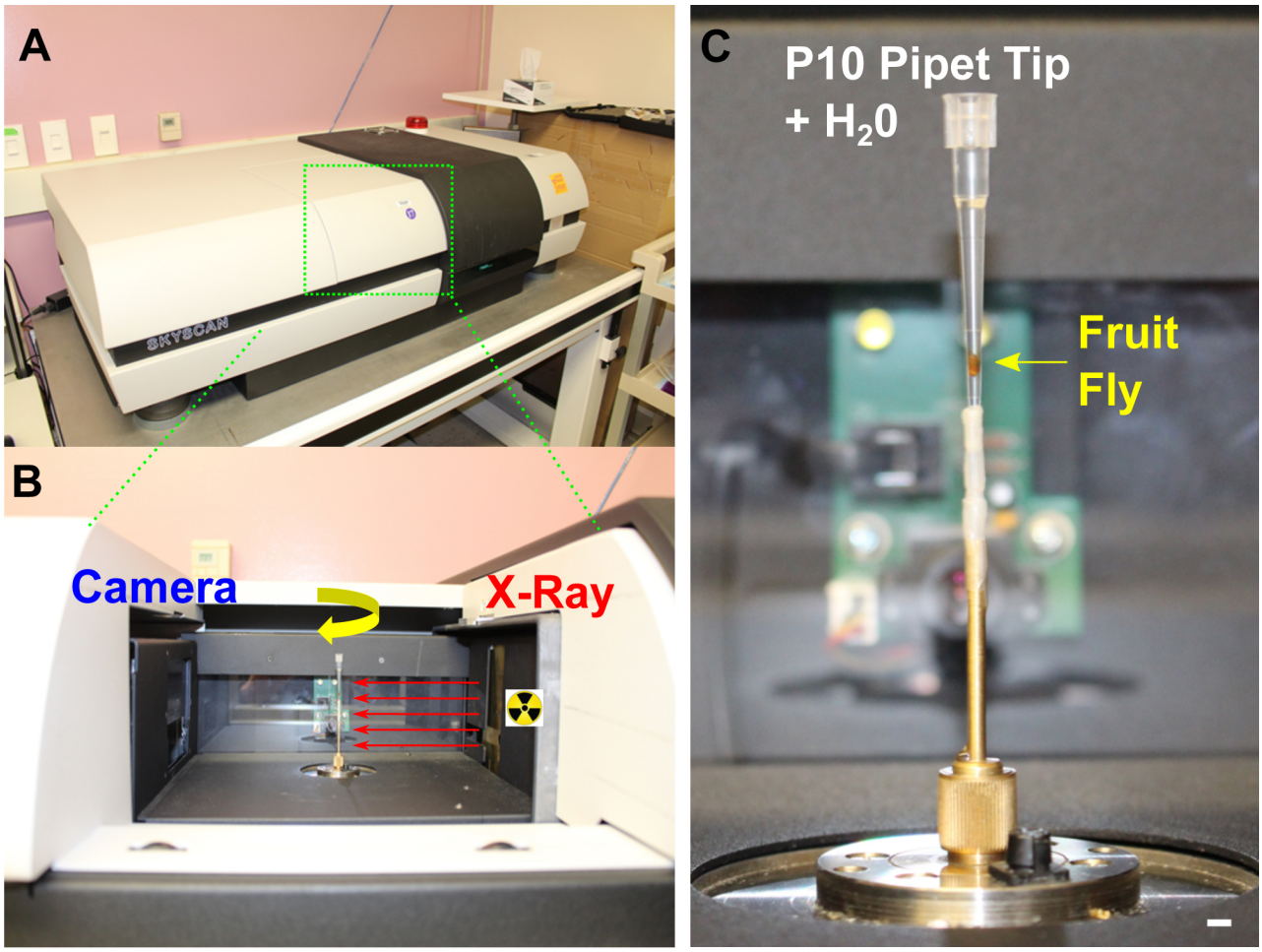


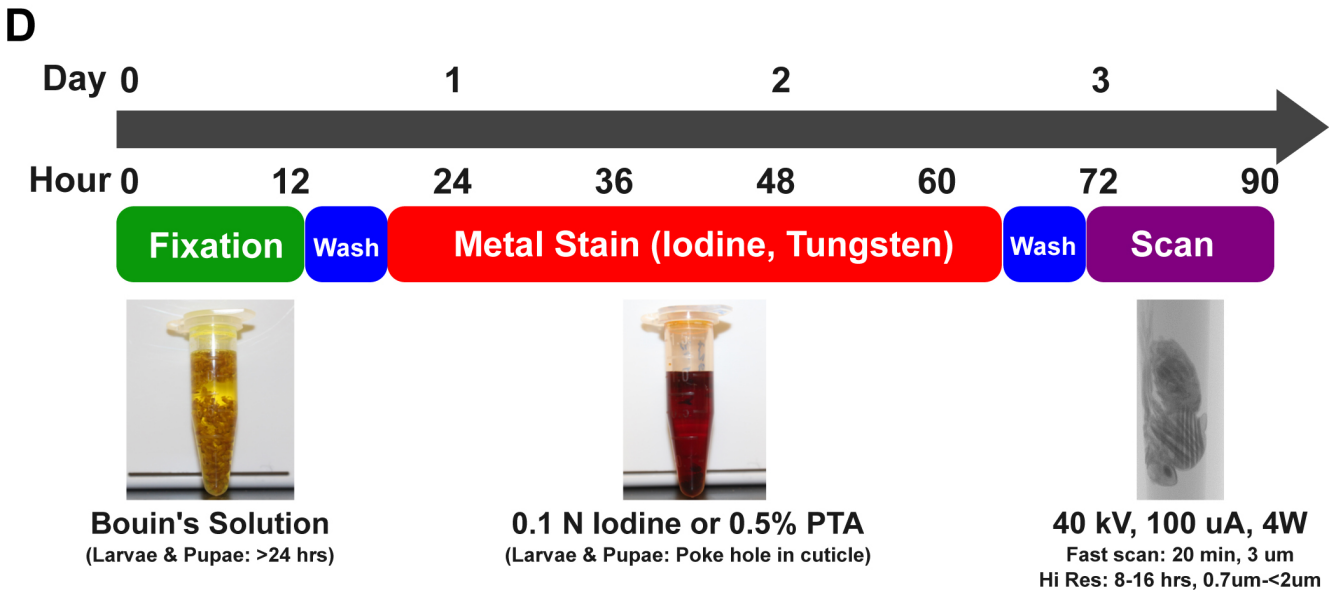


Figure S1. Overview of µ-CT.

(A) The benchtop scanner utilized for this study, a Skyscan 1172 (Bruker). (B) Open door view of the imaging path. X-rays are generated by the source at the right and travel towards the camera (left), which detects attenuation of the X-rays as they pass through a rotating sample in order to generate contrast. (C) Close up view of a sample ready for scanning in the rotating sample holder. Position of the fruit fly is indicated by arrow. Note the pipet tip is not perfectly parallel to the long axis of rotation here, which will result in a lower quality reconstruction. (D) Graphical representation of the labelling protocol outlined in *Methods*. Scale Bars (C) = 2 mm.

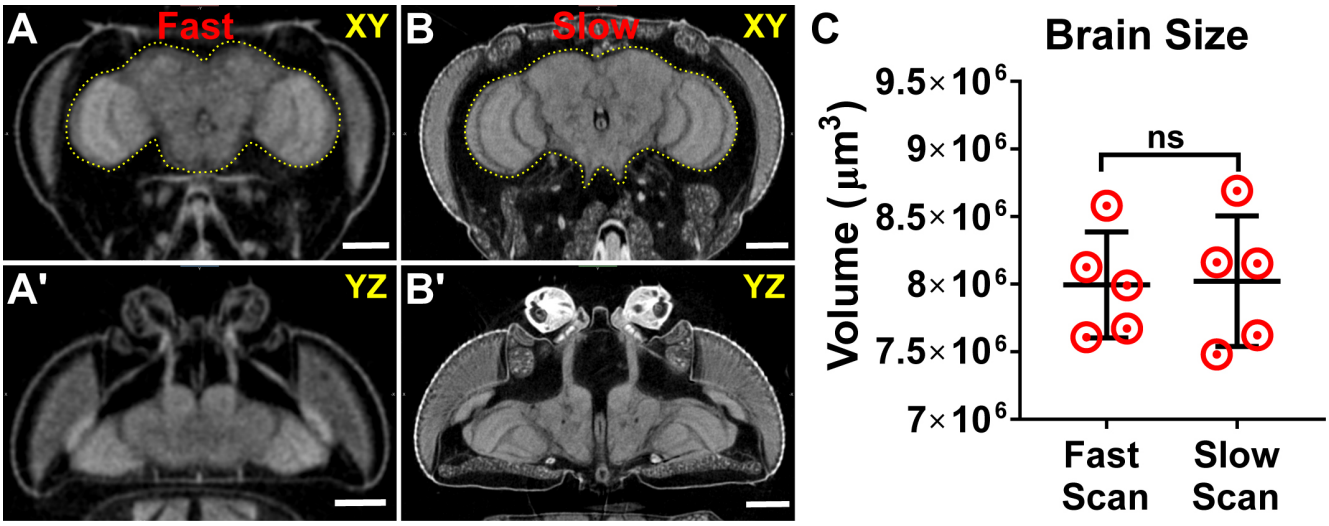


Figure S2. Imaging parameters for µ-CT.

Comparison of adult fly brains scanned in fast mode (~20 min) and slow mode (~8 hrs). (A) XY view of brain scanned in fast mode. (A') YZ view of brain scanned in fast mode. (B) XY view of brain scanned in slow mode. (B') YZ view of brain scanned in slow mode. (C) Volume measurements (μm^3) of brains from slow and fast scans (yellow outline in (A) & (B)). Image pixel sizes: 3 µm (fast); 1 µm (slow). *n= 5* brains, Welch's t-test. *ns = not significant.* Scale Bars = 100 µm.

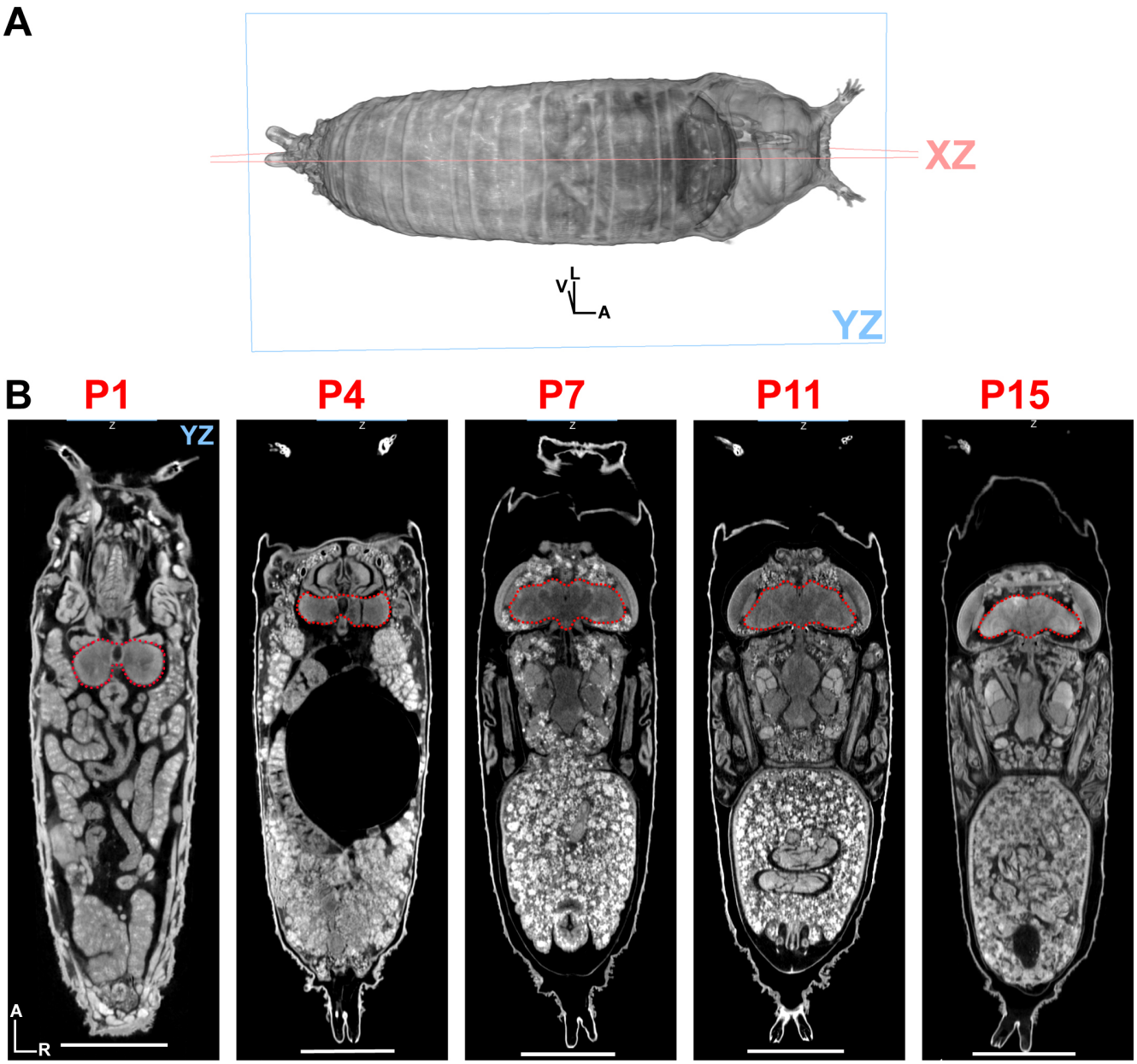


Figure S3, Related to Figure 2.

(A) 3D representation of a late stage (P15) pupae, showing body axes (V (Ventral), A (Anterior), L (Left)) and imaging planes XZ and YZ. (B) 2D representation of pupal development at P1, P3/4, P7, P11 and P15 shown in YZ. Brain is outlined in red. Body axes: anterior (A), right (R) Scale Bars (B) = 500 µm.

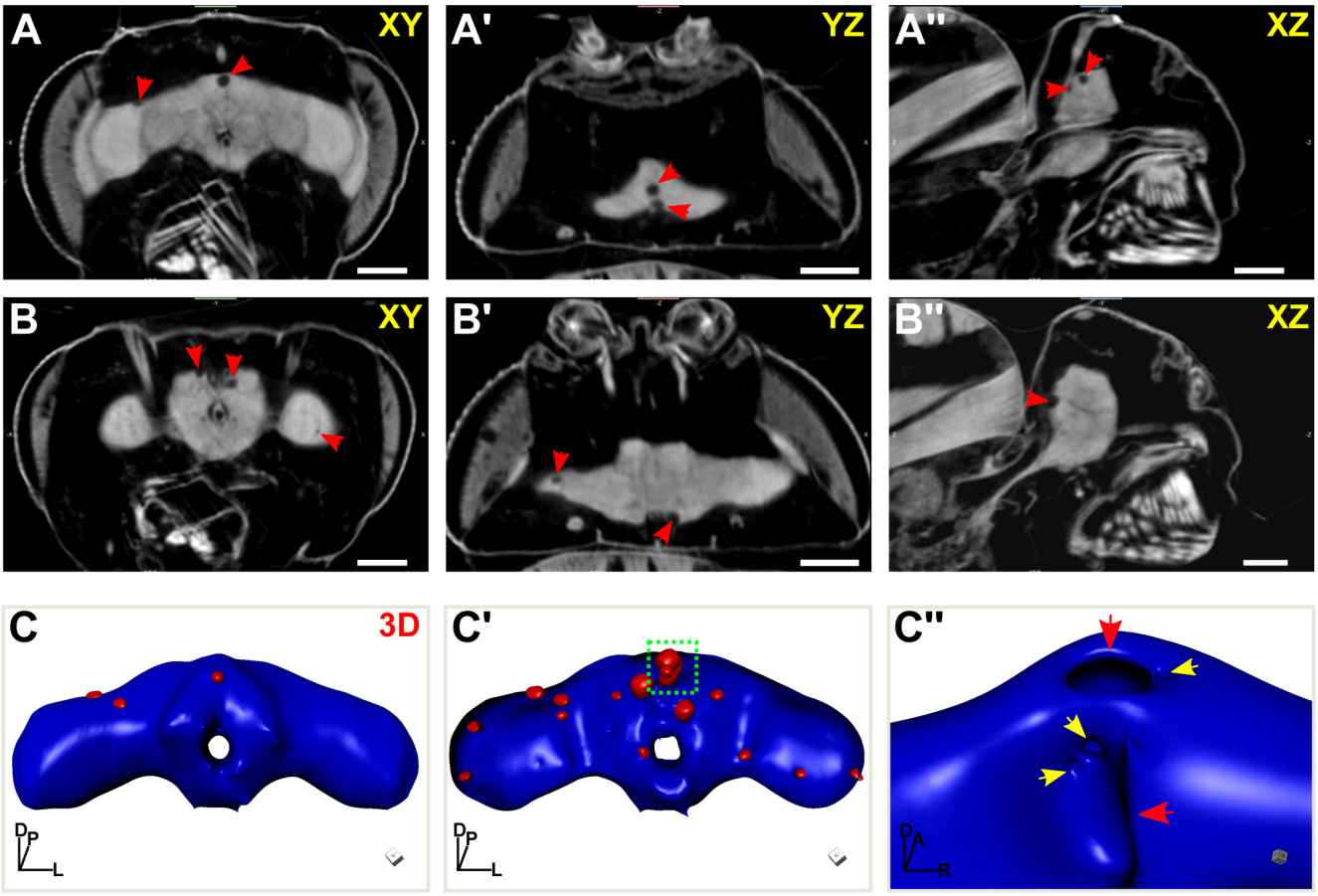
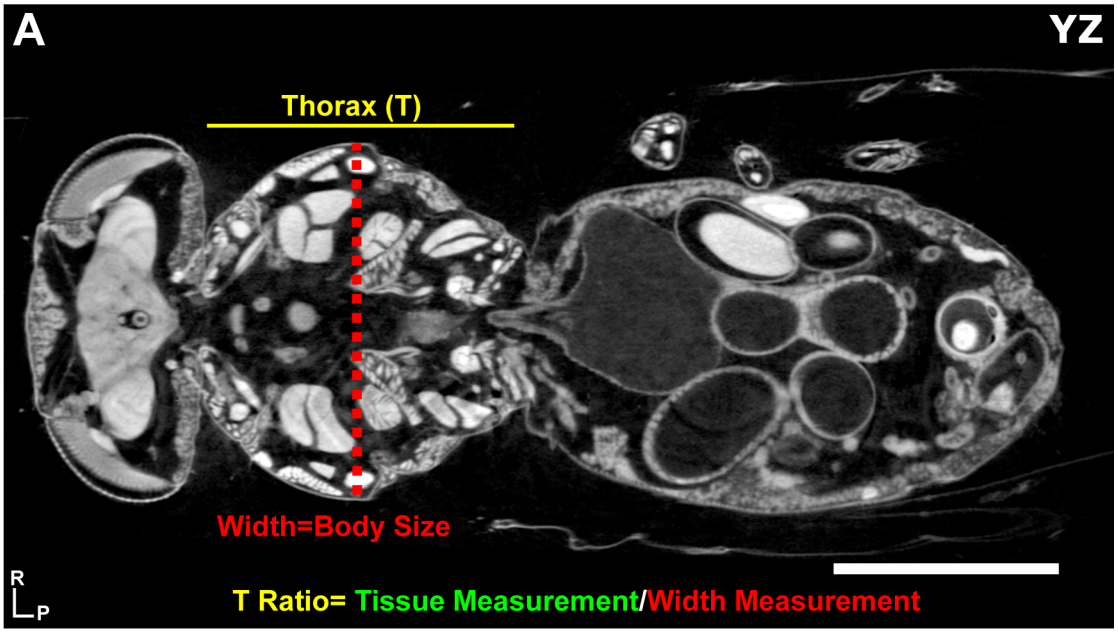
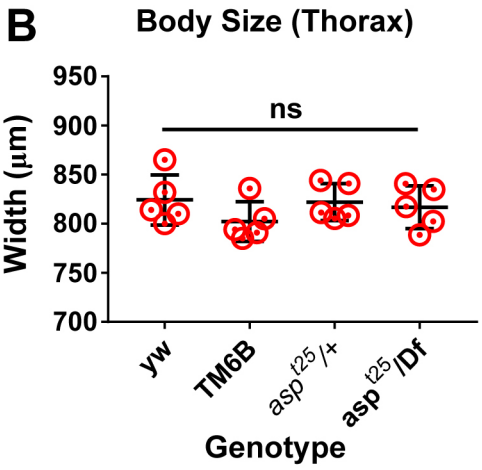
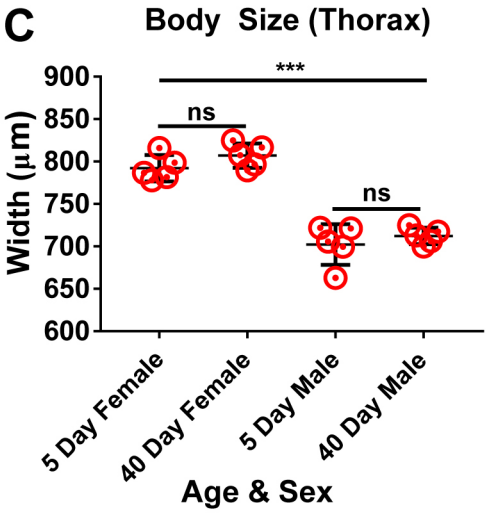


Figure S4. Aging in the adult fly brain.

Neurodegeneration in a 40-day old female adult fruit fly brain, viewed by µ-CT. (A)-(B) Two different XY, (A')-(B') YZ and (A")-(B") XZ 2D views are shown to highlight vacuoles (red arrowheads) present throughout the brain. (C) 3D rendering of brain (blue) with vacuoles shown in red, (C') with the brain partially cut away to reveal vacuole location. (C") Zoom of the green boxed region in (C'), showing large and small vacuoles (red and yellow arrowheads, respectively; 3D rendering of vacuoles has been turned off). (D) Morphometric analysis of vacuole structures and fiber tracts, measuring the "sphericity" of each structure. Sphericity was measured for each of the vacuole structures (n=10) and 2 pairs of fiber tracts (inferior fiber system (IFS) and medial antennal lobe tract (mALT); see Figure 7). Image pixel size: 1.4 µm. Body axes are indicated (D (Dorsal), P (Posterior), A (Anterior), L (Left), R (Right)). Scale Bars = 100 µm.







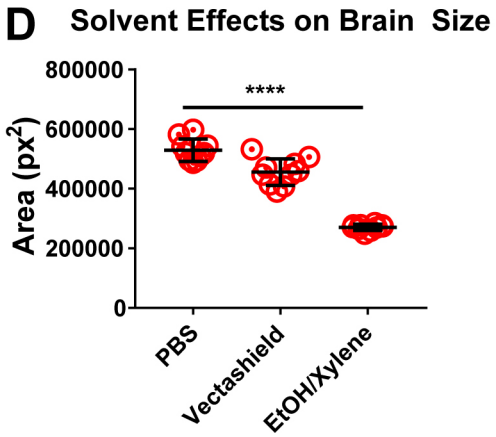


Figure S5, Related to Figure 7.

Thorax width as an accurate predictor of adult *Drosophila* body mass. (A) 2D YZ view (dorsal perspective) of an adult female fly denoting the thorax width measurement that is used as a proxy for body size normalization to derive the T-Ratio. (B) Thorax width measurements from 4 different adult female genotypes, including the asp^{t25}/Df mutant. (C) Thorax width measurements from adult male and female flies, aged for 5 or 40 days. (D) Effect of solvents on brain size. Chemical clearing (EtOH/Xylene) significantly reduces brain size by ~50% compared to untreated tissue._ *n= 5 (B & C), =10 (D)*, Welch's t-test. *ns, P>0.05; ***, P≤0.001; ****, P≤0.0001.* Body axes: right (R), posterior (P). Scale Bar = 500 µm.

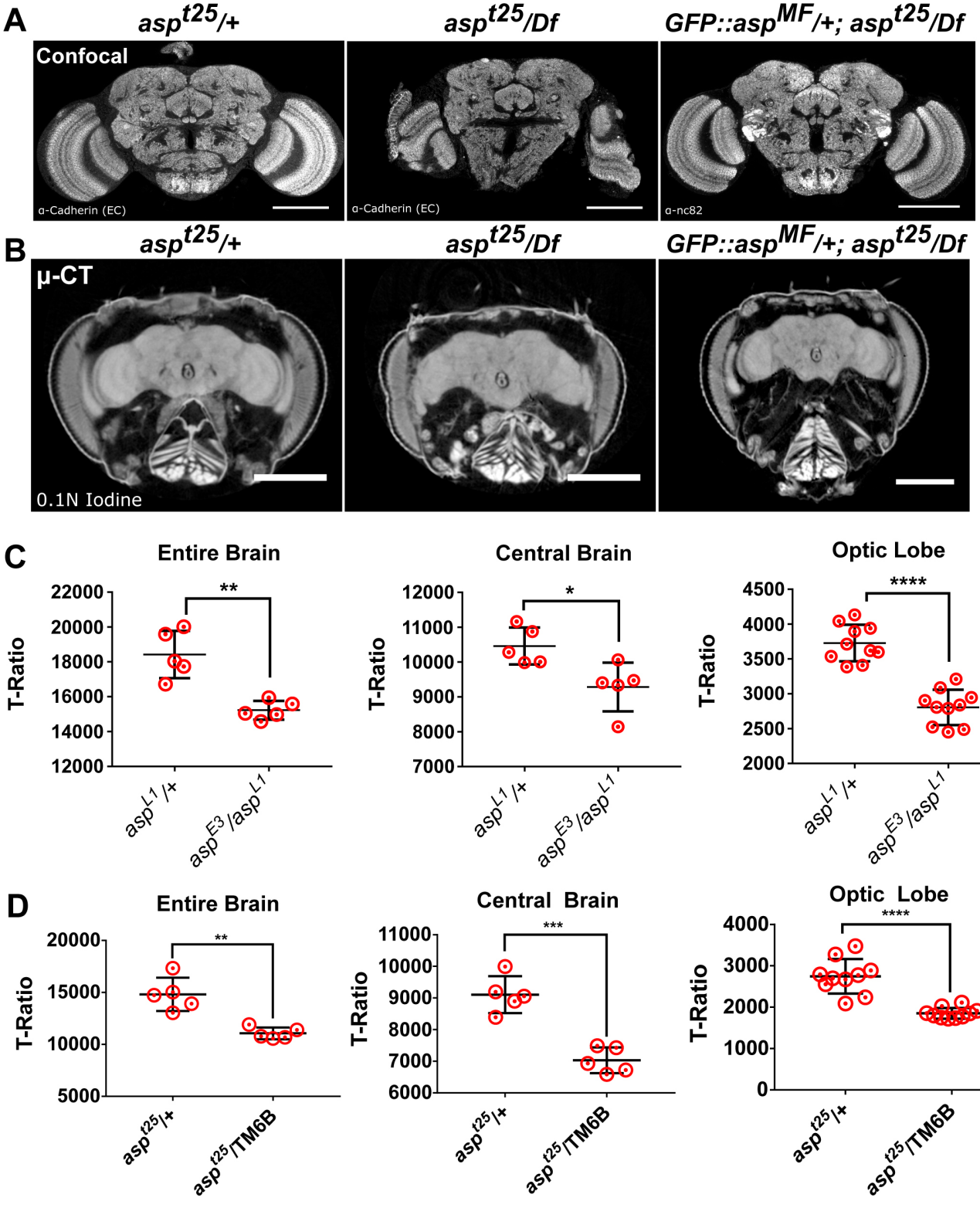


Figure S6, related to Figure 8.

(A) Confocal imaging of control *(asp^{t25}/+), asp* mutant (*asp^{t25}/Df)* and *GFP::asp^{MF}* transgenic rescue flies labelled to visualize the neuropil. (B) High resolution (1.2 µm image pixel size, slow scan) µ-CT imaging of brains of the same genotype labelled with 0.1N iodine. (C) Volume analysis of the *asp^{E3}* and *asp^{L1}* alleles, used as a trans-heterozygote. Entire brain, central brain and optic lobe volume expressed as a T-Ratio are shown. (D) Volume analysis of the entire brain, central brain and optic lobes from two control animals, *asp^{t25}*/+ and *asp^{t25}*/TM6B expressed as a T-Ratio. The presence of the balancer chromosome leads to a reduction in brain size, thus, our main analysis in Figure 6 uses *asp^{t25}*/+ as the control. All µ-CT measurements were obtained from animals scanned in fast mode (20 min) at an image pixel size of 3 µm. *n= 5* brains, Welch's t-test. **, P≤0.05; **, P≤0.01; ***, P≤0.001; ****, P≤0.0001.* Scale Bars: (A) 100 µm; (B) 200 µm.

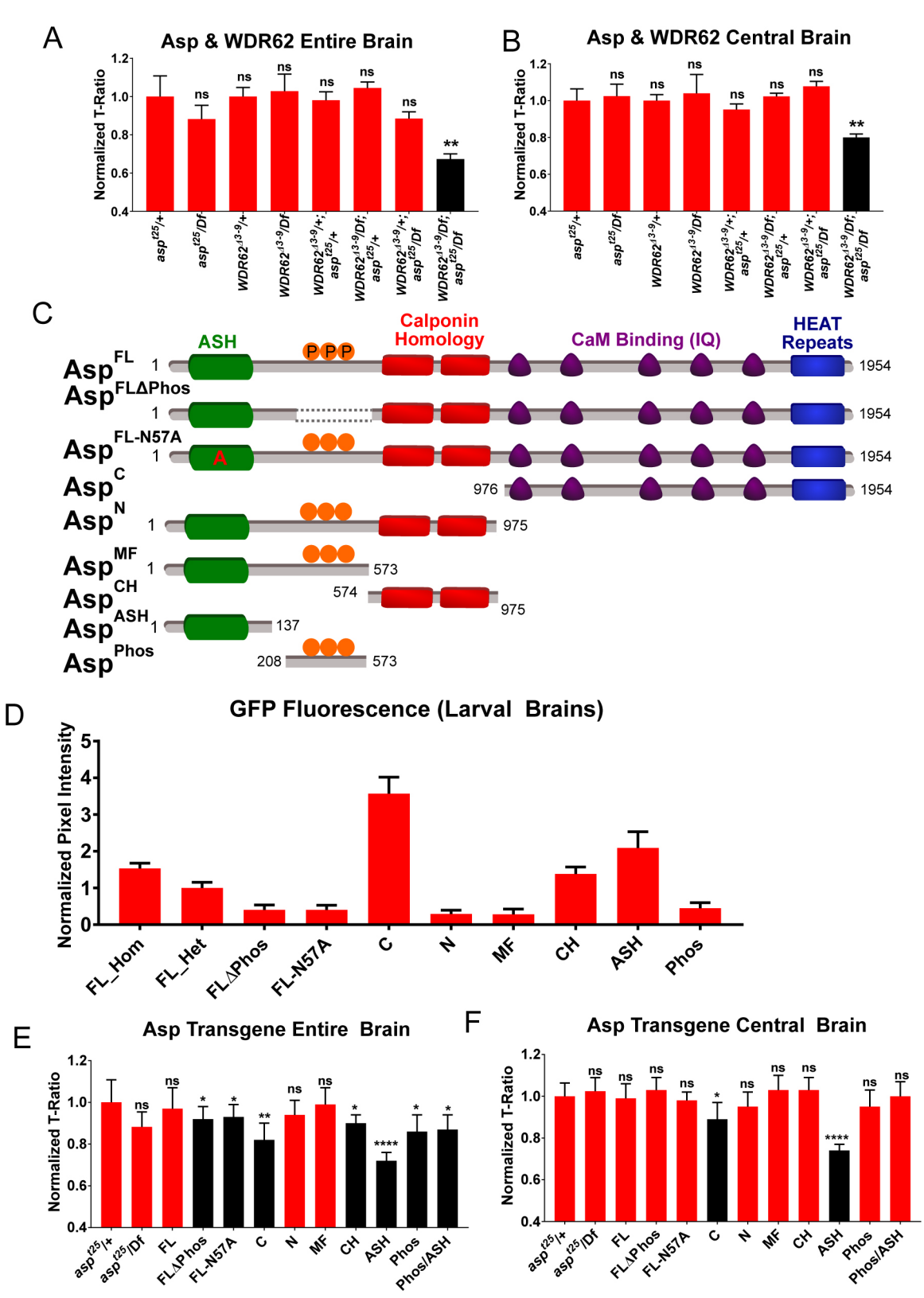


Figure S7, Related to Figure 8.

(A) T-Ratio volume analysis of the entire brain from *wdr62 & asp* adult flies. (B) T-Ratio volume analysis of the central brain from *wdr62 & asp* adult flies. (C) Domain representation of the *asp* protein and the various transgenes used in this study. (D) Expression levels of each transgene in the third instar larval brain. Values represent normalized pixel intensity (Methods). (E) T-Ratio volume analysis of the entire brain from *GFP::asp* transgene rescue flies. (F) T-Ratio volume analysis of the central brain from *GFP::asp* transgene rescue flies. All µ-CT measurements were obtained from animals scanned in fast mode (20 min) at an image pixel size of 3 µm. *n= 5* brains, Welch's t-test. *ns, P>0.05; *, P≤0.05; **, P≤0.01; ***, P≤0.001; ****, P≤0.0001.*

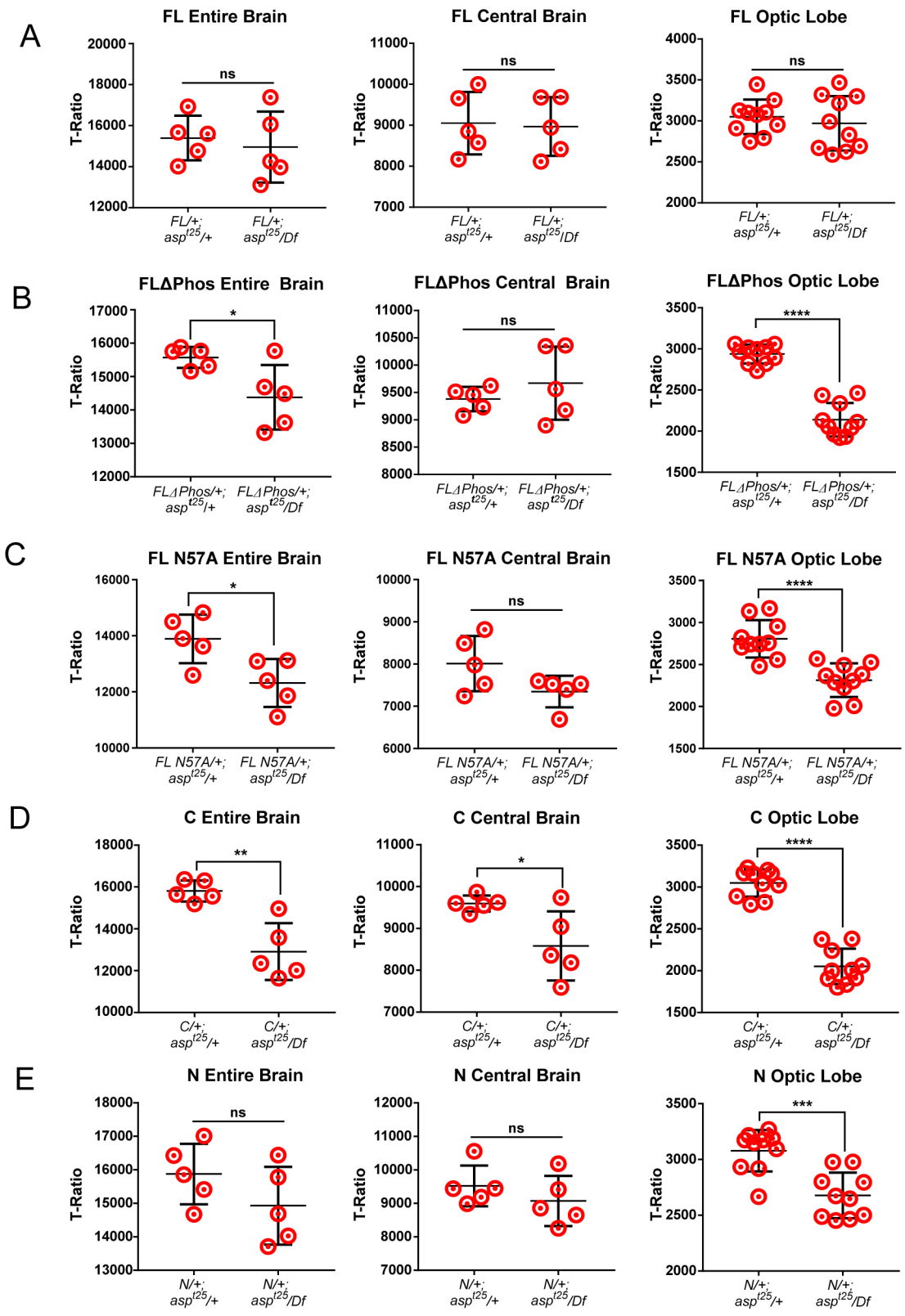


Figure S8, Related to Figure 8.

Pairwise comparisons of entire brain, central brain and optic lobe volume expressed as a T-Ratio for each *GFP::asp* transgenic rescue fragment from both control (GFP::asp/+; asp^{t25}/+) and mutant (*GFP::asp/+; asp^{t25}/Df)* backgrounds. (A) Asp^{FL}, (B) $Asp^{FL\Delta Phos}$, (C) Asp^{FLN57A}, (D) Asp^{C}, (E) Asp^{N}. All µ-CT measurements were obtained from animals scanned in fast mode (20 min) at an image pixel size of 3 µm. *n= 5* brains, Welch's t-test. *ns, P>0.05; *, P≤0.05; **, P≤0.01; ***, P≤0.001; ****, P≤0.0001.*

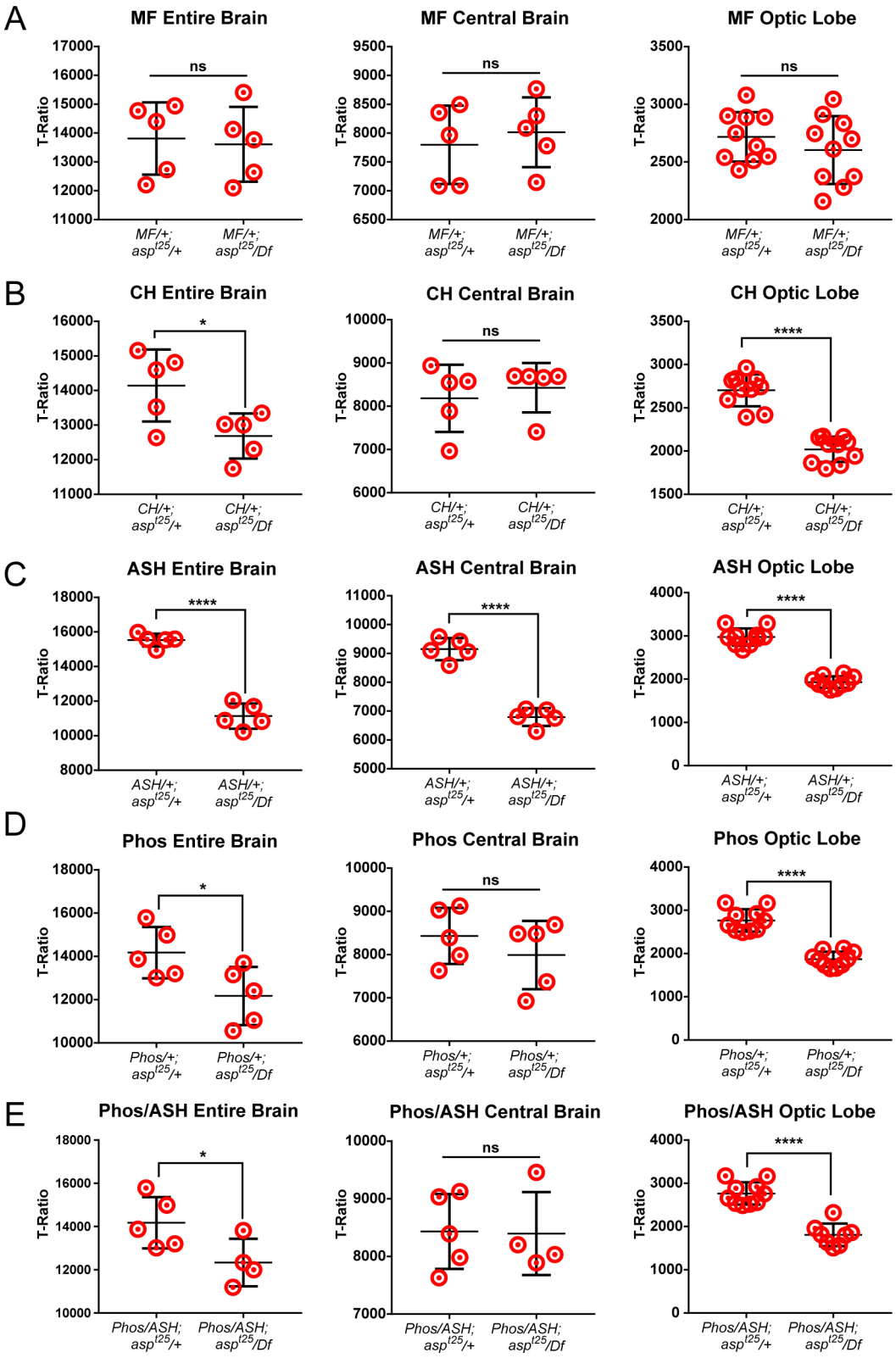


Figure S9, Related to Figure 8.

Pairwise comparisons of entire brain, central brain and optic lobe volume expressed as a T-Ratio for each *GFP::asp* transgenic rescue fragment from both control (GFP::asp/+; asp^{t25}/+) and mutant (*GFP::asp/+; asp^{t25}/Df)* backgrounds. (A) Asp^{MF}, (B) Asp^{CH}, (C) Asp^{ASH}, (D) Asp^{Phos}, (E) $Asp^{Phos/ASH}$. All µ-CT measurements were obtained from animals scanned in fast mode (20 min) at an image pixel size of 3 µm. *n= 5* brains, Welch's t-test. *ns, P>0.05; *, P≤0.05; **, P≤0.01; ***, P≤0.001; ****, P≤0.0001.*

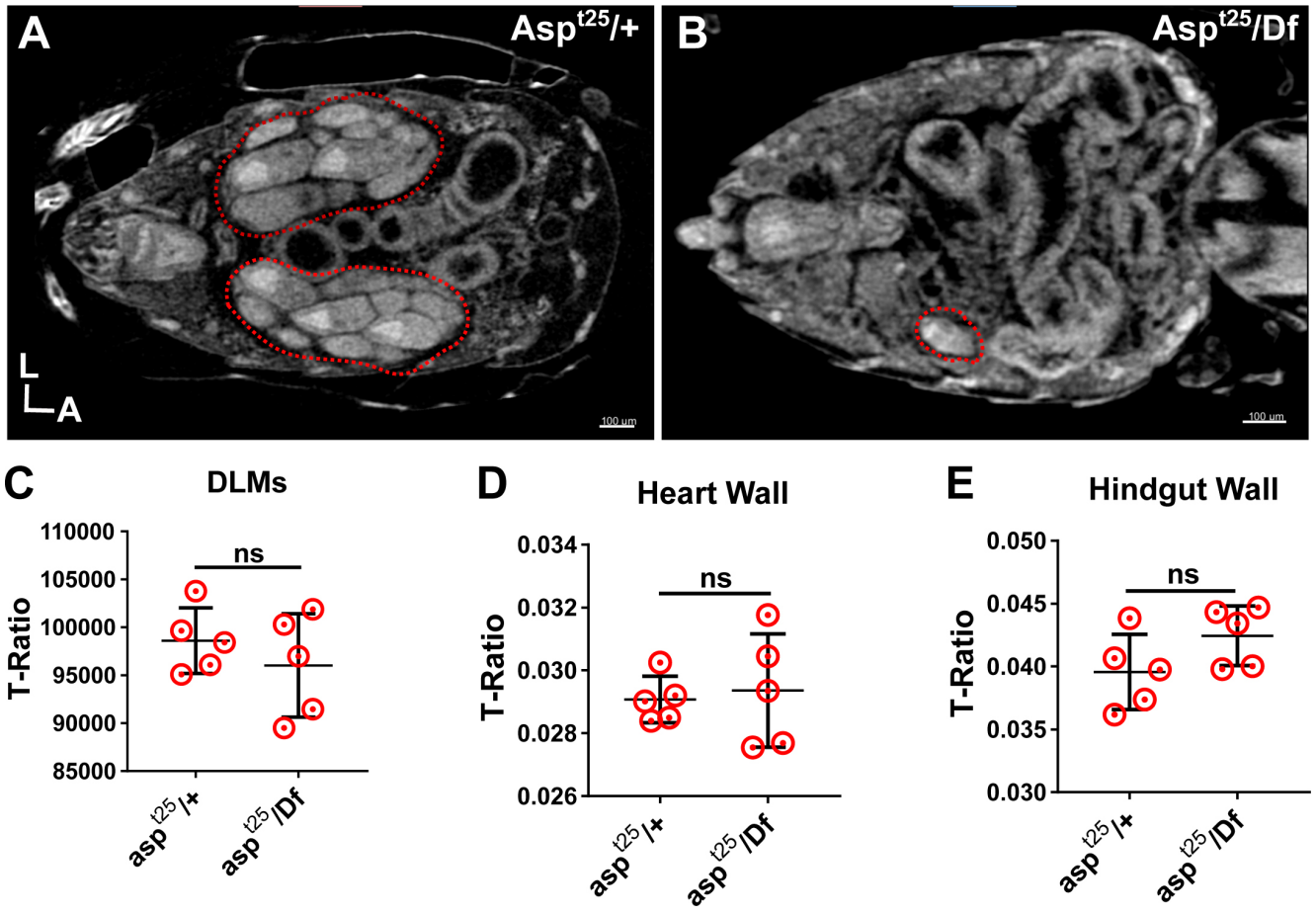
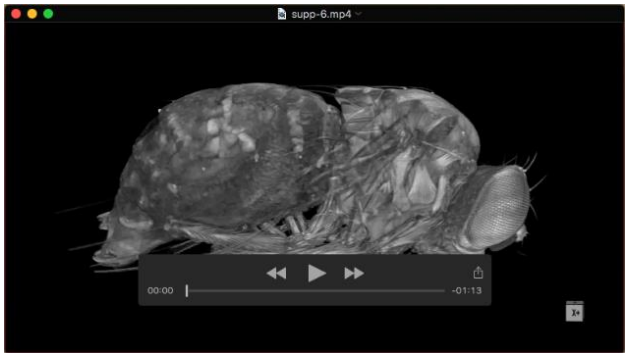


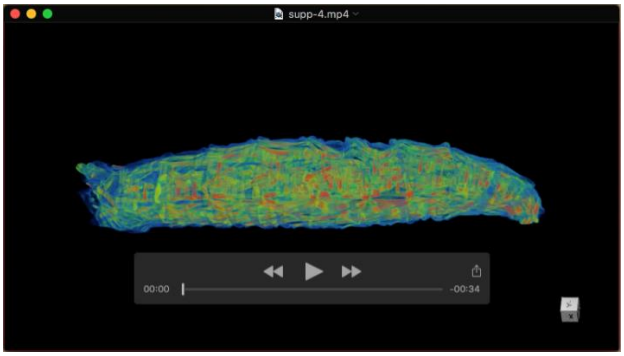
Figure S10, Related to Figure 9.

Phenotyping of asp^{t25}/+ *asp^{t25}/Df* mutant animals by µ-CT. (A) Abdomen of asp^{t25}/+ female showing multiple oocytes at various stages of development in the ovary (red outlines) (B) Abdomen of asp^{t25}/*Df* female showing only a single oocyte in the degenerating ovary (red outline). Morphometric analysis of the (C) dorsal longitudinal flight muscles (DLMs), (D) heart wall and (E) hindgut wall thickness. All µ-CT images and measurements were obtained from animals scanned in fast mode (20 min) at an image pixel size of 3 µm. *n= 5*, Welch's t-test. *ns, P>0.05; *, P≤0.05; **, P≤0.01; ***, P≤0.001; ****, P≤0.0001.* L=left, A=Anterior. Scale Bars = 100 µm.

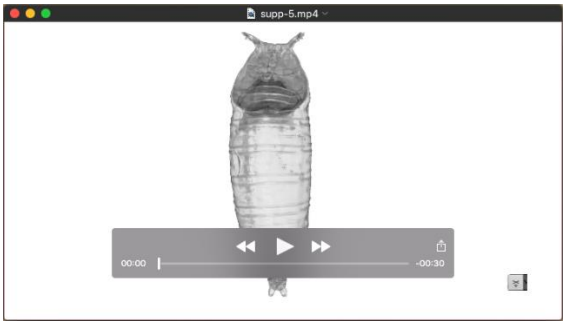


Movie 1. Three-dimensional view of an adult *Drosophila melanogaster* female scanned by µ-CT. The fly is separated along the XZ (2-14 s) and YZ axes (18-30 s) to reveal internal organs. Individual segmented organs are highlighted as follows: ocelli (red, 39 s); brain (blue, 40 s); lamina (yellow, 41 s); retina (red, 42 s); ventral nerve cord (blue, 43 s); esophagus & foregut (green, 44 s); midgut (yellow, 45 s); hindgut (red, 46 s); boli (purple, 47 s); Malpighian tubules (green, 48 s); crop (lavender; 49 s); salivary glands (turquoise, 50 s); early stage egg chamber (germarium-stage 6, blue, 51 s); mid stage egg chamber (stage 7-11, purple, 52 s); late stage egg chamber (stage 12-14; orange, 53 s); gastrulating embryo (purple, 54 s); heart (blue, 55 s); dorsal longitudinal muscles (orange, 56 s); dorsal ventral muscles (yellow, 57 s; green, 58 s; blue-green, 60 s); jump muscle (orange, 59 s); additional thorax and head muscles (61 s). Stain: 0.1N iodine; slow scan at an image pixel size of 1.25 µm.

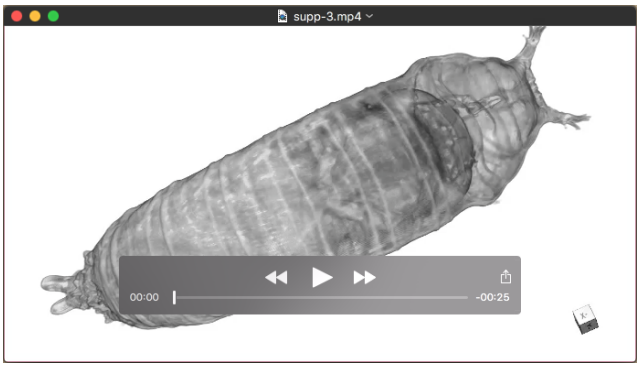
Movie 2. Two-dimensional view of an adult *Drosophila melanogaster* female scanned by µ-CT. The fly is clipped along the X axis to reveal internal structures. Stain: 0.1N iodine; slow scan at an image pixel size of 1.25 µm. Scale Bar: 500 µm.



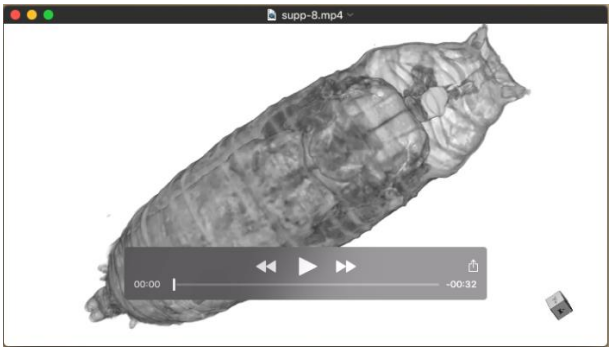
Movie 3. µ-CT of late third instar larva. False-coloring of an intact larvae separated along the XZ axis (0-10 s) and viewed from an anterior-right perspective. 3D rendering of the cuticle and major organ groups: brain (red, 14 s); eye/antennal discs (light blue), leg discs (gold) and haltere discs (yellow) (15 s); wing discs (colored by thickness-see Figure 1, 17 s); gut (foregut, rose; midgut, lavender; hindgut, aqua blue; 18 s); Malpighian tubules (purple, 20 s); fat bodies (blue, 21 s); body wall (green) and mouthpart (yellow) muscles (22 s). Stain: 0.1N iodine; slow scan at an image pixel size of 2 µm.



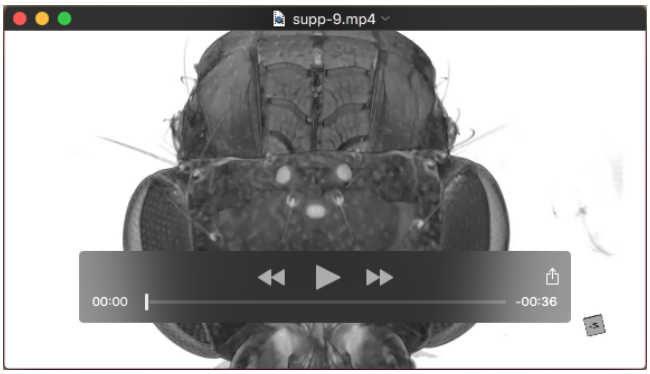
Movie 4. µ-CT of a wildtype (*yw*) pupa at stage P11. The pupa is separated along the YZ axis (5-9 s), followed by clipping along the XY axis from the anterior perspective (15 s). The brain is rendered as a 3D wireframe to reveal its position within the pupal case (18 s). Stain: 0.1N iodine; slow scan at an image pixel size of 1.4 µm.



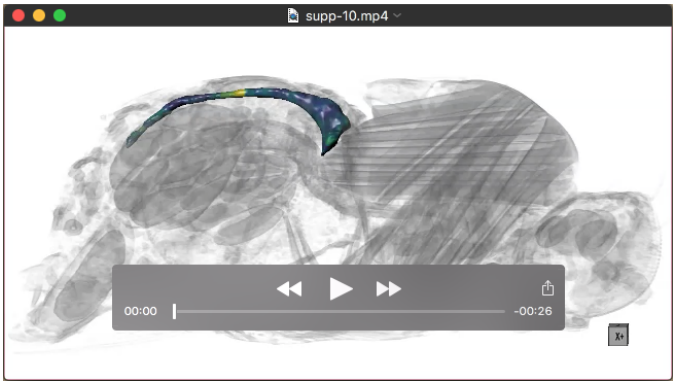
Movie 5. µ-CT of a wildtype (*yw*) pupa at stage P15 highlighting the central nervous system. Position of the ventral nerve cord (VNC, red) and brain (blue) within the thorax and headcase, respectively are highlighted. Stain: 0.1N iodine; slow scan at an image pixel size of 1.4 µm.



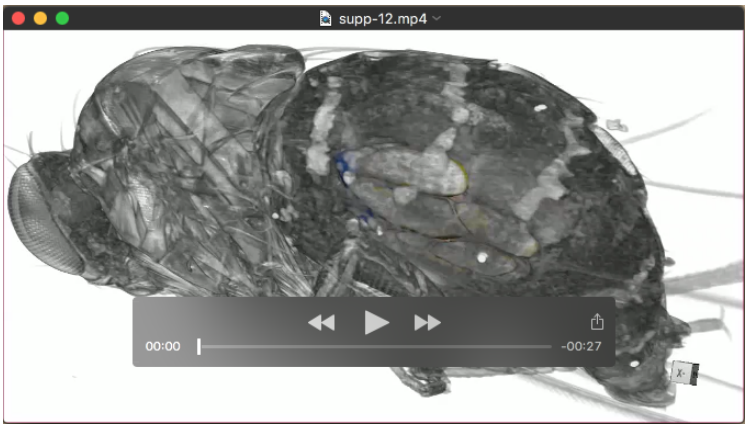
Movie 6. RNAi depletion of *spc105r* leads to loss of head structures and pupal lethality, as revealed by µ-CT. Pupa is initially viewed from the dorsal perspective, anterior is to the right. Position of the ventral nerve cord (VNC, red) and brain (blue) within the thorax is revealed at 10s, the 'head remnant' (HR) structure containing an esophagus and labellelum are rendered as a yellow surface. Note orthogonal attachment of brain to VNC within the thorax. Stain: 0.1N iodine; slow scan at an image pixel size of 1.4 µm.



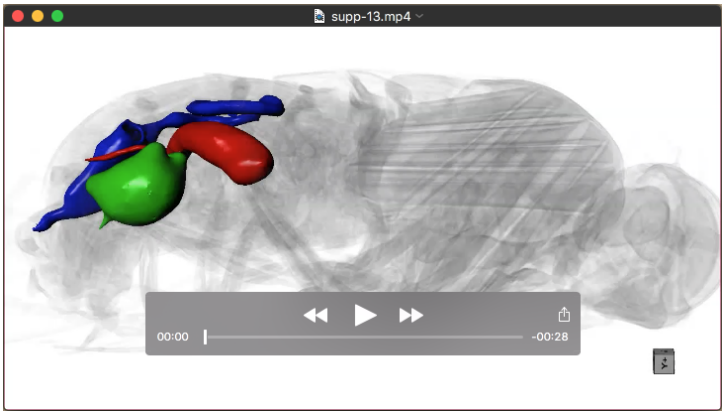
Movie 7. Visualization of the adult thorax muscles by µ-CT. Adult fly clipped along the XY axis from the anterior perspective to the posterior portion of the thorax. 3D rendering of major muscle thorax muscle groups: dorsal longitudinal muscles (red, 22 s); dorsal ventral muscles (yellow, green, blue-green, 24 s); jump muscle (orange, 24 s); additional thorax muscles (25 s). Stain: 0.1N iodine; slow scan at an image pixel size of 1.25 µm.



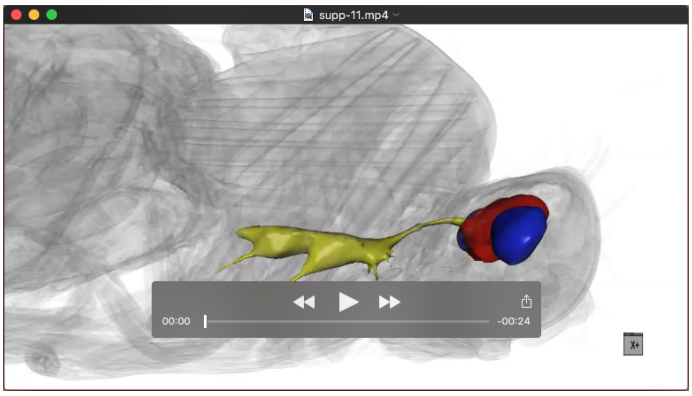
Movie 8. Wall thickness of the adult heart. 3D rendering of the adult heart located dorsally along the abdomen, colored by thickness of the wall (µm) (see Figure 4). Note thickening of the tissue (yellow) at the position of the ostia. Stain: 0.1N iodine; slow scan at an image pixel size of 1.25 µm.



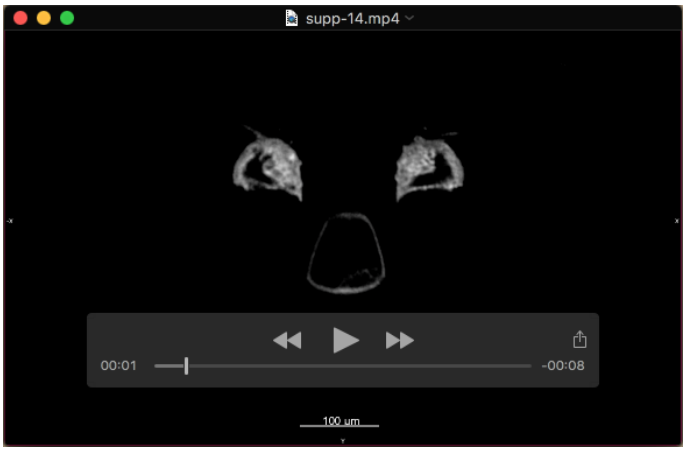
Movie 9. Egg chambers in the adult ovary. The abdomen is clipped from the dorsal surface to reveal the egg chambers within the ovary. Germarium-stage 6, blue; stage 7-11, yellow; stage 12-14, orange. Stain: 0.1N iodine; slow scan at an image pixel size of 1.25 µm.



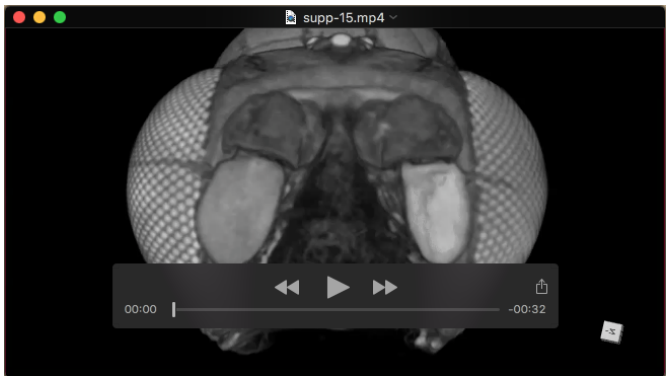
Movie 10. An example of left-right asymmetry in adult flies to probe inter-organ relationships. Directional looping of the male spermiduct (red) over the hindgut (blue). The ejaculatory bulb (green) is shown for reference on the right side of the XZ body axis. Stain: 0.1N iodine; slow scan at an image pixel size of 1.25 µm.



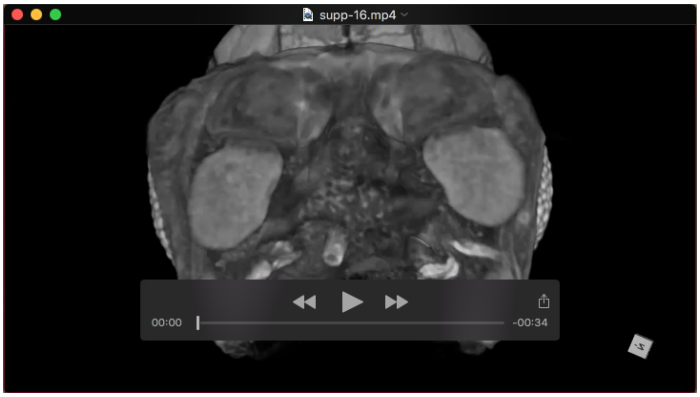
Movie 11. The adult central nervous system. 3D representation of the ventral nerve cord (VNC, yellow) located along the ventral surface of the thorax and the brain, divided into the central brain (red) and optic lobes (blue). Stain: 0.1N iodine; slow scan at an image pixel size of 1.25 µm.



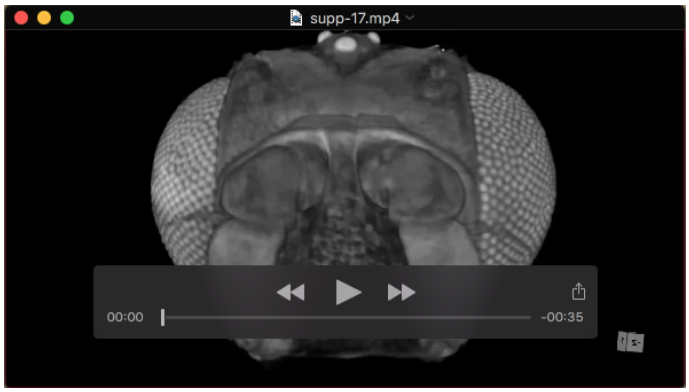
Movie 12. Two-dimensional view of the adult head imaged at submicron resolution by µ-CT. XY clip through the head of an adult fly. Stain: 0.5% PTA; slow scan an image pixel size of 700 nm. Scale Bar: 100 µm.



Movie 13. The visual circuit from an adult *asp^{t25}/+* control fly. 3D representation of the lamina (green), medulla (blue), lobula (yellow) and lobula plate (red). Stain: 0.5% PTA; slow scan at an image pixel size of 1.2 µm.



Movie 14. The visual circuit from an adult *asp^{t25}/Df* mutant fly. 3D representation of the lamina (green), medulla (blue), lobula (yellow) and lobula plate (red). Note extreme disorganization between the medulla, lobula and lobula plate, severely reduced lamina structures and complete absence of the ocelli. Stain: 0.5% PTA; slow scan at an image pixel size of 1.2 µm.



Movie 15. The visual circuit from an adult GFP::Asp^{MF} transgenic rescue fly. 3D representation of the lamina (green), medulla (blue), lobula (yellow) and lobula plate (red) from flies expressing Asp^{MF} in the *asp^{t25}/Df* mutant background. Note complete rescue of morphology defects and the ocelli. Stain: 0.5% PTA; slow scan at an image pixel size of 1.2 µm.