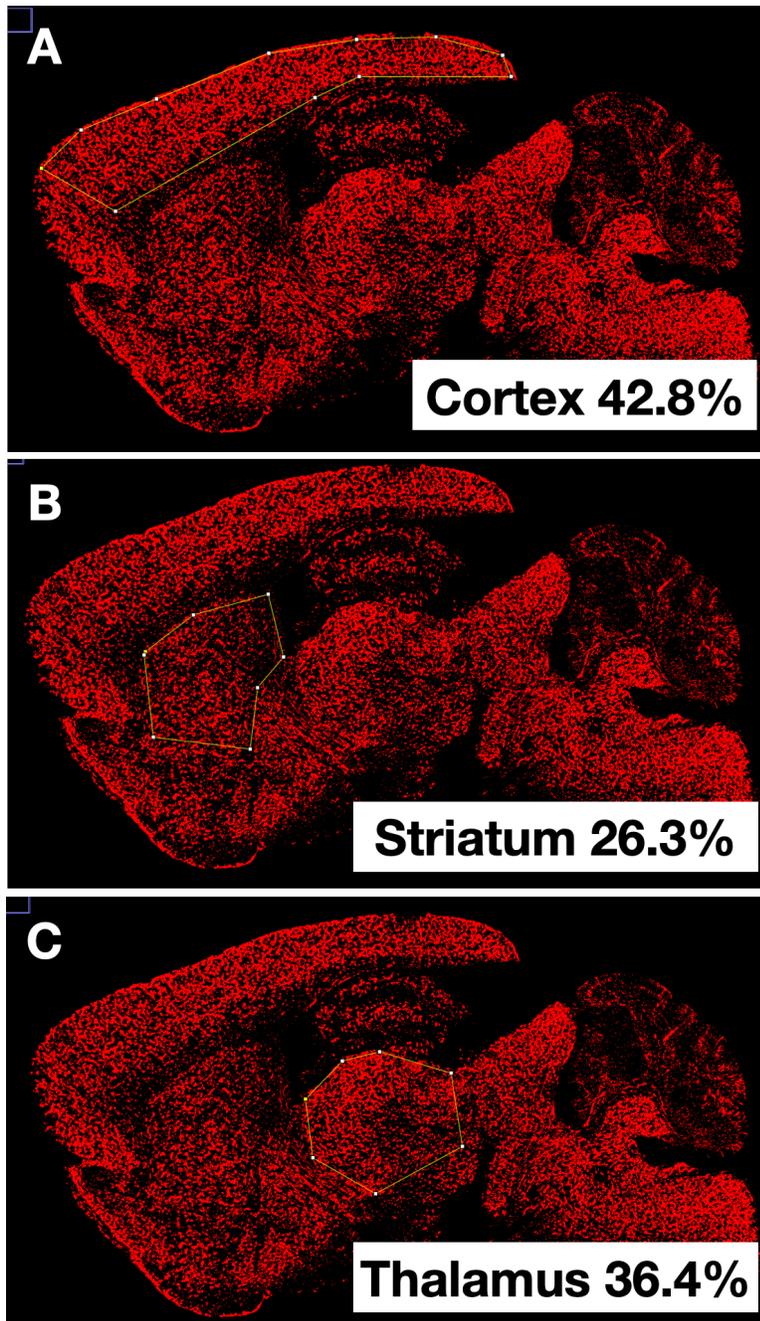
Figure 2. Examples of regions selected for analysis of the cerebral cortex (**A**), striatum (**B**) and thalamus (**C**) are shown along with the percentage of area that has a tdTomato signal above threshold in this example image.