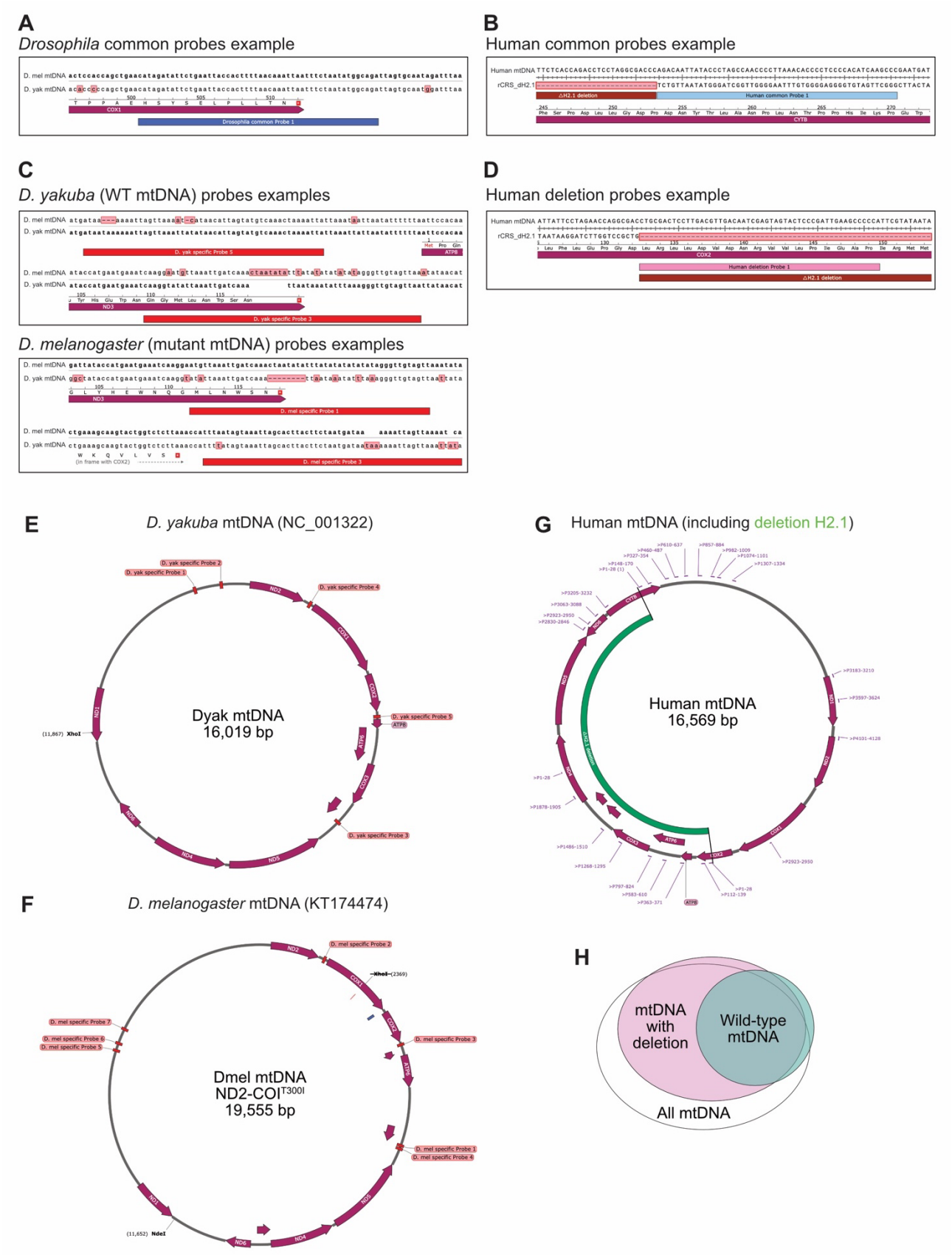
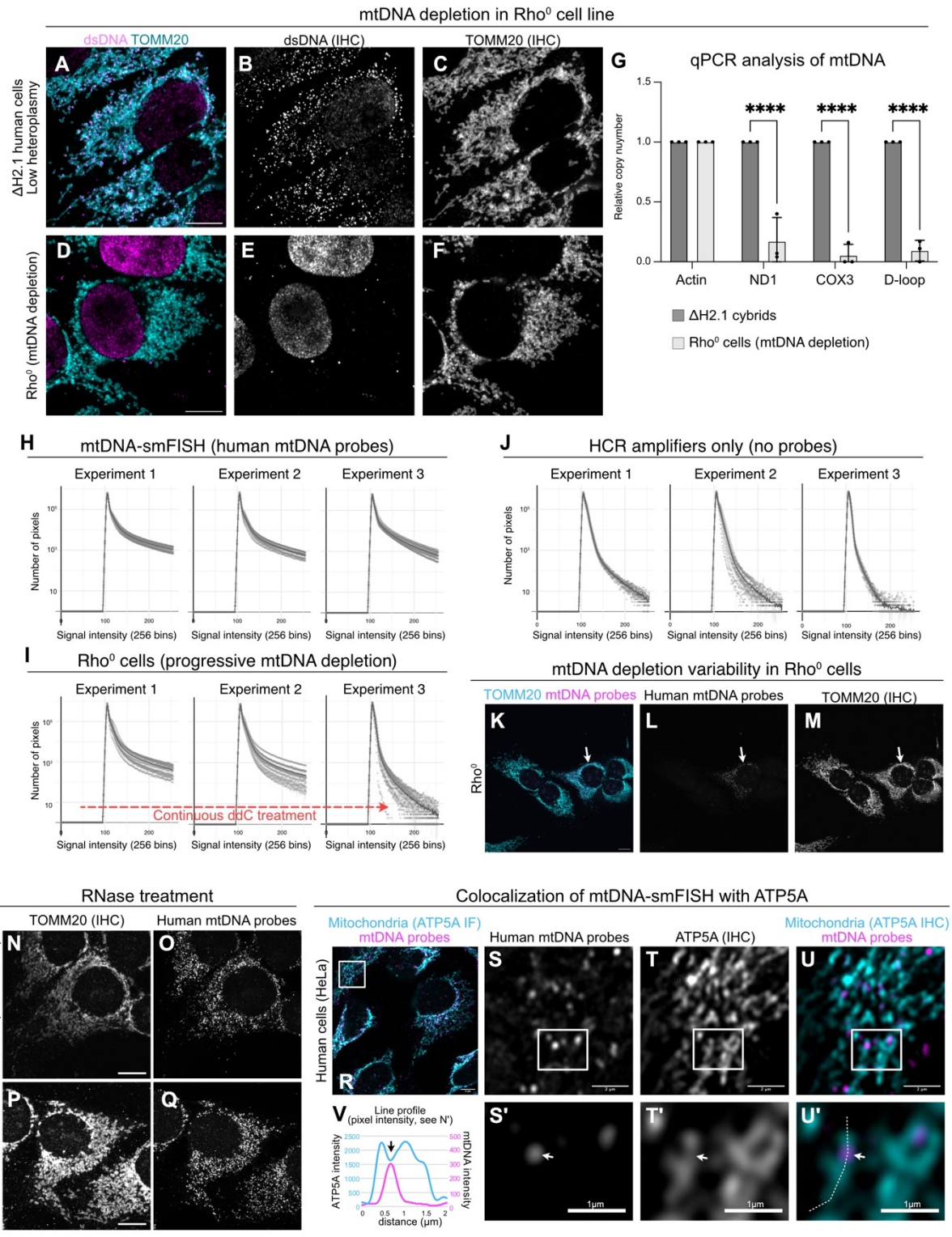
Supplementary figures



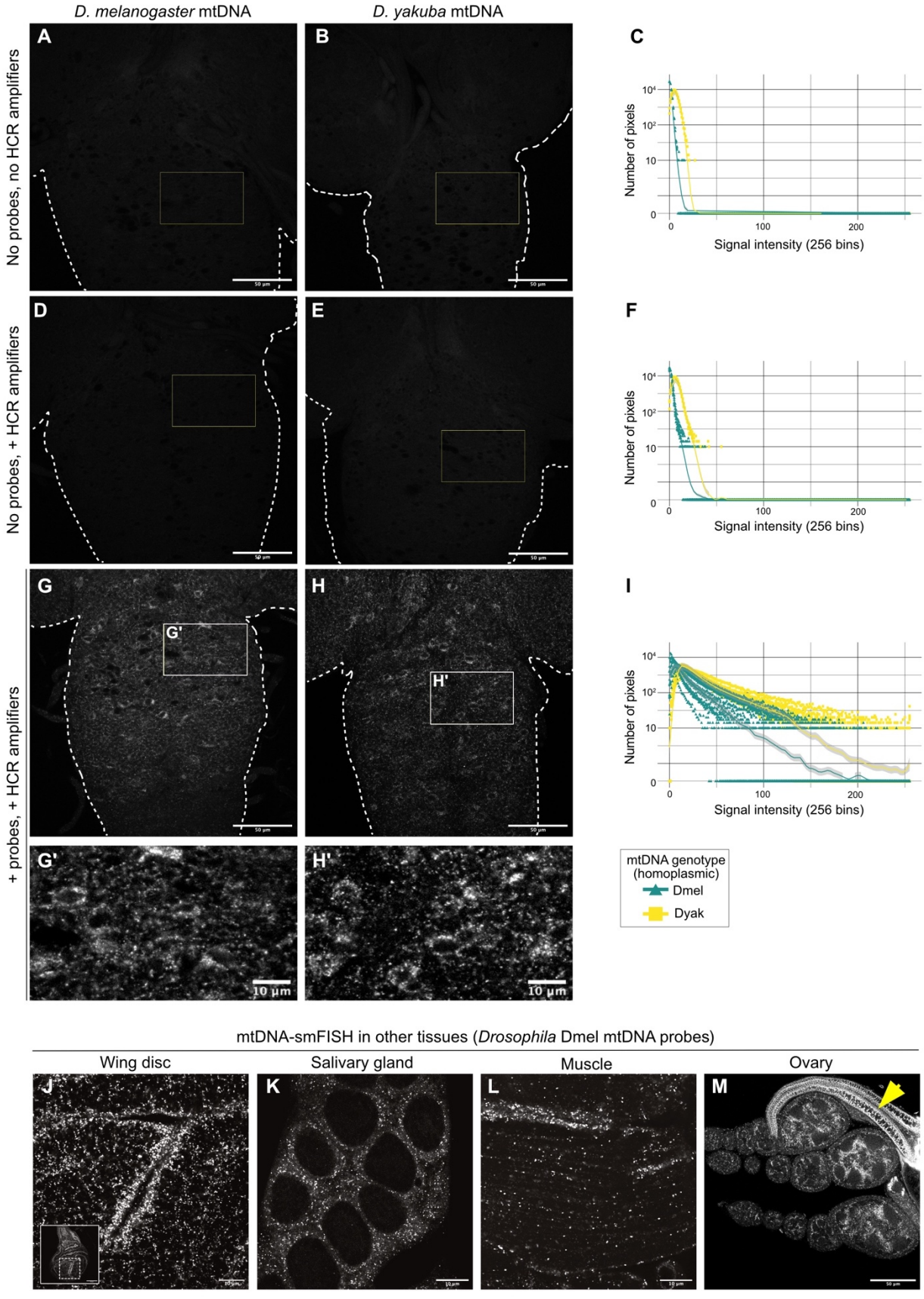
Supplementary Figure 1: Probe Design for mtDNA-smFISH of *Drosophila* and Human mtDNA. (**A-D**) Representative examples of target sequences designed for *Drosophila* mtDNA, common to both *D. melanogaster* and *D. yakuba* (A), specifically for *D. yakuba* (WT, C) or *D. melanogaster*

(mutant, C) mtDNA, or for human mtDNA common to WT and deleted mtDNA (B) or only recognising the WT mtDNA (D). (**E-G**) Schematic of *D. yakuba* (E), and *D. melanogaster* (F) mtDNA and human mtDNA (G) with protein-coding genes in purple and probe target sequences in bright red or pink. Human deleted sequence is indicated in green (G). (**H**) Schematic of how mtDNA-smFISH can distinguish between WT or deleted mtDNA, with deleted molecules only targeted by common mtDNA probes (red) and WT molecules also targeted by deletion-specific probes (green).



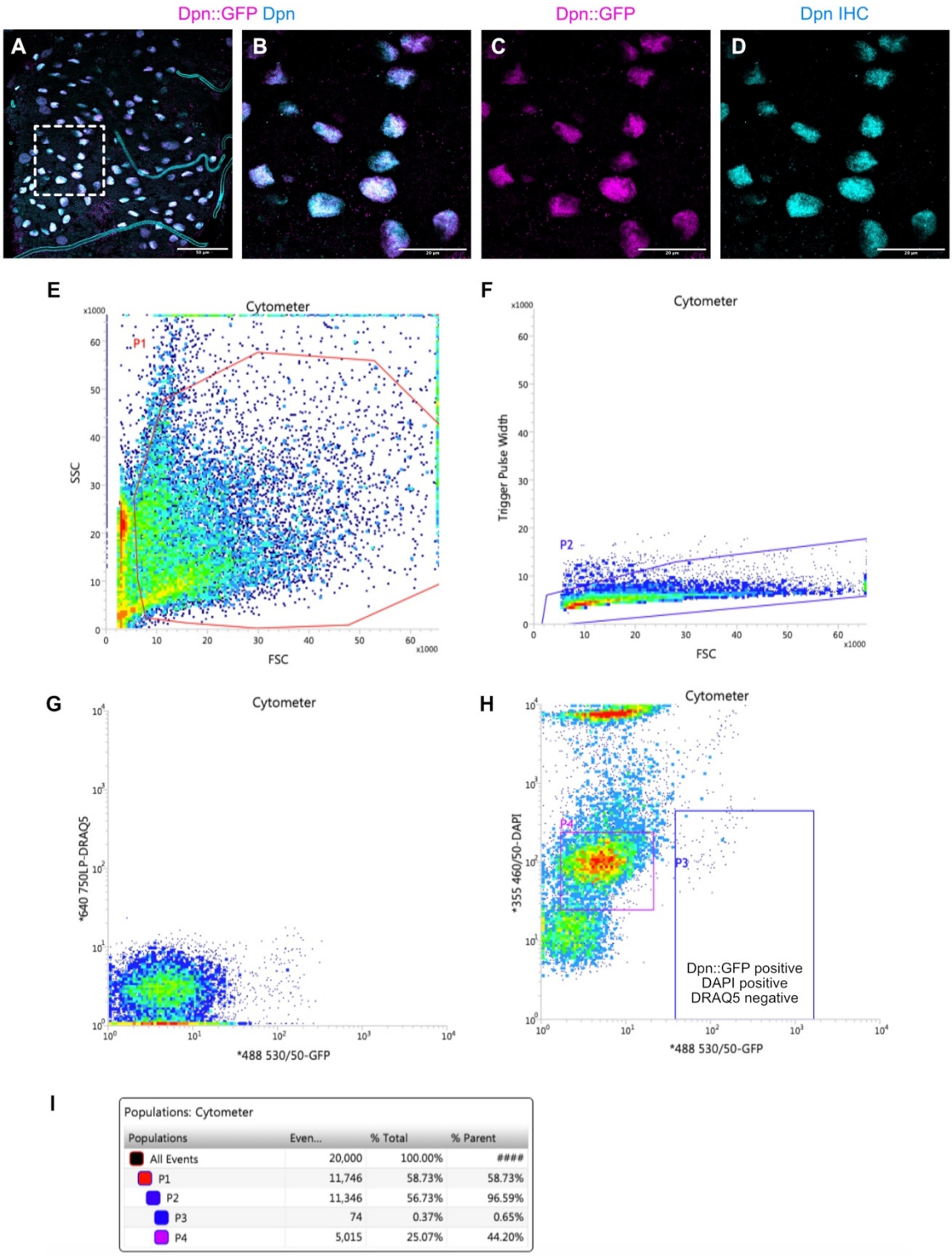
Supplementary Figure 2: mtDNA-smFISH with human mtDNA probes. (**A-F**) Representative confocal images of immunostaining against dsDNA (magenta, B,E) and TOMM20 (cyan, C,F) in a low heteroplasmy ΔH2.1 cybrid cell line (A-C) and a corresponding mtDNA depleted Rho^0 cell line (D-F) generated by ddC treatment. (G) qPCR analysis using ND1, COX3 and D-loop as markers for mtDNA (n = three independent experiments; unpaired t-tests). (**H-J**) mtDNA-smFISH signal (H,I) or background (J, no probes, only HCR amplifiers) quantification in ΔH2.1 cybrid cells upon continuous treatment with control (H) or ddC (I) to generate Rho^0 cells. Experiment 3 is shown in Figure 1Q. (**K-**

M) mtDNA-smFISH and TOMM20 co-immunostaining in Rho^0 cells, where some treated cells retain some mtDNA (arrowhead). (**N-Q**) mtDNA-smFISH and TOMM20 co-immunostaining without (N,O) or with (P,Q) prior RNase treatment. (**R-U**) Airyscan imaging of HeLa cells after mtDNA-smFISH with human mtDNA probes (magenta) with co-immunostaining of ATP5A (cyan), with higher magnification of boxed regions (S-U) in (S'-U'). (V) Line profile analysis of signal along the hatched line in U'. Scale bars are 10μm (A,D,K,N,P), 2μm (S-U), 1μm (S'-U').

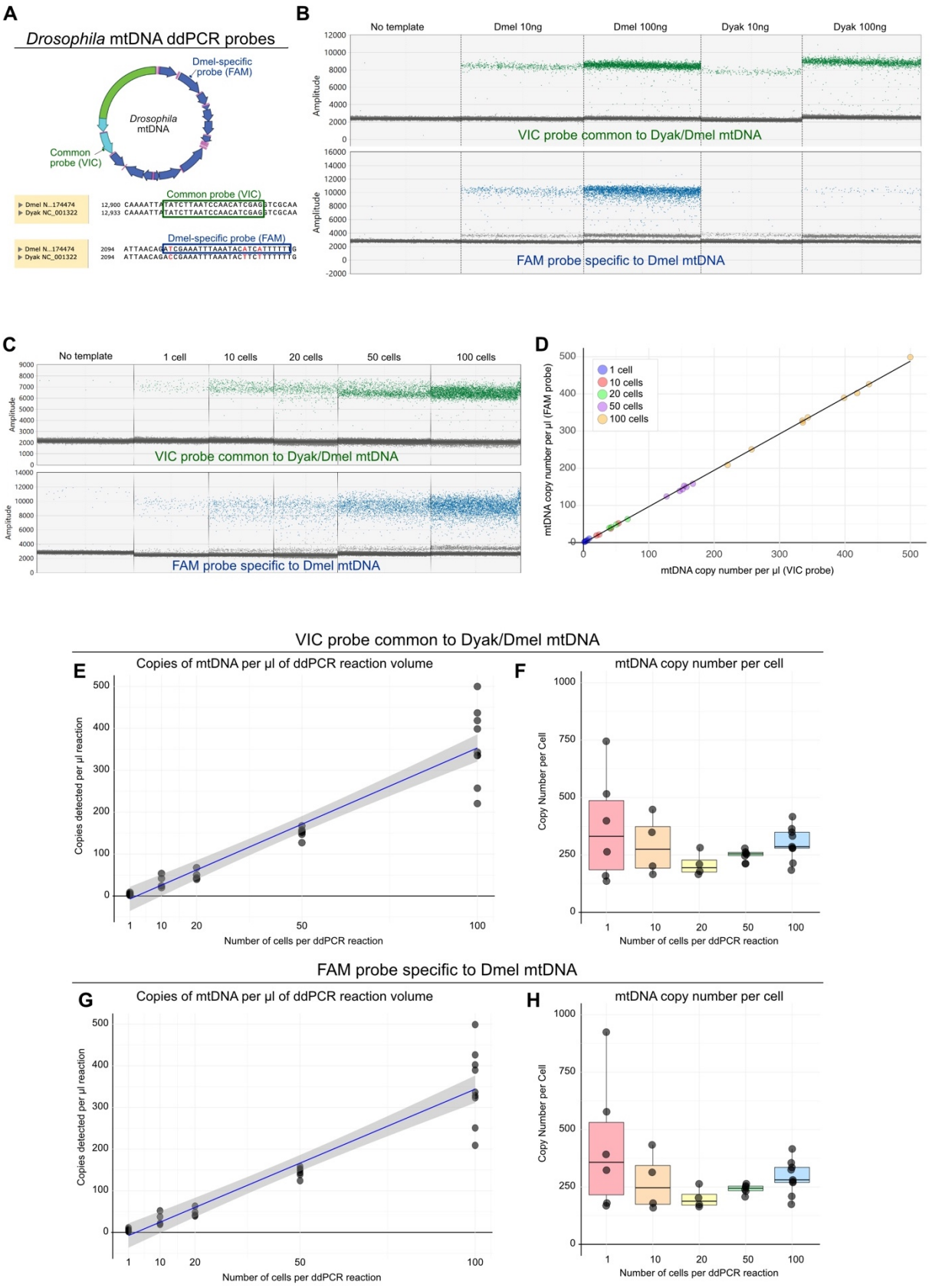


Supplementary Figure 3: mtDNA-smFISH with probes common to *D. melanogaster* and *D. yakuba* mtDNA. (**A-I**) Representative confocal mtDNA-smFISH images (A-H) and signal

quantification (C,F,I) with probes common to both *D. melanogaster* and *D. yakuba* mtDNA in brains of larvae homoplasmic for *D. melanogaster* (A,D,G) or *D. yakuba* (B,E,H) mtDNA, either without probes or amplifiers (A,B,C), without probes (D,E,F), or with both probes and amplifiers added (G,H,I). (G',H') are high magnifications of areas boxed in G,H. (**J-M**) mtDNA-smFISH across various *Drosophila* tissues. Red arrow in (M) points to the dorsal appendage of the maturing egg. Scale bars are 50μm (A,B,D,E,G,H,J-M) or 10μm (G',H').

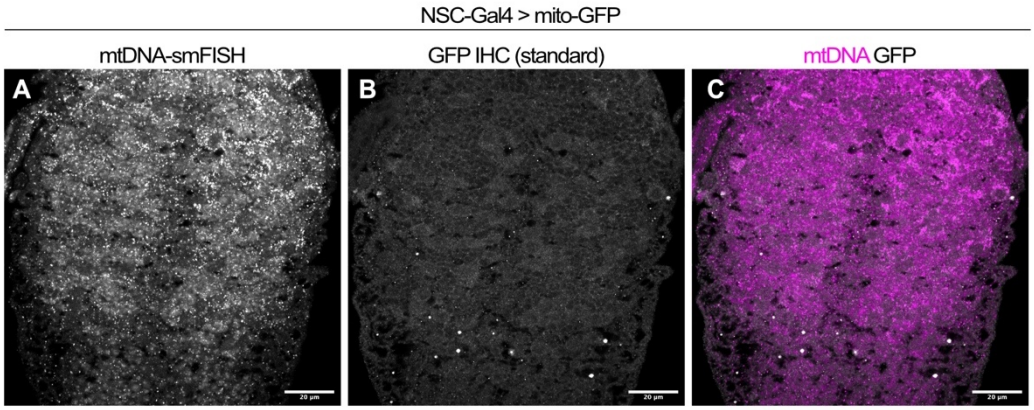


Supplementary Figure 4: FACS-Based Isolation of *Drosophila* NSCs. (**A-D**) Immunostaining of Dpn (cyan) and GFP (magenta) of third instar larval brain from transgenic *Drosophila* with GFP fused to the NSC-marker Dpn (Dpn::GFP). (**E-I**) Representative FACS plots of dissociated third instar larval brains from Dpn::GFP *Drosophila*, with Draq5 and DAPI used to assess viability (G,H), resulting in ~0.37% of all cells retained as NSCs (I).

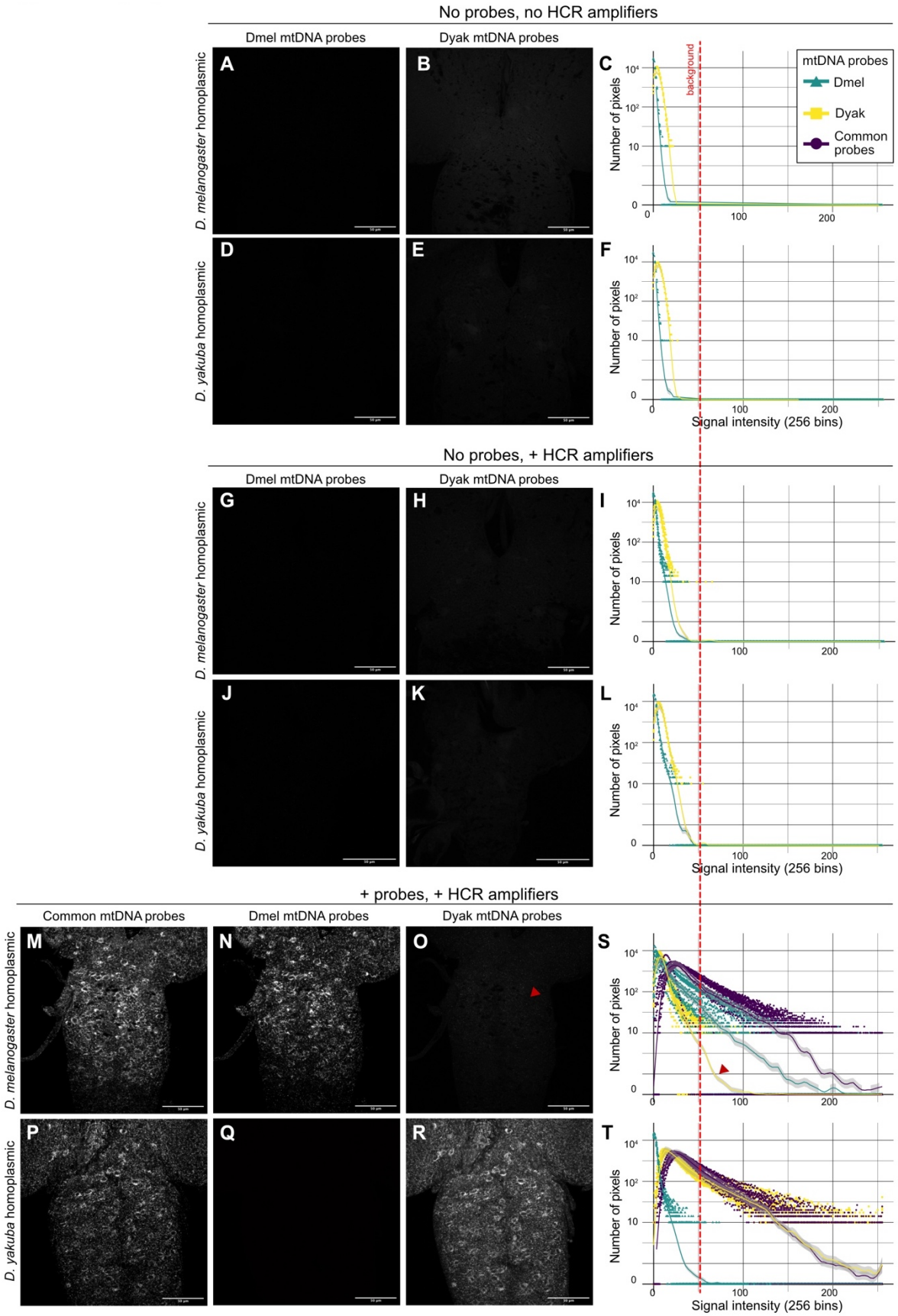


Supplementary Figure 5: Droplet digital PCR (ddPCR) for *Drosophila* mtDNA. (**A**) Schematic representation of VIC and FAM probes for *Drosophila* mtDNA. VIC probe was designed to target both *D. melanogaster* and *D. yakuba* mtDNA. FAM probe was specific to *D. melanogaster* mtDNA.

(**B,C**) ddPCR using VIC (green) and FAM (blue) probes using 0, 10 or 100ng of genomic DNA from homoplasmic *D. melanogaster* or *D. yakuba* (B), or on 1, 10, 20, 50 or 100 sorted NSCs (Dpn::GFP) from homoplasmic *D. melanogaster* (C). (**D**) Correlation between mtDNA CN estimates of VIC and FAM probes on sorted NSCs from homoplasmic *D. melanogaster*. (**E-H**) mtDNA CN measured using VIC probe (E,F) or FAM probe (G,H) on 1, 10, 20, 50 or 100 sorted NSCs from homoplasmic *D. melanogaster*. mtDNA CN increases linearly with increasing cell number (E,G), but larger variability is observed in single-cell samples (F,H).

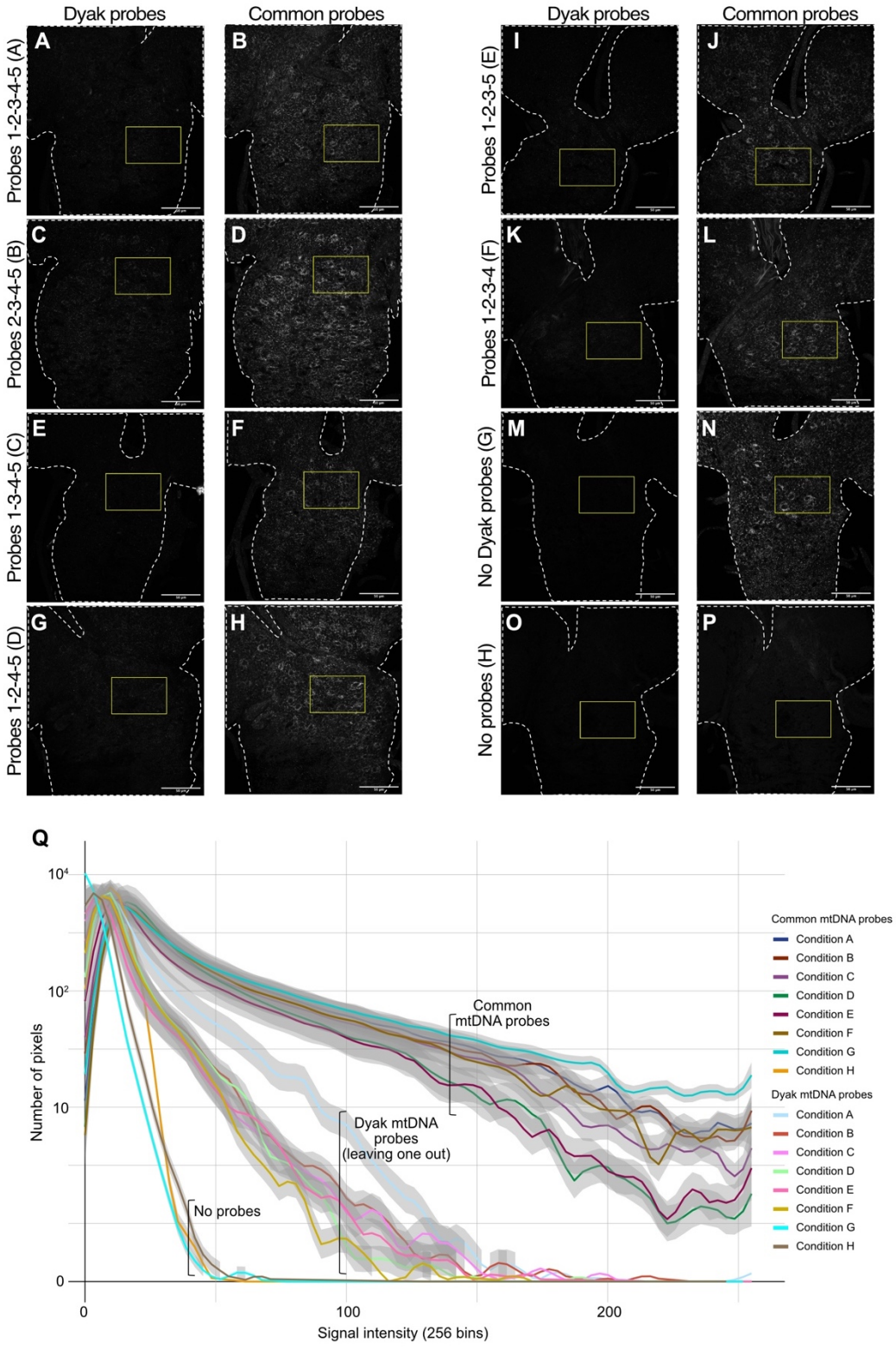


Supplementary Figure 6: Denaturation prevents conventional GFP immunostaining after mtDNA-smFISH. (**A-C**) mtDNA-smFISH (A, magenta) and conventional immunostaining for GFP (Worniu-GAL4 > UAS-mito-GFP) (B, gray) showing no effective GFP staining due to the high denaturation temperature required for mtDNA-smFISH. Scale bars are 50µm.



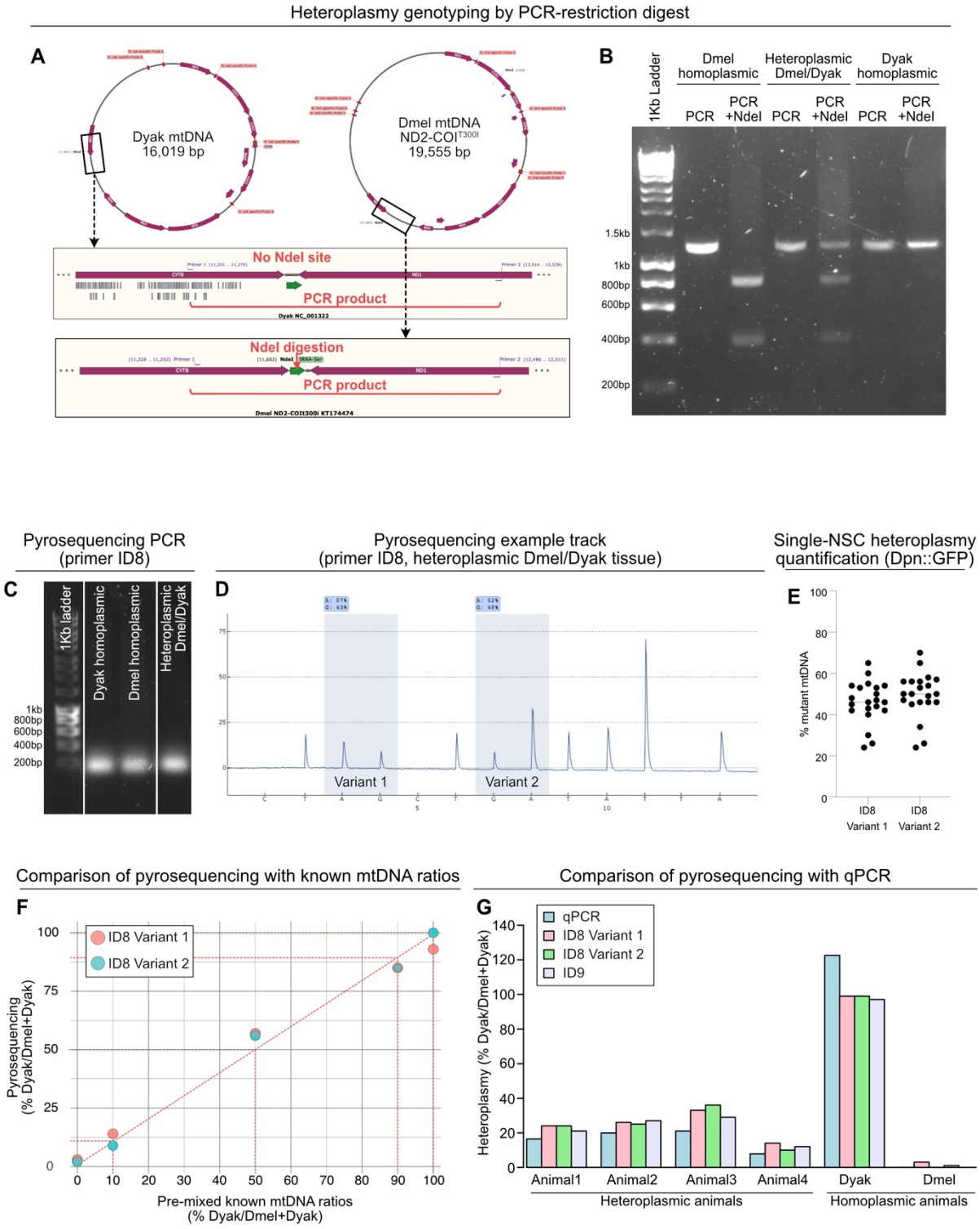
Supplementary figure 7: Specificity of *D. melanogaster* or *D. yakuba* mtDNA-smFISH. (**A-T**) mtDNA-smFISH images (A-E,G-K,M-R) and signal quantification (C,F,I,L,S,T; mean ± s.e.m.) with probes specific to either *D. melanogaster* (mutant, A,D,G,J,N,Q, green graphs), *D. yakuba*

(B,E,H,K,O,R, yellow graphs) or common to both (M,P, purple graphs) mtDNA in brains of *Drosophila* larvae homoplasmic for *D. melanogaster* (A-C,G-I,M-O) or *D. yakuba* (D-F,J-L,P-R) mtDNA, either without probes or amplifiers (A-F), without probes (G-L), or with both probes and amplifiers added (M-R). Scale bars are 50μm. Red arrowheads indicate a low-level non-specific background from the *D.yakuba* probes in *D.melanogaster* homoplasmic (mutant) background. The red line in the graphs separates true signal from background, with a threshold set at 55 bins.



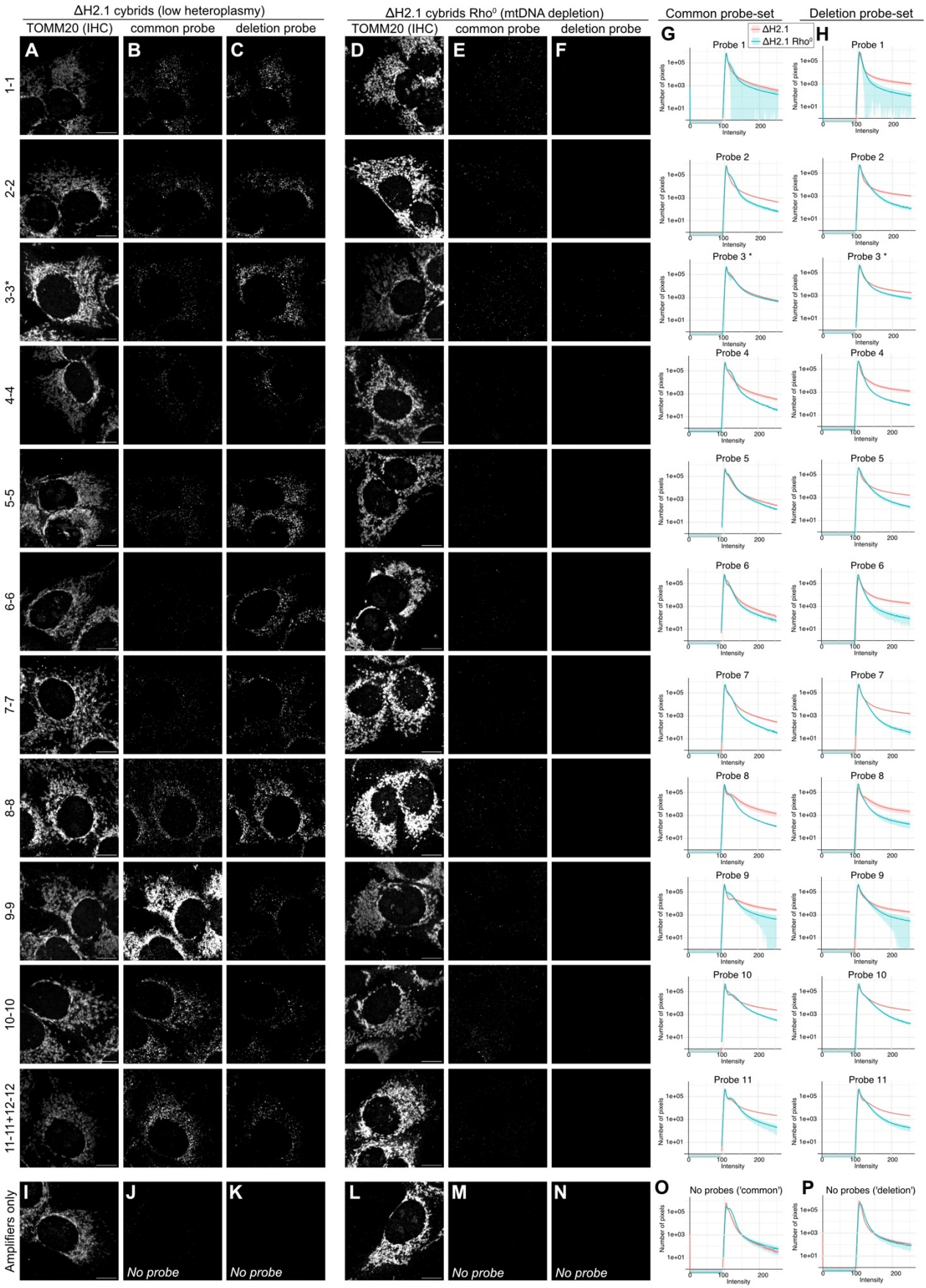
Supplementary Figure 8: Specificity of individual probes for *D. yakuba* mtDNA-smFISH. To assess background of the probe-set specific to *D. yakuba* mtDNA, each of the five individual probe pairs was removed separately from the probe mix used for mtDNA-smFISH on brains from *Drosophila* with homoplasmic *D. melanogaster* mtDNA. Removing individual *D. yakuba* probes (representative confocal images in A,C,E,G,I,K, light colours in Q), all resulted in comparable background levels. mtDNA-smFISH with the common probe set was used as a positive control (representative confocal

images in B,D,F,H,J,L, dark colours in Q). No background or signal was observed in absence of *D. yakuba* probes (M,Q) or any probe (O,P,Q). Scale bars are 50μm.



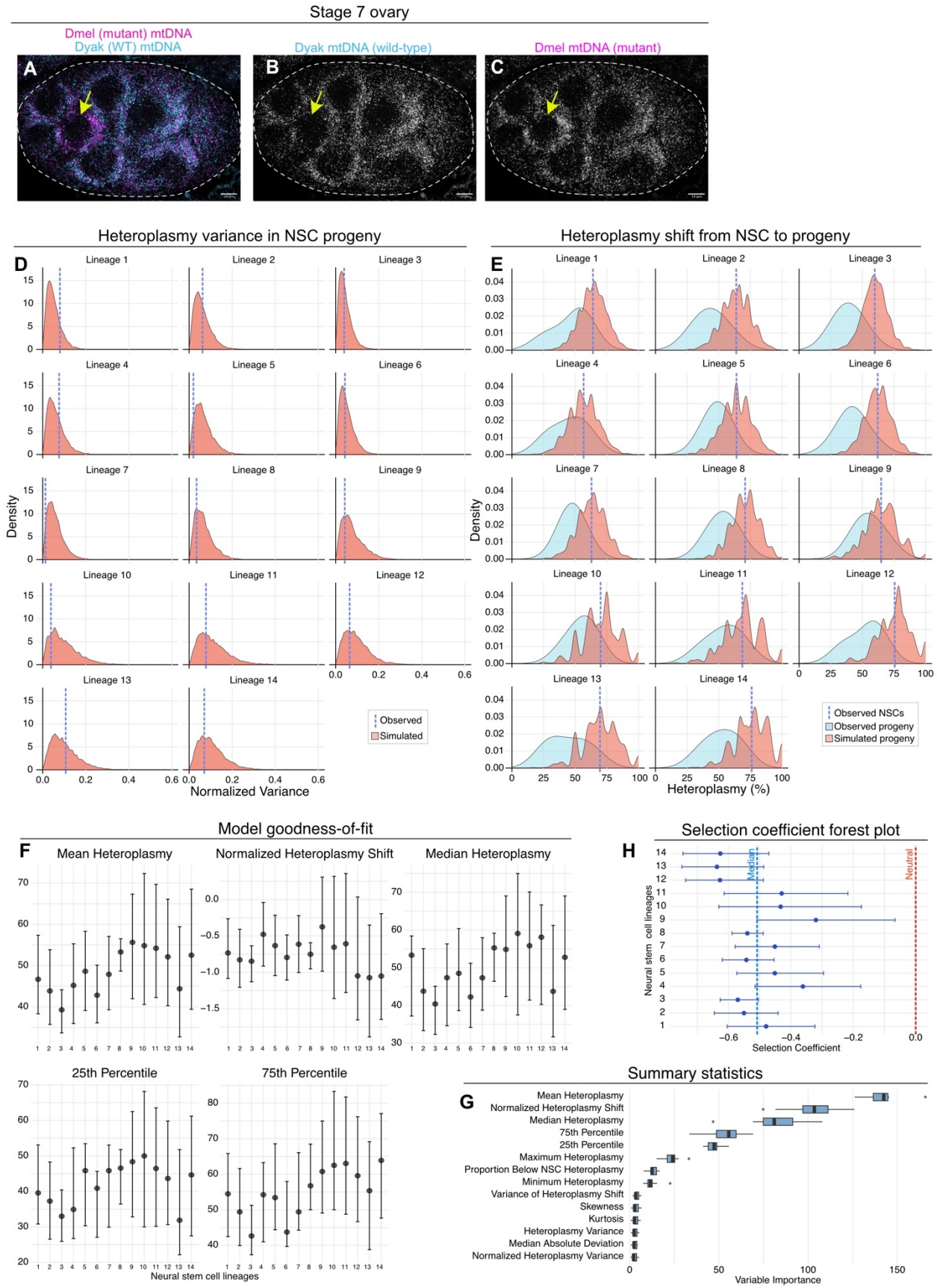
Supplementary figure 9: Heteroplasmy measurement with restriction digestion or pyrosequencing. (**A,B**) Schematic (A) and representative gel image (B) of PCR amplification and NdeI digestion to distinguish *D. yakuba* (WT) from *D. melanogaster* (mutant) mtDNA, with *D. yakuba* lacking the NdeI site. PCR amplification produced a 1284bp (*D. melanogaster*) or 1289bp (*D. yakuba*) product, which, upon digestion with NdeI, resulted in two bands of 425bp and 859bp for the *D. melanogaster* genome only. (**C-G**) Optimisation of pyrosequencing to distinguish *D. yakuba* (WT) from

D. melanogaster (mutant) mtDNA, using primer pairs ID8 (C-G) or ID9 (G). PCR using primer pair ID8 amplifies mtDNA from homoplasmic or heteroplasmic *Drosophila* strains with comparable efficiency (C), and allows quantification of heteroplasmy levels using two independent variants in the same PCR fragment (D). Pyrosequencing detects heteroplasmy levels in single NSCs sorted from Dpn::GFP third instar larvae (E). Pyrosequencing detects known ratios of mixed mtDNA from homoplasmic *D. melanogaster* and *D. yakuba* (F), and detects heteroplasmy levels from heteroplasmic or homoplasmic *Drosophila* with comparable accuracy to a previously validated qPCR method [67] (G).



Supplementary figure 10: Specificity of individual human probes for mtDNA-smFISH. (**A-E**) Representative confocal images of mtDNA-smFISH performed on a low heteroplasmy ΔH2.1 cybrid

cell line (A-C, I-K) and a Rho^0 (D-F, L-M) cell line by using single probes for human mtDNA (common, B,E) or human mtDNA with a single deletion (C,F). Co-immunostaining was performed with anti-TOMM20 antibody (A,D,I,L). Each row represents a single probe from each probe set. The last row (I-N) displays very low background given by the amplifiers when no probes are added during hybridization step. Signal quantification is shown in graphs (G,H,O,P). Probe 3 from the common probe set indicated with an * displayed a high background and was removed for further experiments; probe 3 from the deletion probe set was arbitrarily removed from this set as well to keep equal probe numbers. Scale bars are 10μm.



Supplementary Figure 11: Modelling of mtDNA segregation through a somatic bottleneck. (**A-C**) Representative confocal images of mtDNA-smFISH using probes targeting only *D. melanogaster* (mutant, magenta, C) or *D.yakuba* (WT, cyan, B) mtDNA in stage 7 *Drosophila* egg chamber nurse

cells. Arrow indicates a nurse cell with high mutant heteroplasmy level. Scale bars are 10μm. (**D**) Null distributions for normalized heteroplasmy variance from 10,000 random segregation simulations (red density plots) for each lineage. The corresponding observed normalized variance is marked by the dashed blue line. (**E**) Density plots of the observed progeny heteroplasmy (light blue) overlaid on the null distributions from 10,000 random segregation simulations (red) for each lineage. The heteroplasmy of the parent NSC for each lineage is marked by the dashed dark blue line. (**F**) Model goodness-of-fit assessed via posterior predictive check, comparing observed summary statistics (black dots) against 95% credible intervals of posterior predictive distribution (whiskers). (**G**) Random Forest Variable importance of candidate summary statistics for ABC parameter estimation across all 14 lineages, ranked by median importance. (**H**) Selection coefficient estimates for all lineages from Sequential Monte Carlo ABC; Points show posterior median estimates with 95% credible intervals (whiskers); blue dashed line median selection; red dashed line neutral evolution.