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Enhancer-Driven AAV Reporters Reveal Cell-Type-Specific Expression Differences in Aging
and 5xFAD Mice

THESIS

submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in Biomedical Engineering

by

Haoqi Li

Thesis Committee:

Professor Xiangmin Xu, Chair

Professor Michelle Digman

Professor Gregory Brewer

2026

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Abstract of the Thesis

Enhancer-Driven AAV Reporters Reveal Cell-Type-Specific Expression Differences in Aging
and 5xFAD Mice

by

Haoqi Li

Master of Science in Biomedical Engineering

University of California, Irvine, 2026

Professor Xiangmin Xu, Chair

Enhancer-driven adeno-associated viral (AAV) reporters provide compact tools for targeting genetically defined neuronal populations, but their behavior in aging and Alzheimer's disease model contexts remains uncertain. This thesis evaluated four enhancer-AAV reporter datasets in young wild-type, aged wild-type, and 5xFAD mice: BiCHATe27 for cholinergic neurons, BiPVe3 for PV basket cells, BiPVe4 for PV chandelier cells, and BiSSTe4 for SST Martinotti cells. Cleared brain tissue was imaged by light-sheet microscopy and analyzed within paper-matched regions of interest. Reporter-marker specificity, sensitivity, reporter intensity, and raw cell-count burden were interpreted as descriptive pilot readouts.

Corrected BiCHATe27 ChAT analysis, driven by marker-appropriate image-analysis changes rather than by a new imaging modality, restored young wild-type targeting estimates close to the AAV toolbox reference and suggested broadly stable targeting with reduced signal burden in aged and 5xFAD samples. BiPVe3, BiPVe4, and BiSSTe4 showed lower or more variable targeting, including scan-quality and ROI-sensitivity effects. Whole-section pathology images added tissue context but were not used for specificity, sensitivity, or plaque-proximity calculations. Overall, the dataset supports a cautious cell-type/subtype-dependent model that requires manual counting, atlas registration, direct amyloid-pathology assessment, and chromatin-accessibility validation before stronger disease-level interpretation.

Chapter 1. Introduction

Alzheimer's disease and selective neuronal vulnerability

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by amyloid-beta pathology, tau pathology, synaptic failure, network dysfunction, gliosis, and cognitive decline [1-4]. Although AD pathology becomes widespread, neuronal populations do not respond uniformly. Selective vulnerability is a central principle in AD biology: specific circuits, cortical layers, neurotransmitter systems, and transcriptional cell states are affected earlier or more severely than others [5-7]. This uneven vulnerability motivates experiments that resolve disease-associated changes at the level of genetically defined neuronal populations rather than at the level of gross brain regions alone.

Mouse models remain essential for testing causal and mechanistic hypotheses, even though no single model captures the full complexity of sporadic human AD. The 5xFAD model carries five familial AD mutations and develops early amyloid pathology, gliosis, synaptic impairment, and progressive circuit abnormalities [8-11]. Because aging itself changes neuronal physiology, chromatin state, immune tone, and tissue quality, direct comparison among young WT, aged WT, and 5xFAD mice is useful for separating disease-associated signals from age-associated signals. The present work uses this framework to ask whether enhancer-AAV reporter expression differs across WT, aged, and 5xFAD brains.

Neuronal vulnerability in AD is not limited to excitatory neurons. Inhibitory interneuron dysfunction is increasingly recognized as a contributor to aberrant network activity, impaired oscillatory rhythms, and cognitive decline [12-14]. PV interneurons, including basket and

chandelier subtypes, are positioned to regulate fast cortical inhibition and network timing. Several AD-model studies report PV interneuron dysfunction, PV marker loss, perineuronal net disruption, or altered excitability, although the direction and magnitude of changes depend on region, model, sex, and disease stage [13-20]. SST interneurons also show disease sensitivity, but the literature supports both vulnerability and compensatory activity changes depending on circuit context [14,27-29].

Cholinergic systems are another major axis of AD vulnerability. The cholinergic hypothesis has long linked basal forebrain cholinergic degeneration and reduced cholinergic tone to cognitive impairment in AD [21-24]. More recent work has refined this view by emphasizing circuit-specific cholinergic dysfunction rather than a single uniform loss model [22-26]. For enhancer-reporter analysis, this distinction is important: a lower reporter or marker signal burden may reflect altered cell state, reduced marker expression, altered viral reporter expression, or cell loss. Therefore, this thesis avoids interpreting ChAT signal changes as direct proof of cell death unless independent cell-counting validation supports that conclusion.

Enhancer-AAV reporters as cell-type and subtype readouts

Cell-type-specific genetic access is a major requirement for modern circuit neuroscience and translational gene delivery. Enhancer-AAV strategies use compact cis-regulatory elements to drive reporter or effector expression in molecularly defined neuronal populations [37-42].

Compared with transgenic mouse lines, enhancer-AAVs can be deployed more rapidly, can be combined with systemic or local delivery, and can sometimes label subtypes that are difficult to isolate with conventional promoters. The AAV toolbox paper used in this project provides benchmark specificity and sensitivity values for enhancer reporters targeting interneuron

subtypes, including BiCHATe27, BiPVe3, BiPVe4, and BiSSTe4, in young WT C57BL/6J mouse validation contexts [39].

Enhancer activity is not static. Disease, aging, chromatin accessibility, transcription factor availability, and cell state may alter enhancer output even when the same DNA element and viral backbone are used. This creates both an opportunity and a caveat. On one hand, enhancer-AAV reporter expression may act as a readout of disease-associated transcriptional state. On the other hand, changes in reporter expression can complicate interpretation of targeting specificity if the underlying target marker also changes. This thesis therefore treats enhancer-AAV measurements as a combination of targeting performance and signal burden rather than as a simple one-to-one cell-count assay.

The specific biological question is whether the four selected enhancer-AAV reporters show consistent or divergent changes across young WT, 5xFAD, and aged mice. A consistent disease trend across enhancers would suggest a global technical or viral-delivery effect. Divergent trends across enhancer-defined populations would support a cell-type or subtype-specific model. The current evidence favors cautious enhancer-specific interpretation rather than a single global disease direction: ChAT is the strongest current result, PVe3 is N=2 complete but variable, PVe4 is exploratory, and BiSSTe4 is variable with a Set 1 quality caveat.

Light-sheet imaging context and analysis-pipeline considerations

Tissue clearing and light-sheet microscopy enable mesoscale imaging of large brain volumes while preserving three-dimensional anatomical context [48-55]. In this thesis, light-sheet imaging was essential because it produced the cleared-tissue image volumes and whole-section context used for ROI selection, visual QC, and sample-quality review. However, the main

BiCHATe27 conclusion was not that light-sheet imaging alone solved the ChAT targeting problem. The key correction was analytical: the detection and colocalization pipeline had to be adapted to the morphology of the ChAT marker signal. These approaches are powerful for surveying reporter distributions across cleared tissue, but the sampling and optical resolution used for whole-brain or large-montage imaging differ from high-resolution confocal validation. Coarser sampling can merge nearby puncta, miss dim cells, and reduce accurate double-positive detection. These effects are especially important for specificity and sensitivity calculations, where the double-positive numerator is vulnerable to segmentation and registration error.

Automated image analysis is necessary for scalable quantification, but detection algorithms can introduce threshold-dependent bias [56-60]. In this project, PV and SST analyses used local-maxima detection followed by nearest-neighbor matching between reporter and marker channels. This approach is more appropriate for punctate or soma-like signals. ChAT marker signal was more diffuse and did not consistently produce independent local maxima at the reporter-positive cell center. Therefore, the ChAT pipeline was corrected to use local marker/reporter intensity at detected cell centers. Light-sheet images made this marker-morphology problem visible across the cleared sections, but the conclusion that WT BiCHATe27 targeting was close to the AAV toolbox baseline was primarily driven by the corrected automated analysis pipeline rather than by a change in imaging modality.

Chromatin accessibility as an orthogonal validation axis

Enhancer-AAV reporter changes can be interpreted more strongly if they align with independent evidence of altered enhancer accessibility. ATAC-seq and single-cell epigenomic studies have shown that open chromatin landscapes and enhancer-promoter interactions are highly cell-type-

specific and disease-associated in AD [61-68]. These data support the general premise that disease state can change enhancer output. For this thesis, future comparison of enhancer peak accessibility in 5xFAD versus young WT tissue would be an important orthogonal validation step, especially for PVe3 and SSt4 interpretations that currently require additional validation.

The central hypothesis of this thesis is that enhancer-AAV reporter expression does not change uniformly across all labeled cell types in AD and aging. Instead, reporter targeting and signal burden are expected to show enhancer- and subtype-specific patterns reflecting a mixture of biological cell-state changes and method-dependent measurement constraints. The following chapters describe the current data, the analytical approach, and the conservative interpretation supported by the available evidence.

Thesis Experimental Workflow for Enhancer-AAV Reporter Analysis in WT, Aged, and 5xFAD Mice

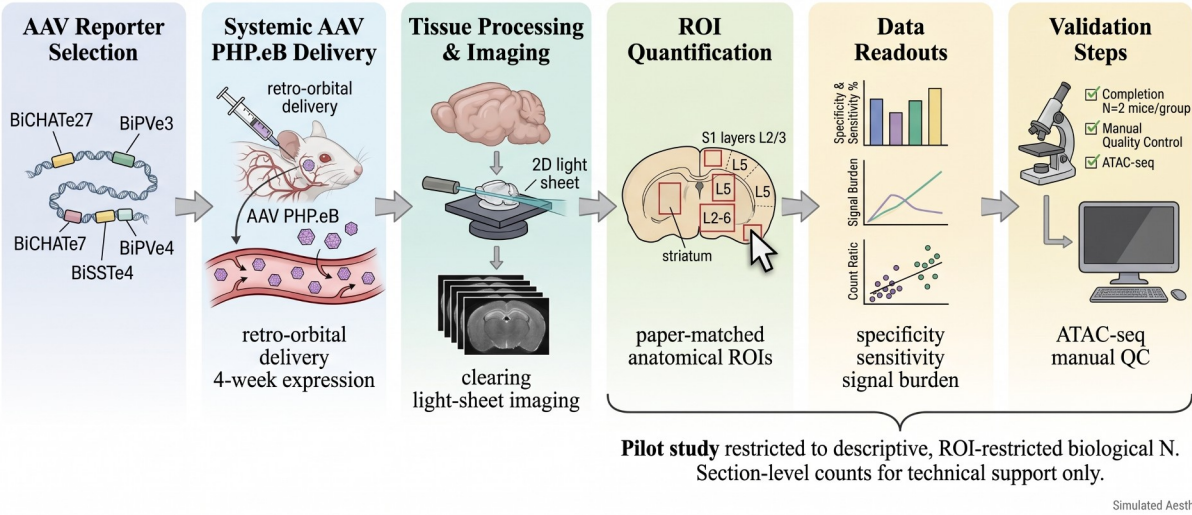


Figure 1. Experimental workflow for enhancer-AAV reporter analysis in WT, aged, and 5xFAD mice. This schematic defines the pipeline used throughout the thesis: enhancer-AAV reporter selection, systemic AAV-PHP.eB delivery, tissue processing, paper-matched ROI quantification, targeting readouts, and validation steps.

Chapter 2. Methods

Experimental design

This pilot study analyzed enhancer-AAV reporter expression across three mouse conditions: young WT, 5xFAD, and aged WT. Four enhancer reporters from the published enhancer-AAV toolbox framework were examined: BiCHATe27, BiPVe3, BiPVe4, and BiSSTe4 [39]. All four enhancers now have N=2 biological samples per group in the primary analyses used in this thesis. Here, N refers to independent biological brains/animals per enhancer per condition, not to tissue sections or imaging fields. Table 2 summarizes the data-lock status and flags which completed datasets remain exploratory because of between-set variability or sample-quality caveats.

Animal- and brain-level metadata were tracked from the hippocampal GABA enhancer experiment log and light-sheet sample log. The recorded fields include experiment-log ID, genotype, sex, age at vector delivery, viral dose, injection date, vector incubation interval, collection date, age at collection, and collection or processing notes. Table 7 summarizes the 24 biological samples used in the quantitative analyses. For aged WT animals with NIA date of birth unavailable in the experiment log, age at collection was approximated from the recorded age at vector delivery plus the vector incubation interval.

AAV-PHP.eB was used for systemic reporter delivery because engineered AAV capsids can support broad CNS transduction after vascular delivery in permissive mouse backgrounds [43-47]. The experimental design followed a retro-orbital injection strategy with approximately four weeks of expression before tissue processing. Brain tissue was cleared and imaged by light-sheet microscopy, a strategy commonly used for mesoscale volume imaging of cleared neural tissue [48-55]. Figure 1 is therefore not only a workflow schematic; it defines the sequence of delivery,

expression, clearing, imaging, ROI quantification, and validation steps used to generate all downstream tables and figures.

Each biological condition has a distinct interpretive role. Young WT is the baseline condition used to determine whether the enhancer-AAV behaves similarly to the published AAV toolbox validation dataset [39]. That published reference used young WT C57BL/6J mouse validation contexts, so it is a baseline-method comparison rather than a disease-background match. Aged WT is included because aging alone can alter tissue quality, marker expression, cell density, and enhancer state; it prevents every non-WT change from being overinterpreted as an AD-model effect [5-7]. The 5xFAD condition is used to test whether an amyloid-pathology-associated genotype changes reporter expression, target-cell burden, or reporter-marker overlap [8-11]. However, the current reporter/marker stains do not directly label amyloid plaques, so this thesis does not claim plaque-proximal effects from the present images. These conditions are not a complete age-matched disease time course. Therefore, this thesis treats the three groups as biologically motivated states and emphasizes descriptive comparisons rather than causal time-course inference. Figure 2 summarizes this timepoint and condition logic, and Table 2 records which enhancers currently support N=2 interpretation.

Regions of interest

ROIs were selected to match the AAV toolbox validation regions as closely as possible because specificity and sensitivity are only interpretable when the sampled anatomy corresponds to the enhancer's intended target population [39]. BiCHATe27 was analyzed in the caudoputamen/striatum, BiPVe3 in S1 somatosensory cortex layers 2-6, BiPVe4 in S1 cortex layers 2/3, and BiSSTe4 in S1 cortex layer 5. Table 1 lists these paper-matched ROI definitions

and explains why each ROI is used. Earlier approximate dorsal-cortex ROIs were treated as insufficiently precise for final interpretation; the current thesis framing uses paper-matched ROIs as the target standard.

Because tissue clearing and large-montage imaging do not fully replace atlas registration, the current analysis remains ROI-restricted rather than whole-brain. The selected sections and ROIs are therefore treated as representative sampling windows, not whole-brain cell-count measurements. In response to advisor feedback, PV and SST datasets were also rerun with an exploratory broader S1 cortical band to test whether conclusions were highly dependent on the narrower layer-specific ROIs. This expanded ROI analysis is used only as a sensitivity analysis; it is not atlas-registered, not layer-resolved, and does not replace the paper-matched primary ROIs. This limitation is carried forward explicitly in Table 6 and motivates atlas-registration validation as a pending internal analysis step.

Cell detection and co-localization

For PV and SST analyses, reporter-positive and marker-positive candidates were detected using difference-of-Gaussian/local-maxima style detection in the selected ROI. Double-positive cells were estimated by nearest-neighbor matching between channels within the expected spatial tolerance of the imaging data. This automated strategy follows the general logic of scalable bioimage analysis but remains threshold-sensitive [56-60]. Specificity was calculated as double-positive cells divided by reporter-positive cells, and sensitivity was calculated as double-positive cells divided by marker-positive cells. The raw reporter-positive, marker-positive, and overlap-positive counts used for these ratios are reported in Table 4, while the section-level QC counts are reported in Table 5.

The ChAT pipeline was modified after inspection showed that ChAT marker signal was diffuse and poorly suited to peak-to-peak local-maxima matching. In the original peak-to-peak approach, a reporter-positive center and a marker-positive local maximum had to be detected as nearby peaks. That assumption works better for compact PV or SST soma-like signals than for diffuse ChAT immunostaining. The corrected ChAT method keeps automated center detection, but evaluates local marker or reporter intensity around detected centers using fixed ROI percentile thresholds. The practical benefit is that ChAT-positive signal is not missed simply because the marker channel is broad or diffuse rather than peak-shaped.

The corrected ChAT method was applied consistently to the BiCHATe27 samples across WT, aged, and 5xFAD conditions and across the two biological sets used for the N=2 ChAT summary. It was not generalized to BiPVe3, BiPVe4, or BiSSTe4 because their marker signals are more compatible with the local-maxima/nearest-neighbor workflow and because mixing method definitions across morphologically different markers would make cross-enhancer comparisons harder to interpret. Figure 4 shows the quantitative consequence of the ChAT correction, and Table 4 provides the animal-level counts behind the corrected ChAT ratios.

Whole-section pathology imaging and display processing

After the primary ROI quantification workflow was finalized, the final pathology scan set was organized for qualitative whole-section review. Raw Olympus cellSens VSI files and their associated ETS sidecar folders were archived for Image_02 through Image_05, and the target 10x_DAPI, cy5, mCH, ORNG series were converted with Bio-Formats into 16-bit, four-channel OME-TIFF masters for provenance. For the final thesis display panel, curated pathology display PNGs from the Thesis data folder (ChAT.png, PVe3.png, PVe4.png, and SSTe4.png) were used because they provide the clearest tissue-level review images. The display images were selected

as upright whole-section pathology-context examples near the cortical/hippocampal levels used for ROI-level review, but they were not atlas-registered to the exact counting sections or to every young-WT counting plane. Minor plane differences therefore reflect the available final pathology sections and tissue orientation rather than an attempt to use Figure 15 as a direct ROI-matched quantification panel. The display mapping is DAPI in blue with Cy5 shown in a magenta/far-red display color; additional reporter/channel colors are preserved from the provided display PNGs. Brightness, contrast, saturation, and gamma adjustments in these display images are used only for visualization. The quantitative specificity and sensitivity analyses in this thesis remain based on the locked ROI/count pipeline rather than on these display-adjusted whole-section images.

Statistical framing

The analyses are descriptive and exploratory. Biological N is reported explicitly for each enhancer in Table 3. In this field, there is no single universal acceptable N for every light-sheet or enhancer-AAV experiment because the required sample size depends on effect size, variance, endpoint, and whether the study is intended as a pilot screen or a definitive biological test. However, N=2 brains per condition is not generally sufficient for conclusive disease-model reporting or inferential group statistics. A stronger confirmatory study would typically require additional independent biological replicates, balanced cohorts, preregistered or power-informed endpoints, and independent validation of key observations. The present N=2 design was used because the thesis was constrained to the processed and scanned biological samples available before submission, and because the immediate goal was to integrate the enhancer-AAV datasets, document sample quality, and identify candidate effects for follow-up rather than to make final disease-mechanism claims. Section-level values are used for quality control and visualization but are not treated as independent biological replicates; this distinction is visible by comparing

animal-level Table 4 with section-level Table 5. When biological N=2 is available, means and standard deviations or standard errors are shown in the relevant figures. No inferential hypothesis testing is used in this thesis because the current biological replication is insufficient for robust group-level statistical inference.

All disease and aging claims are therefore framed conservatively and are tied to explicit sample quantity and quality documentation. Table 2 records claim strength and data-lock status, Table 3 reports biological N and group-level values, Table 4 reports animal-level counts, Table 5 reports section-level QC counts, Table 6 lists limiting caveats and required validation steps, and Table 7 reports animal/brain metadata. Figures 10-15 provide visual QC and tissue-context support for the same samples. BiPVe3 is now N=2 complete but remains exploratory because WT45 improved after rescan but Set 1 and Set 2 still differ substantially for the WT baseline. BiSSTe4 is treated as N=2 but variable because Set 1 and Set 2 disagree directionally; Set 1 may be confounded by poor perfusion or tissue-processing artifacts. BiPVe4 is described as exploratory despite N=2 because its values remain sensitive to scan quality, layer placement, and thresholding. BiCHATe27 is treated as the most methodologically stable result after corrected ChAT colocalization restored WT targeting near the AAV toolbox baseline [39]. Table 6 lists these limitations and the validation actions required before stronger claims are made.

Condition and timepoint interpretation framework

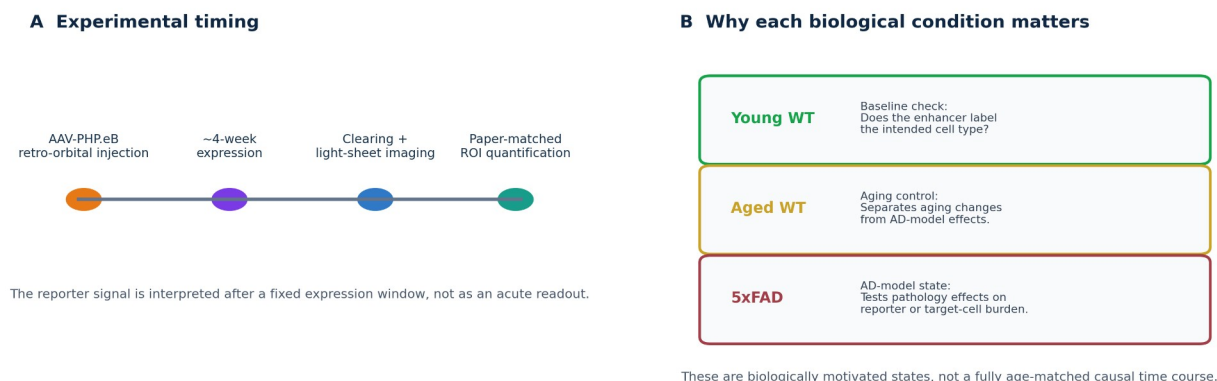


Figure 2. Condition and timepoint interpretation framework. Panel A shows the reporter-expression and imaging timeline, and panel B explains the interpretive role of young WT, aged WT, and 5xFAD. This figure clarifies why the study is treated as a comparison among biologically motivated states rather than as a complete age-matched time course.

Chapter 3. Results

AAV toolbox baseline comparison

The first analytical requirement was to determine whether young WT samples approximate the published AAV toolbox validation values [39]. This comparison is important because disease and aging effects are difficult to interpret if the baseline targeting performance is already far from the reference standard. The toolbox reference is a young WT C57BL/6J method baseline, not a matched 5xFAD or aged-background comparison. Figure 3 compares the paper WT baseline values with the current WT values, while Table 1 defines the paper-matched ROIs used to make that comparison and Table 3 lists the group-level values. After correcting the ChAT counting method, BiCHATe27 WT specificity and sensitivity were close to the paper baseline. BiPVe3, BiPVe4, and BiSSTe4 remained lower than the paper baseline, supporting cautious interpretation of the interneuron enhancers and emphasizing the need to read ratios together with raw counts.

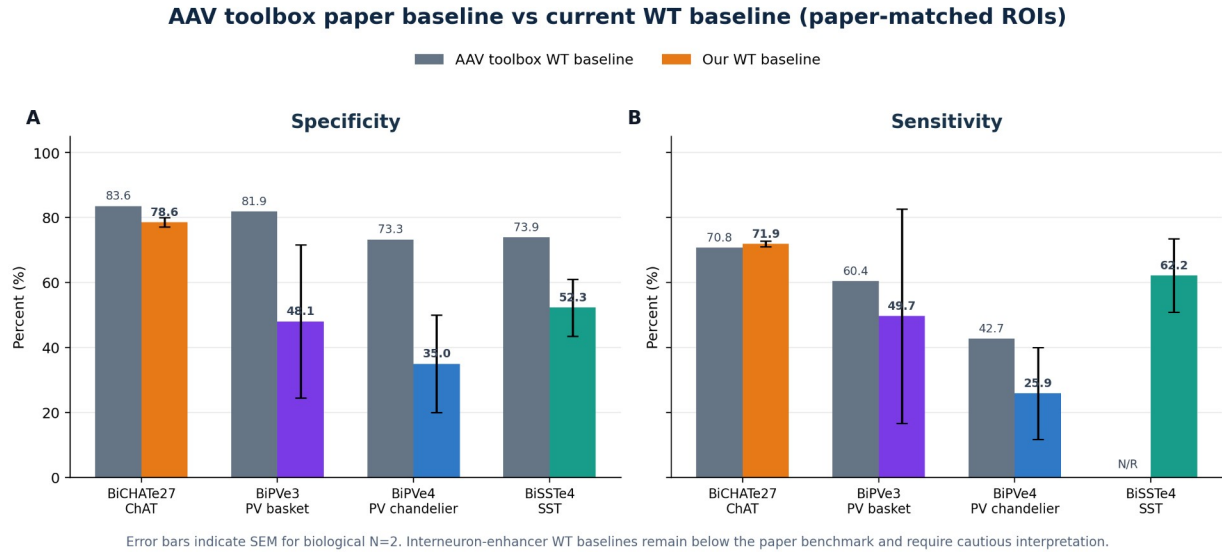


Figure 3. AAV toolbox paper baseline versus current WT baseline. Panel A shows specificity and panel B shows sensitivity for the published WT reference and the current young WT data. This figure is used as the baseline-validation checkpoint: BiCHATE27 is close to the paper reference after ChAT correction, whereas BiPVe3, BiPVe4, and BiSSTe4 remain below the published benchmark and therefore require cautious interpretation. Error bars indicate SEM for biological N=2.

BiCHATE27: corrected ChAT targeting and signal burden

The original ChAT peak-to-peak matching method underestimated co-localization because ChAT marker signal was diffuse, a marker-morphology issue that is consistent with the broader need to adapt image-analysis methods to the biological signal being quantified [21-26,56-60]. Corrected intensity-centered analysis produced WT values close to the paper reference [39], indicating that the earlier discrepancy was primarily methodological. Figure 4A-B shows this corrected targeting comparison, and Table 3 provides the corresponding group-level specificity and sensitivity values. In the primary paper-aligned method, WT specificity and sensitivity were 78.6% and 71.9%, respectively (SD 2.0 and 1.2 percentage points; SEM 1.4 and 0.8 percentage points), compared with the AAV toolbox reference of 83.6% and 70.8%. Aged samples showed similar targeting values, and 5xFAD samples showed larger variance but did not demonstrate a consistent collapse in targeting efficiency.

The stronger biological signal in ChAT was observed in reporter intensity rather than in targeting efficiency. Figure 4C shows that mean reporter intensity decreased from WT to aged and 5xFAD samples, and Table 4 provides the animal-level reporter, marker, and overlap counts used to interpret this signal-burden result. Therefore, the conservative interpretation is that BiCHATe27 targeting remains broadly stable after method correction, while striatal ChAT-associated reporter signal burden is reduced in aged and 5xFAD tissue. This result is consistent with cholinergic vulnerability literature [21-26], but the present data alone do not establish cell loss.

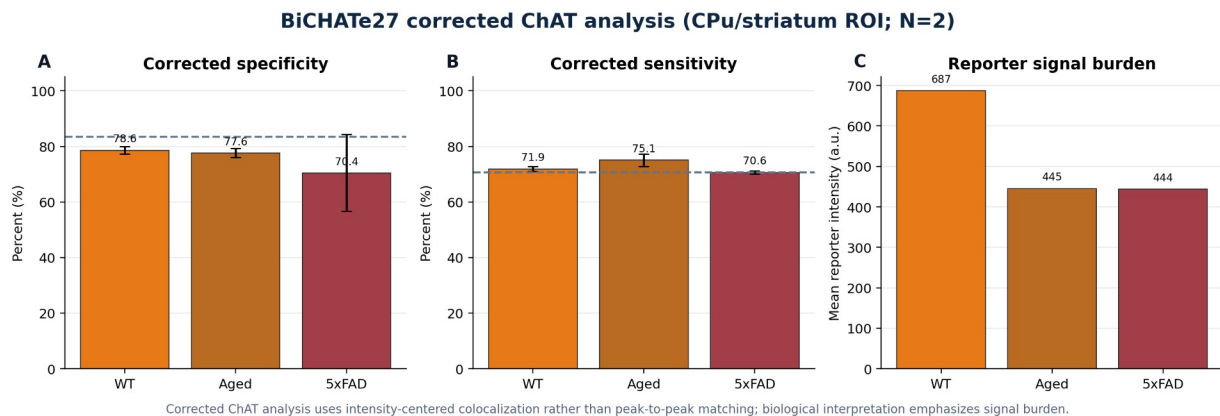


Figure 4. Corrected BiCHATe27 N=2 ChAT analysis. Panel A shows corrected specificity, panel B shows corrected sensitivity, and panel C shows reporter signal burden across WT, aged, and 5xFAD samples. This figure supports the main ChAT interpretation: corrected targeting is broadly stable and close to the AAV toolbox WT baseline, while reporter signal burden decreases in aged and 5xFAD tissue.

BiPVe3: N=2 variable result for PV basket-cell enhancer

BiPVe3 Set 2 was reprocessed after rescanning the WT45 sample. The updated biological N=2 mean +/- SEM values for the S1 cortex layers 2-6 PV basket-cell ROI were WT 48.1 +/- 23.6% specificity and 49.7 +/- 33.0% sensitivity, aged 43.3 +/- 13.1% and 55.7 +/- 2.9%, and 5xFAD 18.3 +/- 3.1% and 20.9 +/- 0.9%. Table 3 lists these group means, Table 4 shows the animal-level counts, Figure 5 shows the updated PVe3 targeting summary, and Figure 11 shows one real QC image from every PVe3 biological sample. The rescanned WT45 sample improved the WT baseline compared with the earlier low-overlap run, but Set 1 and Set 2 still differ substantially.

Therefore, BiPVe3 remains N=2 complete but exploratory rather than a strong monotonic WT > aged > 5xFAD result.

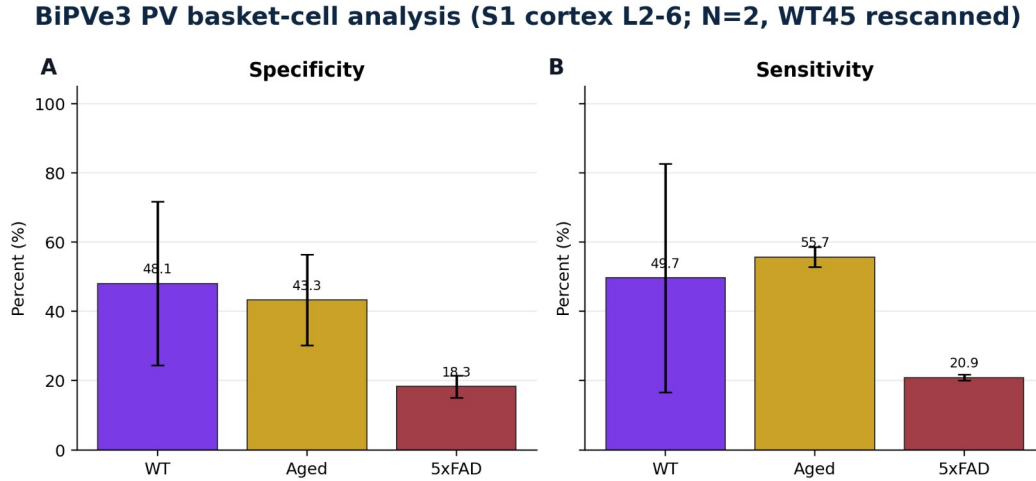


Figure 5. Updated BiPVe3 N=2 analysis after WT45 rescan. Panel A shows specificity and panel B shows sensitivity for the PV basket-cell enhancer in the S1 cortex L2-6 ROI. WT45 improved after rescan, but the WT group still shows high between-set variability, so the PVe3 result remains exploratory pending manual ROI/layer QC and expanded cortical counting. Error bars represent SEM for biological N=2.

BiPVe4: N=2 exploratory result for PV chandelier-cell enhancer

BiPVe4 N=2 values did not reproduce a simple shared PV-enhancer direction with BiPVe3.

Current specificity/sensitivity means were 35.0%/25.9% in WT, 36.0%/45.0% in aged, and 61.1%/44.4% in 5xFAD. Figure 6 shows these N=2 values, Table 3 lists the group summary, and Table 4 shows the animal-level counts behind the ratios. Although the 5xFAD mean specificity is higher, the WT baseline remains far below the AAV toolbox reference [39] and the analysis is sensitive to layer placement and scan quality. Because PV interneuron changes in AD models can be region-, layer-, and subtype-dependent [13-20,30-36], BiPVe4 is treated as exploratory rather than as a strong disease claim.

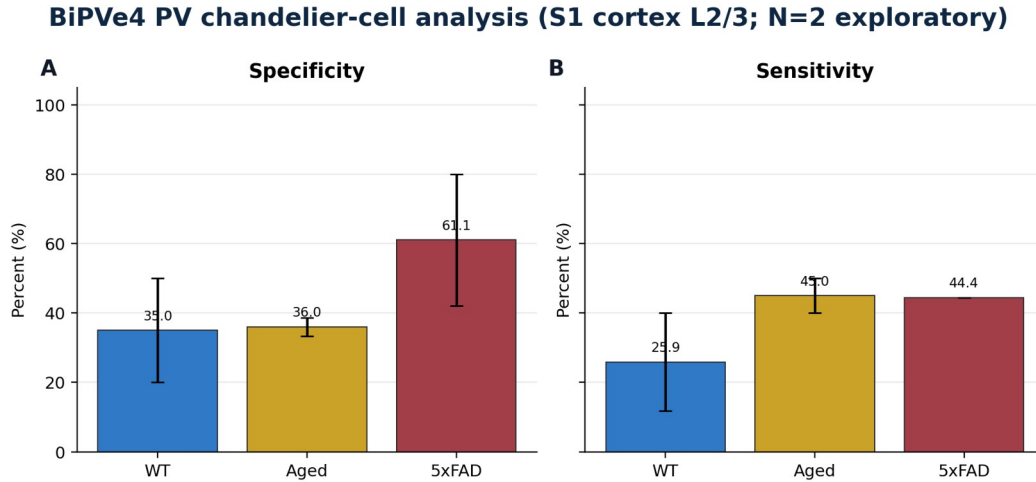


Figure 6. Current BiPVe4 N=2 exploratory analysis. Panel A shows specificity and panel B shows sensitivity for the PV chandelier-cell enhancer in the S1 L2/3 ROI. This figure is used to show why BiPVe4 cannot yet be treated as a strong disease result: 5xFAD specificity is higher, but the WT baseline is low and the measurement is sensitive to ROI/layer placement and scan quality. Error bars represent SEM.

BiSSTe4: N=2 variable result for Martinotti SST enhancer

BiSSTe4 was rerun for Set 1 and Set 2 with the same pipeline. Figure 7 shows that the Set 1 analysis suggested elevated 5xFAD overlap, but this effect was not reproduced in Set 2. Animal-level N=2 mean $\pm$ SEM values were WT 52.3 $\pm$ 8.7% specificity and 62.2 $\pm$ 11.3% sensitivity, aged 45.5 $\pm$ 5.5% and 69.4 $\pm$ 1.2%, and 5xFAD 50.3 $\pm$ 16.4% and 65.6 $\pm$ 18.1%. Table 3 gives the group summary, Table 4 gives the animal-level counts, and Table 5 gives the section-level QC counts. The Set 1 5xFAD sample may be confounded by poor perfusion or tissue-processing artifacts, consistent with the original internal PPT annotation. Because SST interneuron responses in AD and aging can vary by circuit and disease stage [14,27-29], BiSSTe4 is interpreted as variable and exploratory rather than evidence for stable SST enhancer upregulation in 5xFAD.

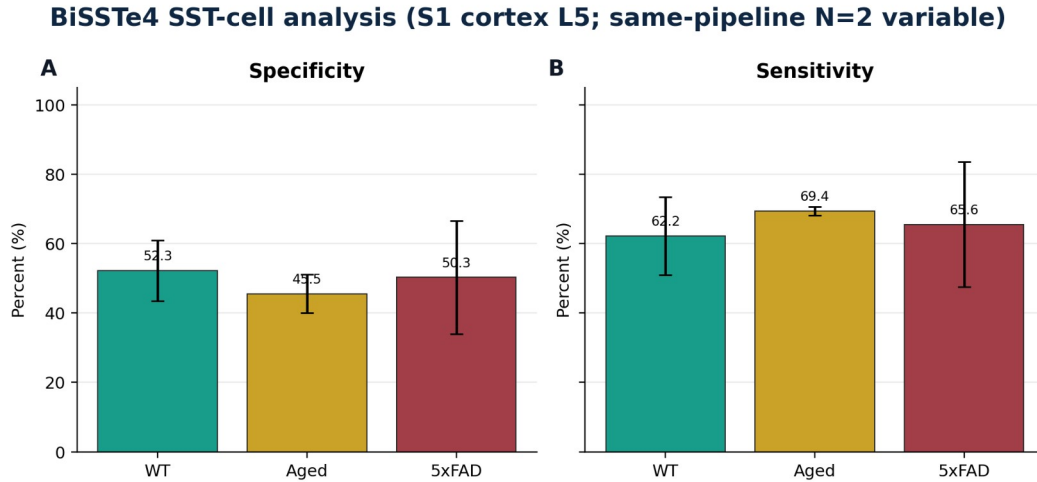


Figure 7. BiSSTe4 N=2 same-pipeline analysis. Panel A shows specificity and panel B shows sensitivity for the Martinotti SST enhancer in the S1 L5 ROI. This figure documents the key SST limitation: Set 1 and Set 2 do not reproduce the same direction, so the combined N=2 result is variable and should not be presented as a stable 5xFAD increase. Error bars represent SEM for biological N=2.

Animal-level count burden: reporter labeling, baseline marker burden, and overlap

Specificity and sensitivity are ratios, so they can change because reporter counts, marker-positive target-cell counts, or overlap counts change. To make the raw count logic explicit, Figure 8 compares animal-level reporter-positive, marker-positive, and overlap-positive counts within each enhancer, and Table 4 reports the exact animal-level counts used to generate this figure. Reporter-positive counts estimate how strongly the enhancer labels cells in the sampled ROI. Marker-positive counts estimate the baseline target-cell burden in that ROI. Overlap-positive counts estimate the cells that are both reporter positive and marker positive. This count-level view is important because a lower ratio can reflect reduced target labeling, altered marker burden, or both. Table 5 provides the section-level version of the same logic for QC, but those section-level values are not treated as biological replicates.

Animal-level cell-count burden by enhancer and condition (paper-matched ROIs)

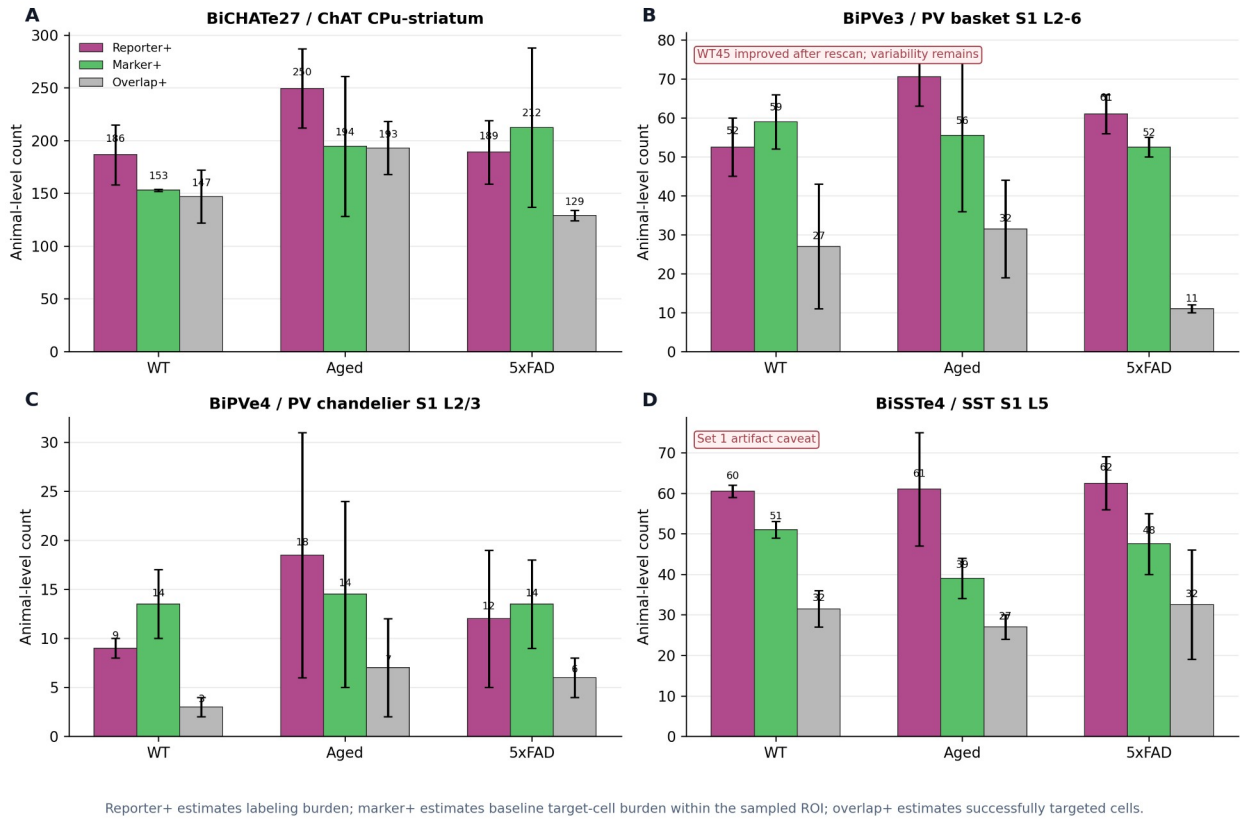


Figure 8. Animal-level cell-count burden by enhancer and condition. Panel A shows BiCHATE27/ChAT counts in the CPu/striatum ROI, panel B shows BiPVe3/PV basket counts in S1 cortex L2-6, panel C shows BiPVe4/PV chandelier counts in S1 cortex L2/3, and panel D shows BiSSTe4/SST counts in S1 cortex L5. This figure separates reporter-positive, marker-positive, and overlap-positive counts so that specificity and sensitivity changes can be traced back to actual count components.

Cross-enhancer synthesis

Across enhancers, the current data do not support a single global direction of disease effect.

Figure 9 summarizes this cross-enhancer pattern: BiCHATE27 shows stable corrected targeting but reduced signal burden, BiPVe3 is N=2 complete but variable with low 5xFAD overlap efficiency, BiPVe4 remains variable and exploratory, and BiSSTe4 is variable across sets with a specific Set 1 artifact caveat. Table 3 provides the numerical group-level summary behind the heatmap, and Table 6 lists the interpretation limits that prevent overclaiming. This pattern is

more consistent with enhancer- and subtype-specific behavior plus technical sensitivity than with a simple uniform imaging or viral-delivery effect, which is consistent with the broader premise that enhancer activity and interneuron vulnerability are cell-type dependent [30-42,61-68].

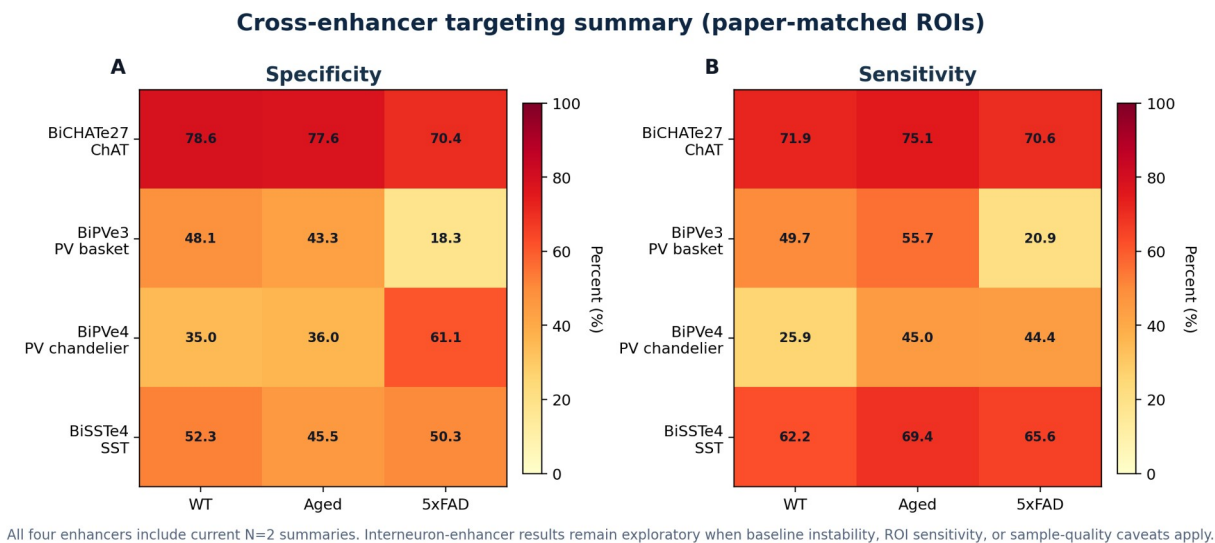


Figure 9. Cross-enhancer targeting summary. Panel A shows specificity and panel B shows sensitivity across enhancers and conditions in the paper-matched ROIs. This heatmap is used to compare whether the dataset supports a global disease effect or enhancer-specific behavior. All four enhancers include N=2 summaries, but PVe3, PVe4, and BiSSTe4 remain exploratory because of baseline instability, ROI/layer sensitivity, or set-specific artifact caveats.

All-sample tissue-image QC and expanded cortical ROI sensitivity analysis

Representative tissue images were added for every biological sample because the quantitative ratios are more convincing when the reader can inspect real tissue quality, ROI placement, and detected signal patterns. Figures 10-13 show one real image panel for each sample used in the N=2 biological summaries. These images are visual QC support for Tables 4-5, not additional independent biological replicates.

All-sample BiCHATe27 real-image QC sheet

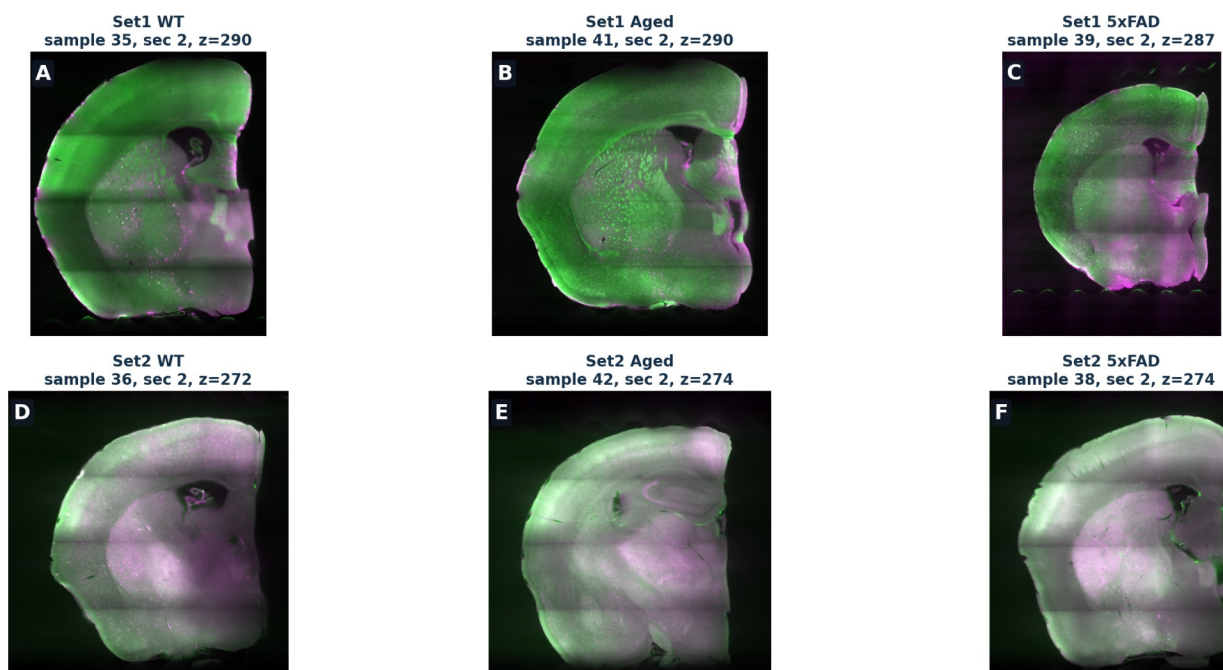


Figure 10. All-sample BiCHATe27 real-image QC sheet. Panels A-F show one representative z-plane from each biological sample used for corrected ChAT analysis: Set 1 WT35, aged41, 5xFAD39 and Set 2 WT36, aged42, 5xFAD38. Magenta marks the AAV reporter channel and green marks the ChAT marker channel. These panels show real sample signal context for the corrected intensity-centered ChAT quantification.

All-sample BiPVe3 real-image QC sheet

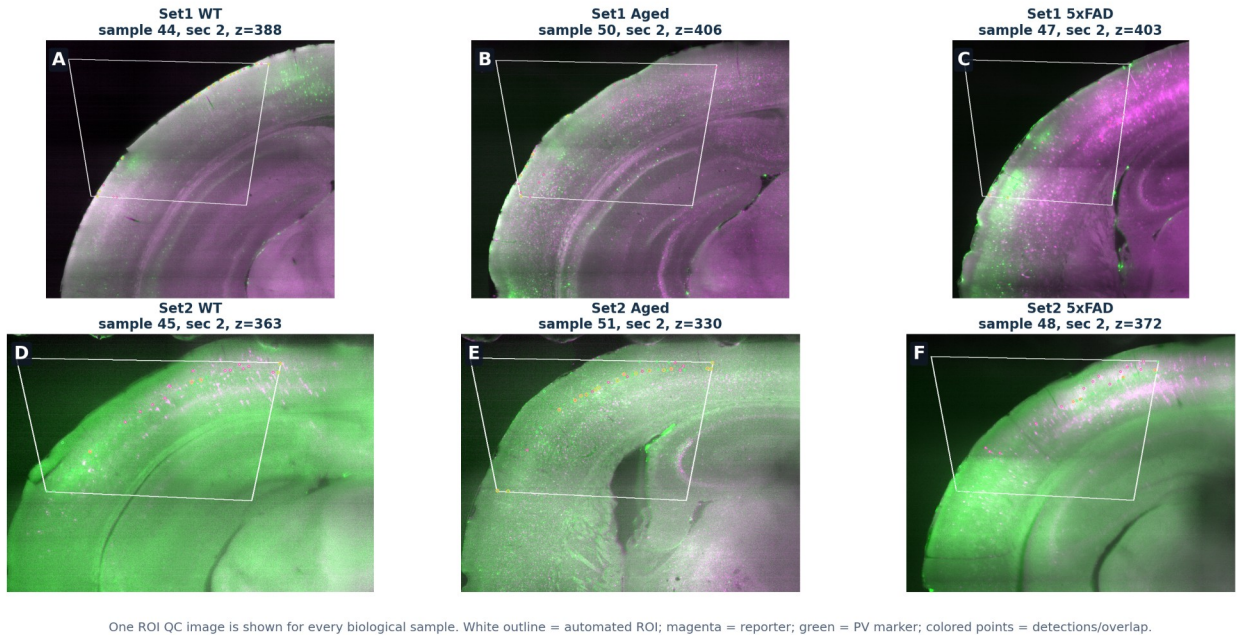
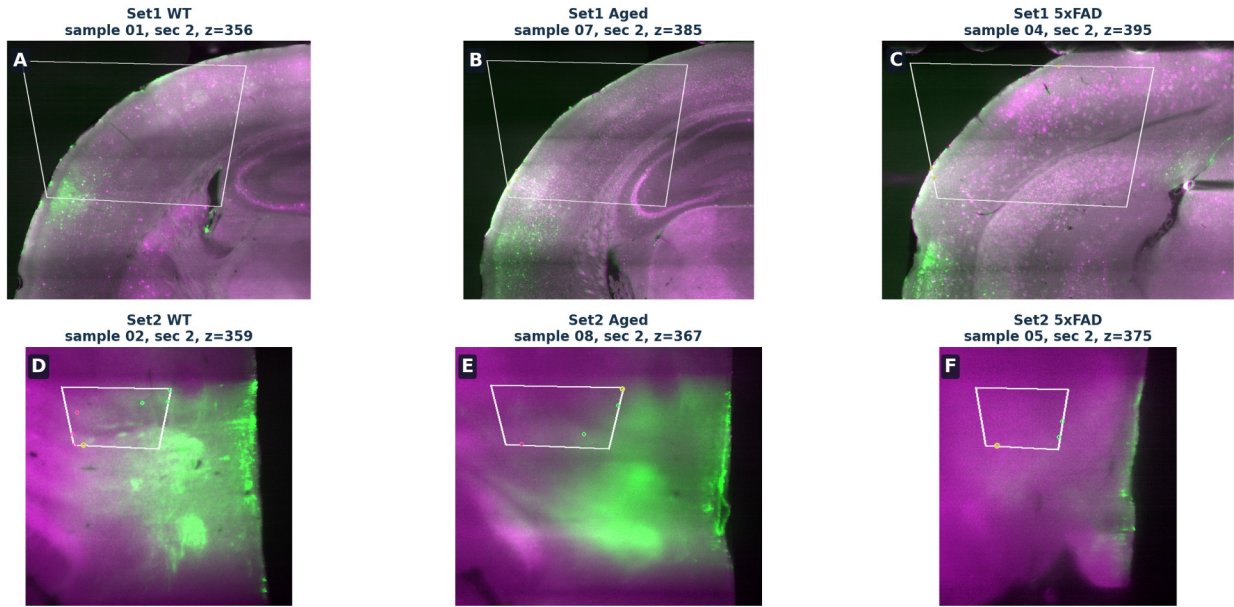


Figure 11. All-sample BiPVe3 real-image QC sheet. Panels A-F show one ROI QC image from each BiPVe3 biological sample: Set 1 WT44, aged50, 5xFAD47 and Set 2 WT45, aged51, 5xFAD48. The white outline marks the automated S1 cortical ROI; magenta marks reporter signal; green marks PV marker signal; colored points mark detected candidates and overlap calls.

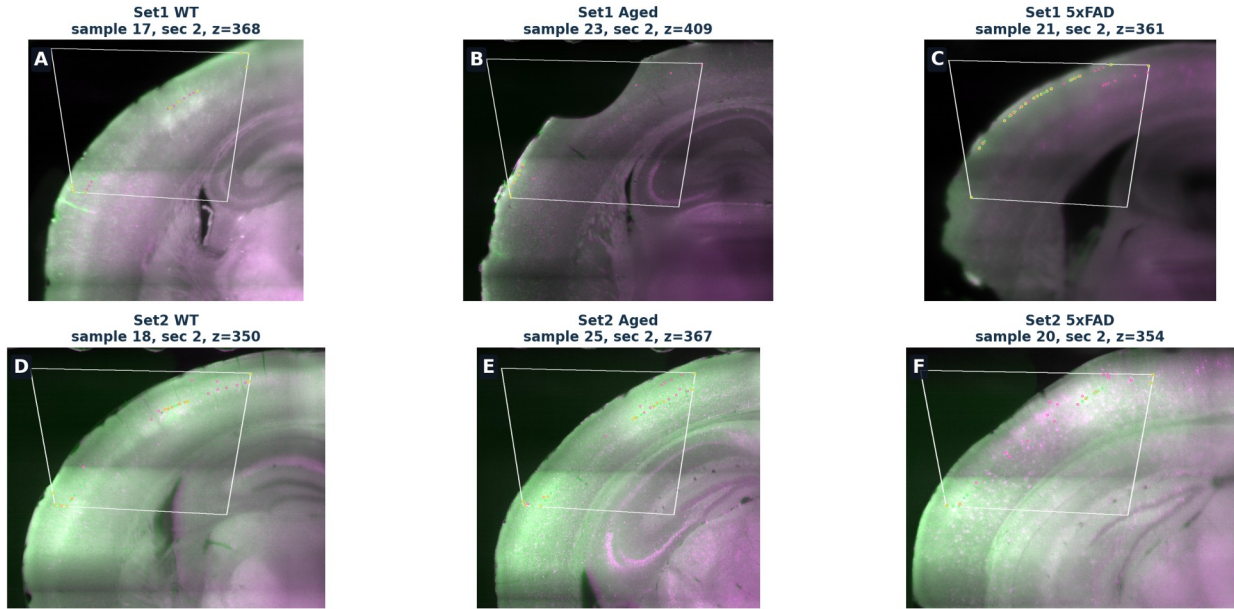
All-sample BiPVe4 real-image QC sheet



One ROI QC image is shown for every biological sample. These panels document the low-count and ROI/layer-sensitive PVe4 dataset.

Figure 12. All-sample BiPVe4 real-image QC sheet. Panels A-F show one ROI QC image from each BiPVe4 biological sample: Set 1 WT01, aged07, 5xFAD04 and Set 2 WT02, aged08, 5xFAD05. These panels make the low-count and ROI/layer sensitivity of the PVe4 analysis visible.

All-sample BiSSTe4 real-image QC sheet



One ROI QC image is shown for every biological sample. These panels provide sample-level context for the variable SST result.

Figure 13. All-sample BiSSTe4 real-image QC sheet. Panels A-F show one ROI QC image from each BiSSTe4 biological sample: Set 1 WT17, aged23, 5xFAD21 and Set 2 WT18, aged25, 5xFAD20. These panels document sample-level visual context for the variable SST result and the Set 1 artifact caveat.

The expanded cortical ROI analysis tested whether PV/SST conclusions changed when the sampled cortical band was broadened toward a more toolbox-like S1 cortical sampling window. Figure 14 shows that PVe3 5xFAD overlap remains low even in the broad ROI, but WT and aged values remain variable. For PVe4, the broader ROI removes the apparent high 5xFAD specificity seen in the narrow L2/3 ROI, supporting the interpretation that PVe4 is strongly ROI/layer sensitive. For BiSSTe4, broad-ROI sensitivity remains similar across groups but still variable. Therefore, expanded counting supports caution rather than strengthening a disease-level claim.

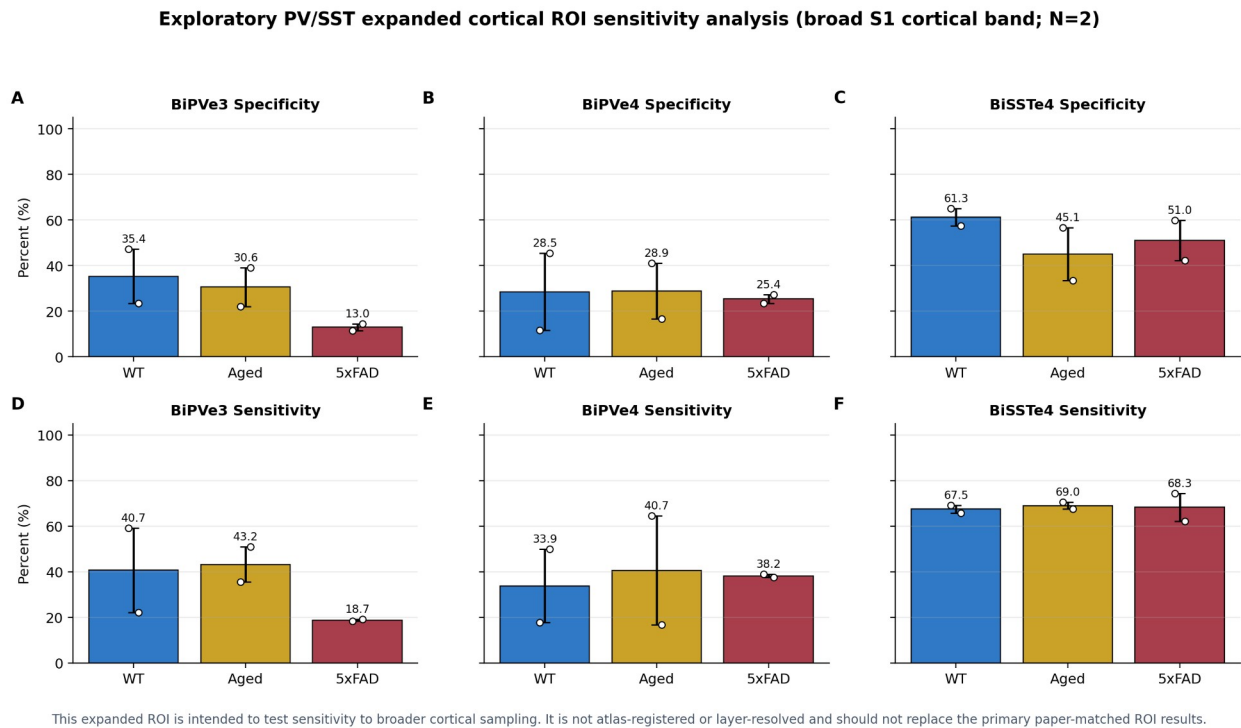


Figure 14. Exploratory PV/SST expanded cortical ROI sensitivity analysis. Panels A-C show specificity for BiPVe3, BiPVe4, and BiSSTe4, and panels D-F show sensitivity for the same enhancers using a broad S1 cortical band. This analysis addresses whether the narrow paper-matched ROI is driving the PV/SST conclusions. It is a sensitivity analysis only: the broad ROI is not atlas-registered or layer-resolved and should not replace the primary paper-matched results.

Whole-section pathology image review

The final pathology scan set adds a qualitative whole-section image layer to the ROI-level QC panels. Figure 15 shows upright representative 10x whole-section images for the four enhancer datasets, providing tissue-level context for the final scan set and connecting the raw VSI/ETS files to the thesis-level display images. The planes were chosen from the final pathology images to show intact tissue context near the anatomical levels used for cortical/hippocampal ROI review, but they were not treated as one-to-one registered counting planes. A quick display-PNG threshold QC of magenta plaque-like particles estimated similar whole-section particle densities across panels: 145.3, 144.7, 140.0, and 147.3 particles per tissue megapixel for ChAT, PVe3, PVe4, and SSTe4, respectively. These images and exploratory display-level counts are included for pathology review, tissue-context assessment, and data traceability; they are not used to calculate specificity, sensitivity, or formal plaque-proximity measurements.

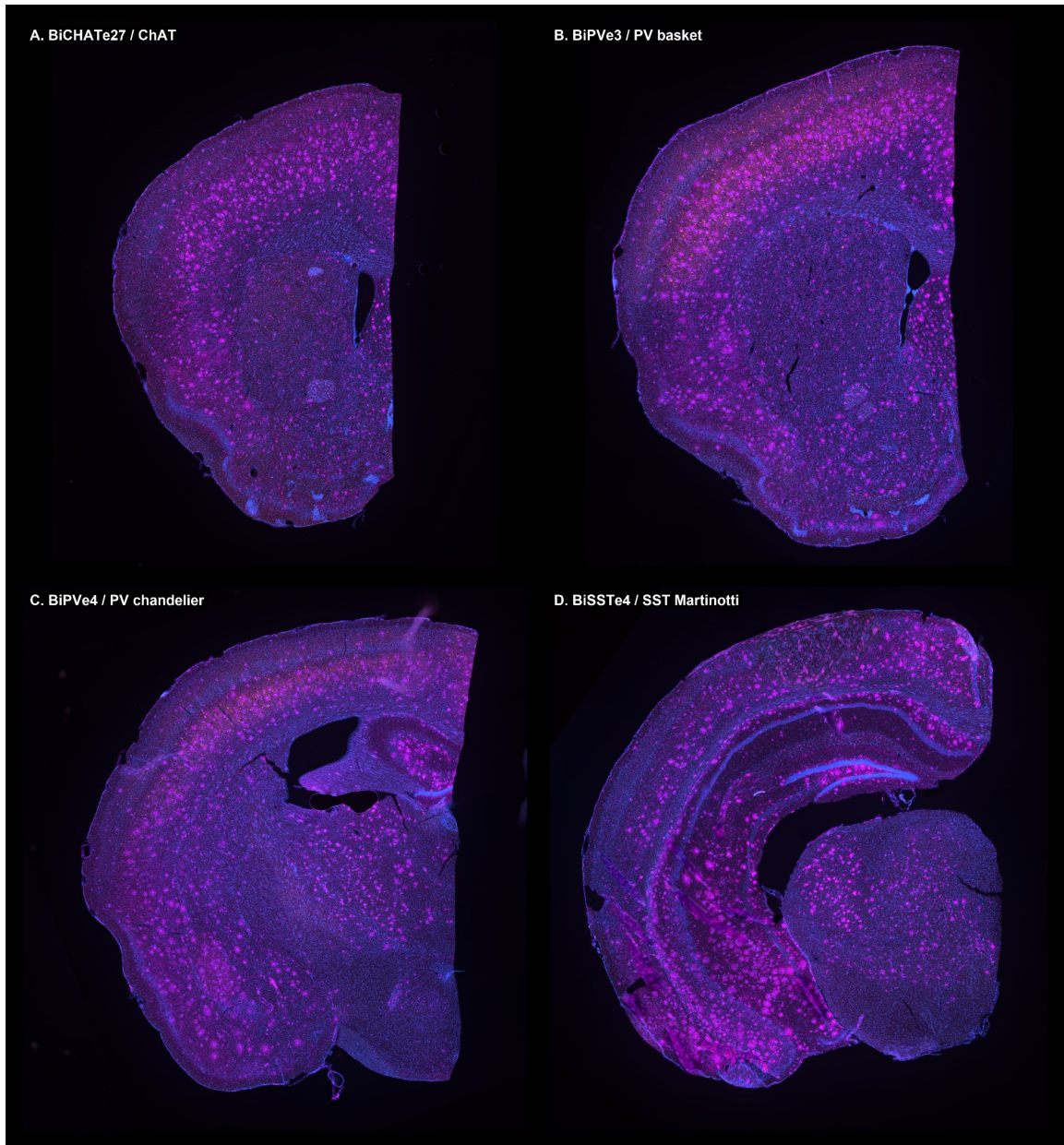


Figure 15. Whole-section pathology/immunofluorescence review images for the final pathology scan set. Panels A-D show curated pathology display PNGs from the Thesis data folder for BiCHATe27/ChAT, BiPVe3/PV basket, BiPVe4/PV chandelier, and BiSSTe4/SST Martinotti samples. The panels preserve the colors and orientation of the provided display images: DAPI is shown in blue and Cy5 is shown in a magenta/far-red display color, with other reporter/channel colors retained from the source PNGs. The selected planes provide whole-section pathology context near the cortical/hippocampal levels used for ROI-level review, but they are not atlas-registered one-to-one to the exact counting sections. Exploratory display-PNG threshold QC estimated plaque-like particle densities of 145.3, 144.7, 140.0, and 147.3 particles per tissue megapixel for panels A-D, respectively. These qualitative panels provide tissue context and were not used for specificity, sensitivity, or formal plaque-proximity calculations.

Chapter 4. Discussion and Conclusions

Main interpretation

The current dataset supports a conservative pilot model, not a conclusive disease-mechanism result, in which enhancer-AAV reporter behavior differs across cell types and interneuron subtypes in aging and 5xFAD mice. Each primary condition/enhancer comparison is based on N=2 independent brains, with sample quantity, animal-level counts, section-level QC counts, and sample-quality caveats documented in Tables 2-7 and Figures 10-15. Figure 9 and Table 3 provide the cross-enhancer basis for this pilot interpretation. The strongest methodological correction occurred in BiCHATe27: once ChAT-specific diffuse marker signal was handled with intensity-centered colocalization, WT targeting became close to the AAV toolbox reference [39]. Figure 4 and Table 4 show that this correction changes the interpretation from failed targeting to apparently stable targeting with reduced signal burden in the sampled ROI. This result indicates that counting method can strongly affect apparent specificity and sensitivity, especially when marker morphology differs across cell types [56-60].

For the all-data version, the BiCHATe27/ChAT dataset is the most internally consistent result, whereas PVe3, PVe4, and BiSSTe4 remain exploratory for different reasons. PVe3 is N=2 complete but still has between-set WT baseline variability after WT45 rescanning, as shown in Figure 5 and Table 4. Figures 10-13 add real sample images for every biological sample so that the quantitative results can be evaluated alongside visible tissue quality, ROI placement, and signal quality. Figure 14 adds an expanded cortical ROI sensitivity analysis; it supports caution because the PV/SST results remain sensitive to ROI breadth and layer definition. The same-pipeline BiSSTe4 N=2 rerun is also important because Figure 7 and Table 4 show that the Set 1

5xFAD increase is not stable after Set 2 is added. Because Set 1 may be confounded by poor perfusion or tissue-processing artifacts, the SST result should be used as a cautionary example of sample-quality sensitivity rather than as a firm disease mechanism. This caution is consistent with the broader literature showing that interneuron and SST-related changes in AD models are region- and context-dependent [14,27-29].

Why PVe3 and PVe4 may diverge

BiPVe3 and BiPVe4 both relate to PV-lineage populations, but they target different PV-associated subtypes and layers. PVe3 is interpreted as a PV basket-cell enhancer in S1 layers 2-6, while PVe4 is interpreted as a PV chandelier-cell enhancer in S1 layers 2/3 [39]. Table 1 makes this ROI distinction explicit, Figure 5 shows that PVe3 5xFAD overlap remains low after WT45 rescanning, and Figure 6 shows why the current PVe4 result should remain exploratory. The key point is methodological and biological: PV interneuron dysfunction in AD models can be subtype- and region-dependent [13-20,30-36]. Therefore, PVe3 and PVe4 should not be collapsed into one generic PV conclusion; both require manual ROI validation before mechanistic interpretation, as listed in Table 6.

AAV toolbox comparison and cortical ROI breadth

The AAV toolbox paper is used here as a WT method baseline, not as a matched disease-control dataset. The relevant toolbox validation was performed in young WT C57BL/6J mouse contexts with toolbox-specific injection, imaging, and quantification settings [39]. The present thesis metadata show that viral dose was held constant at 2.0×10^{11} genome copies across the thesis cohorts (Table 7), so viral dose should not be invoked as an explanation for within-thesis group differences. Differences between the current WT values and the published baseline can still

reflect animal age and cohort source, subtle genetic-background or colony differences among JAX, NIA, and lab 5xFAD animals, vector incubation interval, clearing and imaging conditions, ROI/layer sampling, or counting method. Because the mouse-background differences are expected to be relatively small compared with the larger methodological and age/pathology variables, they should be treated as a caveat rather than as the default explanation for the observed effects.

To test whether the PV and SST counting window should be broader and more comparable with toolbox-style cortical sampling, Figure 14 addresses this point as a sensitivity analysis. The broad ROI result does not replace the primary paper-matched ROIs, but it helps show whether the PV/SST interpretation is sensitive to layer placement and ROI breadth.

Interpreting ratios together with raw cell counts

Specificity and sensitivity are useful because they summarize targeting efficiency, but they are not sufficient by themselves. In this thesis, lower specificity does not mean that a PV, SST, or ChAT marker-positive cell is biologically the wrong identity. It means that, within the sampled ROI and marker channel, a larger fraction of reporter-positive objects are not also counted as marker-positive target cells. That pattern can occur because the enhancer labels marker-negative or weak-marker cells in the ROI, because staining or imaging makes marker detection less reliable, or because segmentation separates reporter and marker signals differently across samples. Sensitivity can fall when fewer marker-positive target cells are labeled, but it can also shift when the number of marker-positive cells in the sampled ROI changes. Figure 8 was added to address this problem visually, and Table 4 was added to show the exact animal-level counts behind the ratios. Therefore, this thesis now treats reporter-positive counts, marker-positive

counts, and overlap-positive counts as separate readouts that must be interpreted together [56-60].

This count-based view clarifies the biological question motivating the comparison. For each enhancer, the WT marker-positive count in Table 4 is the local baseline target-cell burden in the selected ROI. Reporter-positive counts ask whether the enhancer successfully labeled cells in that ROI. Overlap-positive counts ask whether labeling occurred in the intended target population. Table 5 adds section-level transparency for QC, but these sections remain technical samples rather than independent animals. A disease or aging effect is most convincing when the direction of ratio changes in Figures 4-14 is supported by a coherent count-level pattern in Tables 3-5 and by visual QC. When ratios and raw counts disagree, the result should be treated as method-sensitive until manual counting or atlas registration confirms it.

Broader use of enhancer-AAV workflows in disease models

Beyond the specific pilot dataset, this workflow could be useful as a cross-sectional screening strategy for disease models. When enhancer-AAV labeling is combined with marker staining, metadata control, and matched ROI or atlas-registered sampling, the same pipeline can ask whether disease state or age is associated with changes in target-cell abundance, marker detectability, reporter expression, or reporter-marker overlap. This is especially relevant for heterogeneous interneuron populations because broad PV or SST labels may hide subtype- and layer-specific vulnerability. The current PVe3/PVe4 contrast illustrates this point: two PV-associated enhancer readouts can behave differently, so enhancer-level analysis may provide a more refined screen than a single pan-PV measurement.

The same enhancer-AAV logic can also connect structural or histological findings to functional experiments. The enhancer-AAV toolbox was designed not only for fluorescent reporter labeling

but also for activity readout and manipulation using cargo such as GCaMP or optogenetic effectors [39]. Therefore, once a reproducible disease-associated change is identified for a cell type or subtype, a matched enhancer could in principle be used to monitor activity in that population and then perturb the same population to test whether restoring or compensating for its function improves circuit-level phenotypes. This thesis does not perform those functional rescue experiments, but it defines the validation requirements needed before moving from anatomical screening to activity tracking or causal manipulation.

Amyloid pathology interpretation

The 5xFAD comparison is biologically motivated by amyloid pathology. The final whole-section pathology scans add qualitative tissue-level image context for the four enhancer datasets (Figure 15), but they are not registered to the exact ROI counting sections. As a quick check requested during thesis review, a display-PNG threshold-and-particle-count workflow was applied to the magenta pathology-like signal in Figure 15 after tissue masking. This exploratory QC estimated 145.3, 144.7, 140.0, and 147.3 plaque-like particles per tissue megapixel for the ChAT, PVe3, PVe4, and SSt4 panels, respectively. Because the PVe3 and PVe4 display-level plaque-like particle densities were not higher than the SSt4 panel, the current Figure 15 images do not support a simple explanation in which the lower PV sensitivity is driven by uniquely higher gross plaque burden in the PV pathology panels. However, this is not a validated plaque-burden analysis; formal plaque segmentation, raw-channel thresholding, and registration to the counted ROIs would still be required before making a plaque-proximal mechanism claim.

This point matters for committee interpretation. If the final thesis needs a stronger plaque-specific mechanism claim, the next analysis should include validated amyloid-pathology

segmentation or plaque-dye/antibody quantification on the same or adjacent sections, followed by registration to the same S1 layers and ROIs used for PVe3, PVe4, and BiSSTe4. That analysis would allow plaque burden, plaque proximity, reporter-positive cells, marker-positive cells, and overlap-positive cells to be compared within the same anatomical framework. The display-level Figure 15 QC is therefore useful for detecting whether one pathology panel is grossly different from the others, but it cannot replace same-plane plaque quantification.

Technical limitations

The major limitation is that N=2 still provides limited biological replication and should not be treated as sufficient for conclusive disease-model reporting. Table 2 shows that every enhancer has N=2, but PVe3, PVe4, and BiSSTe4 remain exploratory because completion does not eliminate baseline instability, ROI/layer sensitivity, or sample-quality artifacts. A second limitation is that the analysis uses ROI-restricted section sampling rather than full atlas-registered whole-brain quantification, as explained in Table 1 and tested by the broad-ROI sensitivity analysis in Figure 14. A third limitation is algorithmic: local-maxima detection and nearest-neighbor matching are sensitive to image resolution, marker signal-to-noise ratio, threshold choice, and tissue artifacts [56-60]. A fourth limitation is pathology: Figure 15 now includes a quick display-level plaque-like particle QC, but amyloid burden was not validated with raw-channel plaque segmentation or ROI-registered plaque-proximity analysis. These issues are particularly important when comparing the current light-sheet montage analysis with the high-resolution validation datasets in the AAV toolbox paper [39,48-55].

BiSSTe4 has an additional sample-quality limitation: Set 1 may be confounded by poor perfusion or tissue-processing artifacts. This caveat is not a minor formatting note; it changes the

interpretation. Figure 7 shows the unstable group-level result, Table 4 shows the animal-level disagreement, and Table 6 records the required mitigation. The combined N=2 result should not be presented as a stable 5xFAD increase because the Set 1 direction is not reproduced by Set 2.

The analysis also separates targeting efficiency from signal burden. A sample can appear visually brighter or have more reporter-positive objects while showing lower specificity or sensitivity if the reporter signal is no longer well aligned with the target marker. This distinction is central to interpreting the Xu-lab PPT and the current automated results. Figure 8 and Table 4 make this distinction explicit by separating reporter-positive, marker-positive, and overlap-positive counts. Bright 5xFAD reporter signal does not automatically imply better targeting; conversely, lower targeting ratios do not by themselves prove biological loss.

Future validation

The highest-priority next step is manual ROI QC now that all N=2 datasets are complete. Table 6 lists the specific risk-mitigation actions rather than presenting them as a separate results figure. Manual counting should be performed on matched ROIs to validate automated specificity and sensitivity estimates, particularly for PVe3 WT Set 2, PVe4, and BiSSTe4. Whole-brain atlas registration would reduce FOV-selection bias and allow regional patterns to be described more systematically. If committee discussion requires a pathology-level claim, supplemental amyloid staining or adjacent-section amyloid labeling should be evaluated with raw-channel segmentation and ROI registration before final submission. Finally, comparison of enhancer ATAC-seq peaks in 5xFAD versus young WT tissue would help test whether disease-associated chromatin-accessibility changes are compatible with the observed reporter trends [61-68].

Conclusion

In summary, this thesis asks whether enhancer-AAV reporters maintain the same targeting and expression behavior in young WT, aged WT, and 5xFAD mouse brains. The answer from the current N=2-per-condition dataset should be interpreted as a descriptive pilot result rather than as a conclusive group-level claim. The dataset does not show a single uniform disease effect.

Instead, Figures 3-14 and Tables 1-7 support a conservative model in which enhancer-AAV readouts are cell-type dependent, ROI dependent, and technically sensitive to counting strategy and sample quality.

The strongest current conclusion is methodological and biological for BiCHATe27. Once ChAT was analyzed with a marker-appropriate intensity-centered method, WT specificity and sensitivity moved close to the published AAV toolbox baseline [39]. Figure 4 and Tables 3-4 support the validity of the corrected ChAT pipeline. Within that corrected framework, the main disease/aging-associated signal is not a collapse of targeting specificity, but a lower ChAT-associated reporter signal burden in aged and 5xFAD samples. This should be described as altered reporter/marker signal burden, not as direct proof of cholinergic cell loss [21-26].

The PV and SST enhancer results should remain cautious because sample quantity and quality limit claim strength. BiPVe3 is N=2 complete but exploratory because the WT baseline remains variable across sets, even though WT45 improved after rescan and 5xFAD overlap efficiency remains low, as shown in Figures 5, 8, 9, 11, and 14 and Tables 3-4. BiPVe4 is N=2 but exploratory because the WT baseline remains low and the sampled L2/3 ROI is technically sensitive, as shown in Figures 6, 12, and 14 and Table 3. BiSSTe4 is also N=2 but variable because Set 1 and Set 2 disagree, and Set 1 may be confounded by poor perfusion or tissue-processing artifacts, as shown in Figures 7, 13, and 14 and Tables 4-5. Therefore, the current

thesis claim is that enhancer-AAV reporter measurements can nominate candidate cell-type/subtype-specific differences across aging and AD-model states, but those candidates require larger biological replication, manual counting, matched ROI QC, atlas registration, direct amyloid-pathology validation if disease mechanism is claimed, and, ideally, chromatin-accessibility validation before being advanced toward disease-mechanism claims (Table 6).

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Appendix

Current data status and update checklist

This appendix preserves the current state of the thesis dataset so that later N=2 updates can be made without losing the provenance of the thesis. Tables 1-7 are cited in the Methods, Results, and Discussion chapters so that the appendix functions as a transparent data source rather than as disconnected supplementary material.

Table 1 explains why each ROI was chosen and how each thesis ROI maps onto the AAV toolbox validation region. This table is referenced in the Methods because ROI choice determines whether the quantitative results can be compared to the paper baseline.

Table 1. Paper-matched ROI definitions and why each ROI is used. This methods-orientation table explains how each enhancer was mapped to the anatomical region used for quantification and why that ROI is necessary for comparison with the AAV toolbox baseline. It is not a results table.

Enhancer	Target cell	Paper ROI	Thesis ROI	Why this ROI matters	Paper spec./sens.
BiCHATe27	ChAT	Caudoputamen / striatum	Current CPu/striatal ROI	Defines whether cholinergic reporter targeting is comparable to the AAV toolbox baseline.	83.6% / 70.8%
BiPVe3	PV basket	S1 somatosensory cortex, L2-6	Current same-pipeline S1 L2-6 ROI	Tests the broad cortical PV basket-cell enhancer; N=2 is complete but baseline variability requires caution.	81.9% / 60.4%
BiPVe4	PV chandelier	S1 cortex, L2/3	Current S1 L2/3 ROI	Restricts analysis to the layer where chandelier-cell targeting should be most relevant.	73.3% / 42.7%
BiSSTe4	Martinotti SST	S1 cortex, L5	Current S1 L5 ROI	Restricts analysis to the expected cortical layer for Martinotti SST targeting.	73.9% / N/R

Table 2 is the reader's key for interpreting claim strength. All four enhancer datasets now have N=2, but the table flags which completed datasets remain exploratory because of baseline instability, ROI/layer sensitivity, or sample-quality caveats.

Table 2. Current data status by enhancer. This table defines claim strength for each enhancer by separating the stable corrected ChAT result from completed but exploratory interneuron-enhancer datasets that should not yet be overinterpreted.

Enhancer	Current status	Interpretive action
BiCHATe27	N=2 complete	Use corrected intensity-centered ChAT method as primary.
BiPVe3	N=2 complete but variable	WT45 improved after rescan; keep exploratory due to between-set variability and need for expanded cortical/manual ROI QC.
BiPVe4	N=2 complete	Keep exploratory due to low WT baseline and method sensitivity.
BiSSTe4	N=2 complete but variable	Set 1 may be confounded by poor perfusion or tissue-processing artifacts; keep exploratory.

Table 3 contains the group-level specificity and sensitivity values that correspond to the main Results figures. It now includes all four N=2 enhancer datasets.

Table 3. Current group-level quantitative summary used in this thesis. This table provides the numerical specificity and sensitivity values corresponding to the main Results figures for all four N=2 enhancer datasets.

Enhancer	Group	Biological N	Specificity (%)	Sensitivity (%)	Interpretation status
BiCHATE27	WT	2	78.6	71.9	Corrected N=2 primary
BiCHATE27	Aged	2	77.6	75.1	Corrected N=2 primary
BiCHATE27	5xFAD	2	70.4	70.6	Corrected N=2 primary
BiPVe3	WT	2	48.1	49.7	N=2 complete; exploratory after WT45 rescan because between-set variability remains
BiPVe3	Aged	2	43.3	55.7	N=2 complete; exploratory after WT45 rescan because between-set variability remains
BiPVe3	5xFAD	2	18.3	20.9	N=2 complete; exploratory after WT45 rescan because between-set variability remains
BiPVe4	WT	2	35.0	25.9	N=2 exploratory
BiPVe4	Aged	2	36.0	45.0	N=2 exploratory
BiPVe4	5xFAD	2	61.1	44.4	N=2 exploratory
BiSSTe4	WT	2	52.3	62.2	N=2 variable; Set 1 artifact caveat
BiSSTe4	Aged	2	45.5	69.4	N=2 variable; Set 1 artifact caveat
BiSSTe4	5xFAD	2	50.3	65.6	N=2 variable; Set 1 artifact caveat

Table 4 is the primary raw-count appendix for biological interpretation. It is the numerical source for Figure 8 and allows the reader to see whether ratio changes come from reporter-positive counts, marker-positive counts, overlap-positive counts, or a combination of these factors.

Table 4. *Animal-level raw count table used for specificity, sensitivity, and count-burden interpretation. This table is the primary raw-count source for Figure 8 and shows whether ratio changes arise from reporter-positive counts, marker-positive counts, overlap-positive counts, or a combination of these components. For BiCHATE27, Overlap+ is the reporter-centered positive count from the corrected intensity-centered method.*

Enhancer	Set	Sample	Group	Reporter+	Marker+	Overlap+	Spec. (%)	Sens. (%)
BiCHATE27	Set1	35	WT	158	152	122	77.2	71.1
BiCHATE27	Set2	36	WT	215	154	172	80.0	72.7
BiCHATE27	Set1	41	Aged	287	261	218	76.0	72.8
BiCHATE27	Set2	42	Aged	212	128	168	79.2	77.3
BiCHATE27	Set1	39	5xFAD	219	288	124	56.6	71.2
BiCHATE27	Set2	38	5xFAD	159	137	134	84.3	70.1
BiPVe3	Set1	44	WT	60	52	43	71.7	82.7
BiPVe3	Set2	45	WT	45	66	11	24.4	16.7
BiPVe3	Set1	50	Aged	63	36	19	30.2	52.8
BiPVe3	Set2	51	Aged	78	75	44	56.4	58.7
BiPVe3	Set1	47	5xFAD	66	50	10	15.2	20.0
BiPVe3	Set2	48	5xFAD	56	55	12	21.4	21.8
BiPVe4	Set1	01	WT	10	17	2	20.0	11.8
BiPVe4	Set2	02	WT	8	10	4	50.0	40.0
BiPVe4	Set1	07	Aged	31	24	12	38.7	50.0
BiPVe4	Set2	08	Aged	6	5	2	33.3	40.0
BiPVe4	Set1	04	5xFAD	19	18	8	42.1	44.4
BiPVe4	Set2	05	5xFAD	5	9	4	80.0	44.4
BiSSTe4	Set1	17	WT	62	53	27	43.5	50.9
BiSSTe4	Set2	18	WT	59	49	36	61.0	73.5
BiSSTe4	Set1	23	Aged	47	34	24	51.1	70.6
BiSSTe4	Set2	25	Aged	75	44	30	40.0	68.2
BiSSTe4	Set1	21	5xFAD	69	55	46	66.7	83.6
BiSSTe4	Set2	20	5xFAD	56	40	19	33.9	47.5

Table 5 reports the section-level counts used for QC. These values provide transparency for ROI and section selection, but they are not treated as independent biological replicates in the Results or Discussion. For readability, the section-level table is split by enhancer as Table 5A-D.

Table 5A. Section-level raw count table for BiCHATE27. These section-level values support ROI QC and transparency for the automated counting workflow, but they are technical samples and are not treated as independent biological replicates.

Enhancer	Set	Sample	Group	Sec.	Z	ROI mm2	Reporter+	Marker+	Overlap+
BiCHATE27	Set1	35	WT	1	258	3.45	62	65	56
BiCHATE27	Set1	35	WT	2	290	3.54	57	57	45
BiCHATE27	Set1	35	WT	3	322	1.79	39	30	21
BiCHATE27	Set2	36	WT	1	242	2.64	75	52	63
BiCHATE27	Set2	36	WT	2	272	2.84	81	46	58
BiCHATE27	Set2	36	WT	3	302	3.00	59	56	51
BiCHATE27	Set1	41	Aged	1	258	2.97	84	88	59
BiCHATE27	Set1	41	Aged	2	290	3.12	94	81	75
BiCHATE27	Set1	41	Aged	3	322	3.28	109	92	84
BiCHATE27	Set2	42	Aged	1	243	2.46	67	45	51
BiCHATE27	Set2	42	Aged	2	274	2.53	82	41	60
BiCHATE27	Set2	42	Aged	3	304	2.60	63	42	57
BiCHATE27	Set2	38	5xFAD	1	243	2.30	50	43	45
BiCHATE27	Set2	38	5xFAD	2	274	2.58	61	46	52
BiCHATE27	Set2	38	5xFAD	3	304	2.74	48	48	37
BiCHATE27	Set1	39	5xFAD	1	255	2.82	66	91	36
BiCHATE27	Set1	39	5xFAD	2	287	3.12	69	87	34
BiCHATE27	Set1	39	5xFAD	3	318	3.46	84	110	54

Table 5B. Section-level raw count table for BiPVe3. These section-level values support ROI QC and transparency for the automated counting workflow, but they are technical samples and are not treated as independent biological replicates.

Enhancer	Set	Sample	Group	Sec.	Z	ROI mm2	Reporter+	Marker+	Overlap+
BiPVe3	Set1	44	WT	1	324	1.10	19	16	12
BiPVe3	Set1	44	WT	2	388	1.08	22	18	15
BiPVe3	Set1	44	WT	3	421	1.03	19	18	16
BiPVe3	Set2	45	WT	1	302	0.92	16	25	2
BiPVe3	Set2	45	WT	2	363	0.86	17	17	5
BiPVe3	Set2	45	WT	3	393	0.90	12	24	4
BiPVe3	Set1	50	Aged	1	338	1.08	23	11	6
BiPVe3	Set1	50	Aged	2	406	1.04	22	7	5
BiPVe3	Set1	50	Aged	3	439	0.98	18	18	8
BiPVe3	Set2	51	Aged	1	275	0.92	31	28	20
BiPVe3	Set2	51	Aged	2	330	0.82	26	26	17
BiPVe3	Set2	51	Aged	3	358	0.77	21	21	7
BiPVe3	Set1	47	5xFAD	1	336	1.74	32	19	3
BiPVe3	Set1	47	5xFAD	2	403	1.52	19	21	2
BiPVe3	Set1	47	5xFAD	3	436	1.06	15	10	5
BiPVe3	Set2	48	5xFAD	1	310	0.89	25	18	7
BiPVe3	Set2	48	5xFAD	2	372	0.94	17	19	4
BiPVe3	Set2	48	5xFAD	3	403	0.89	14	18	1

Table 5C. Section-level raw count table for BiPVe4. These section-level values support ROI QC and transparency for the automated counting workflow, but they are technical samples and are not treated as independent biological replicates.

Enhancer	Set	Sample	Group	Sec.	Z	ROI mm2	Reporter+	Marker+	Overlap+
BiPVe4	Set1	01	WT	1	297	0.42	5	7	0
BiPVe4	Set1	01	WT	2	356	0.38	2	5	0
BiPVe4	Set1	01	WT	3	386	0.37	3	5	2
BiPVe4	Set2	02	WT	1	300	0.10	3	4	3
BiPVe4	Set2	02	WT	2	359	0.11	4	4	1
BiPVe4	Set2	02	WT	3	389	0.07	1	2	0
BiPVe4	Set1	07	Aged	1	321	0.40	14	10	5
BiPVe4	Set1	07	Aged	2	385	0.37	7	8	4
BiPVe4	Set1	07	Aged	3	417	0.42	10	6	3
BiPVe4	Set2	08	Aged	1	306	0.13	3	1	1
BiPVe4	Set2	08	Aged	2	367	0.11	2	3	1
BiPVe4	Set2	08	Aged	3	398	0.03	1	1	0
BiPVe4	Set1	04	5xFAD	1	329	0.30	6	6	2
BiPVe4	Set1	04	5xFAD	2	395	0.26	6	7	3
BiPVe4	Set1	04	5xFAD	3	428	0.32	7	5	3
BiPVe4	Set2	05	5xFAD	1	312	0.05	1	3	1
BiPVe4	Set2	05	5xFAD	2	375	0.05	1	3	1
BiPVe4	Set2	05	5xFAD	3	406	0.13	3	3	2

Table 5D. Section-level raw count table for BiSSTe4. These section-level values support ROI QC and transparency for the automated counting workflow, but they are technical samples and are not treated as independent biological replicates.

Enhancer	Set	Sample	Group	Sec.	Z	ROI mm2	Reporter+	Marker+	Overlap+
BiSSTe4	Set1	17	WT	1	306	0.89	20	16	8
BiSSTe4	Set1	17	WT	2	368	0.93	18	16	10
BiSSTe4	Set1	17	WT	3	398	0.91	24	21	9
BiSSTe4	Set2	18	WT	1	292	0.88	15	12	10
BiSSTe4	Set2	18	WT	2	350	0.84	24	16	13
BiSSTe4	Set2	18	WT	3	380	0.83	20	21	13
BiSSTe4	Set1	23	Aged	1	340	0.91	17	16	9
BiSSTe4	Set1	23	Aged	2	409	0.98	13	9	8
BiSSTe4	Set1	23	Aged	3	443	1.01	17	9	7
BiSSTe4	Set2	25	Aged	1	306	0.72	30	13	8
BiSSTe4	Set2	25	Aged	2	367	0.78	25	18	12
BiSSTe4	Set2	25	Aged	3	398	0.80	20	13	10
BiSSTe4	Set2	20	5xFAD	1	295	0.79	15	14	5
BiSSTe4	Set2	20	5xFAD	2	354	0.75	20	13	6
BiSSTe4	Set2	20	5xFAD	3	384	0.74	21	13	8
BiSSTe4	Set1	21	5xFAD	1	300	0.84	15	14	12
BiSSTe4	Set1	21	5xFAD	2	361	1.36	31	19	18
BiSSTe4	Set1	21	5xFAD	3	391	1.62	23	22	16

Table 6 lists the limitations that directly constrain the thesis claim. These actions are handled as internal validation tasks rather than as a standalone results figure.

Table 6. Limitations and validation steps. This table links each major interpretation risk to the validation step needed before the corresponding result can be promoted from pilot observation to stronger thesis claim.

Limitation	Risk	Planned mitigation
Low biological N	All enhancers are N=2, but N=2 is still limited	Use descriptive interpretation; avoid inferential statistics.
PVe3 baseline instability	WT45 improved after rescan, but Set1/Set2 WT variability remains	Manual ROI QC, expanded cortical counting, and matched-layer review before strong PV-basket claim.
SSTe4 sample quality	Set 1 poor perfusion or tissue-processing artifact risk	Manual ROI QC; avoid stable 5xFAD-increase claim.
Technical section sampling	Sections are not independent animals	Report as technical support only.
ROI/layer uncertainty	Most relevant for PVe4	Manual layer-matched counting.
Expanded cortical ROI sensitivity	Broad PV/SST ROI is not atlas-registered or layer-resolved	Use Figure 14 as sensitivity analysis only; keep primary paper-matched ROI results.
Amyloid pathology not directly measured	5xFAD interpretation is genotype/model-based rather than plaque-burden-based	Consider A-beta or amyloid-dye staining on same/adjacent sections before making plaque-level claims.
Resolution/downsampling	May depress double-positive ratios	Interpret paper comparison cautiously.
No chromatin validation yet	Enhancer mechanism unconfirmed	Compare ATAC-seq peaks if available.

Table 7 summarizes the animal- and brain-level metadata needed to interpret the biological sample provenance. Entries were extracted from the hippocampal GABA enhancer experiment log and cross-checked against the light-sheet sample log where matching processing batches were present.

Table 7. Animal and brain metadata for biological samples included in quantitative analyses. This table lists experiment-log identifiers, genotype, sex, age at vector delivery, viral dose, injection date, vector incubation, collection date, age at collection, and collection/processing notes for the 24 biological samples used in the thesis analyses.

Enhancer	Sample / set / log ID	Group / genotype / sex	Age at vector delivery	Vector delivery	Collection / processing
BiCHATe27	Set1 35 / hipp-GABAe_M35	WT / C57BL6/J / M	66 d (9.4 wk)	2.0E+11 GC; 2024-11-27; incubation 26 d	92 d (13.1 wk); 2024-12-23; heparinized PBS +4%PFA (halved)
BiCHATe27	Set2 36 / hipp-GABAe_M36	WT / C57BL6/J / M	66 d (9.4 wk)	2.0E+11 GC; 2024-11-27; incubation 26 d	92 d (13.1 wk); 2024-12-23; heparinized PBS +4%PFA
BiCHATe27	Set1 41 / hipp-GABAe_M41	Aged / C57BL6/J / M	94 wk (DOB not recorded)	2.0E+11 GC; 2024-11-27; incubation 26 d	~97.7 wk (DOB not recorded); 2024-12-23; heparinized PBS +4%PFA (halved)
BiCHATe27	Set2 42 / hipp-GABAe_M42	Aged / C57BL6/J / M	94 wk (DOB not recorded)	2.0E+11 GC; 2024-11-27; incubation 26 d	~97.7 wk (DOB not recorded); 2024-12-23; heparinized PBS +4%PFA
BiCHATe27	Set1 39 / hipp-GABAe_M39	5xFAD / 5xFAD / M	428 d (61.1 wk)	2.0E+11 GC; 2024-11-27; incubation 26 d	454 d (64.9 wk); 2024-12-23; heparinized PBS +4%PFA (halved)
BiCHATe27	Set2 38 / hipp-GABAe_M38	5xFAD / 5xFAD / M	428 d (61.1 wk)	2.0E+11 GC; 2024-11-27; incubation 26 d	454 d (64.9 wk); 2024-12-23; heparinized PBS +4%PFA
BiPVe3	Set1 44 / hipp-GABAe_M44	WT / C57BL6/J / M	83 d (11.9 wk)	2.0E+11 GC; 2024-12-26; incubation 28 d	111 d (15.9 wk); 2025-01-23; heparinized PBS +4%PFA (halved)
BiPVe3	Set2 45 / hipp-GABAe_M45	WT / C57BL6/J / M	83 d (11.9 wk)	2.0E+11 GC; 2024-12-26; incubation 28 d	111 d (15.9 wk); 2025-01-23; heparinized PBS +4%PFA
BiPVe3	Set1 50 / hipp-GABAe_M50	Aged / C57BL6/J / M	94 wk (DOB not recorded)	2.0E+11 GC; 2024-12-26; incubation 28 d	~98.0 wk (DOB not recorded); 2025-01-23; heparinized PBS +4%PFA (halved)
BiPVe3	Set2 51 / hipp-GABAe_M51	Aged / C57BL6/J / M	94 wk (DOB not recorded)	2.0E+11 GC; 2024-12-26; incubation 28 d	~98.0 wk (DOB not recorded); 2025-01-23; heparinized PBS +4%PFA
BiPVe3	Set1 47 / hipp-GABAe_M47	5xFAD / 5xFAD / M	437 d (62.4 wk)	2.0E+11 GC; 2024-12-26; incubation 28 d	465 d (66.4 wk); 2025-01-23; heparinized PBS +4%PFA (halved)
BiPVe3	Set2 48 / hipp-GABAe_M48	5xFAD / 5xFAD / M	437 d (62.4 wk)	2.0E+11 GC; 2024-12-26; incubation 28 d	465 d (66.4 wk); 2025-01-23; heparinized PBS +4%PFA
BiPVe4	Set1 01 / hipp-GABAe_M1	WT / C57BL6/J / M	59 d (8.4 wk)	2.0E+11 GC; 2024-11-08; incubation 25 d	84 d (12.0 wk); 2024-12-03; heparinized PBS +4%PFA (halved)
BiPVe4	Set2 02 / hipp-GABAe_M2	WT / C57BL6/J / M	58 d (8.3 wk)	2.0E+11 GC; 2024-11-08; incubation 25 d	83 d (11.9 wk); 2024-12-03; heparinized PBS +4%PFA
BiPVe4	Set1 07 / hipp-GABAe_M7	Aged / C57BL6/J / M	104 wk (DOB not recorded)	2.0E+11 GC; 2024-11-08; incubation 25 d	~107.6 wk (DOB not recorded); 2024-12-03; heparinized PBS +4%PFA (halved)

Enhancer	Sample / set / log ID	Group / genotype / sex	Age at vector delivery	Vector delivery	Collection / processing
BiPVe4	Set2 08 / hipp-GABAe_M8	Aged / C57BL6/J / M	104 wk (DOB not recorded)	2.0E+11 GC; 2024-11-08; incubation 25 d	~107.6 wk (DOB not recorded); 2024-12-03; heparinized PBS +4%PFA
BiPVe4	Set1 04 / hipp-GABAe_M4	5xFAD / 5xFAD / M	409 d (58.4 wk)	2.0E+11 GC; 2024-11-08; incubation 25 d	434 d (62.0 wk); 2024-12-03; heparinized PBS +4%PFA (halved)
BiPVe4	Set2 05 / hipp-GABAe_M5	5xFAD / 5xFAD / M	409 d (58.4 wk)	2.0E+11 GC; 2024-11-08; incubation 25 d	434 d (62.0 wk); 2024-12-03; heparinized PBS +4%PFA
BiSSTe4	Set1 17 / hipp-GABAe_M17	WT / C57BL6/J / M	78 d (11.1 wk)	2.0E+11 GC; 2024-11-27; incubation 26 d	104 d (14.9 wk); 2024-12-23; suboptimal perfusion noted, heparinized PBS +4%PFA (halved)
BiSSTe4	Set2 18 / hipp-GABAe_M18	WT / C57BL6/J / M	78 d (11.1 wk)	2.0E+11 GC; 2024-11-27; incubation 26 d	104 d (14.9 wk); 2024-12-23; heparinized PBS +4%PFA
BiSSTe4	Set1 23 / hipp-GABAe_M23	Aged / C57BL6/J / M	94 wk (DOB not recorded)	2.0E+11 GC; 2024-11-27; incubation 26 d	~97.7 wk (DOB not recorded); 2024-12-23; heparinized PBS +4%PFA (halved)
BiSSTe4	Set2 25 / hipp-GABAe_M25	Aged / C57BL6/J / M	94 wk (DOB not recorded)	2.0E+11 GC; 2024-11-27; incubation 26 d	~97.7 wk (DOB not recorded); 2024-12-23; heparinized PBS +4%PFA
BiSSTe4	Set1 20 / hipp-GABAe_M20	5xFAD / 5xFAD / M	428 d (61.1 wk)	2.0E+11 GC; 2024-11-27; incubation 26 d	454 d (64.9 wk); 2024-12-23; heparinized PBS +4%PFA
BiSSTe4	Set2 21 / hipp-GABAe_M21	5xFAD / 5xFAD / M	428 d (61.1 wk)	2.0E+11 GC; 2024-11-27; incubation 26 d	454 d (64.9 wk); 2024-12-23; heparinized PBS +4%PFA (halved)

Note. All rows received 2.0E+11 genome copies of enhancer-AAV reporter. For aged WT mice without date of birth recorded in the experiment log, collection age is approximated from the recorded age at vector delivery plus the incubation interval.