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Supplementary Materials for

SpotMAX: A generalist framework for multidimensional automatic spot detection and quantification

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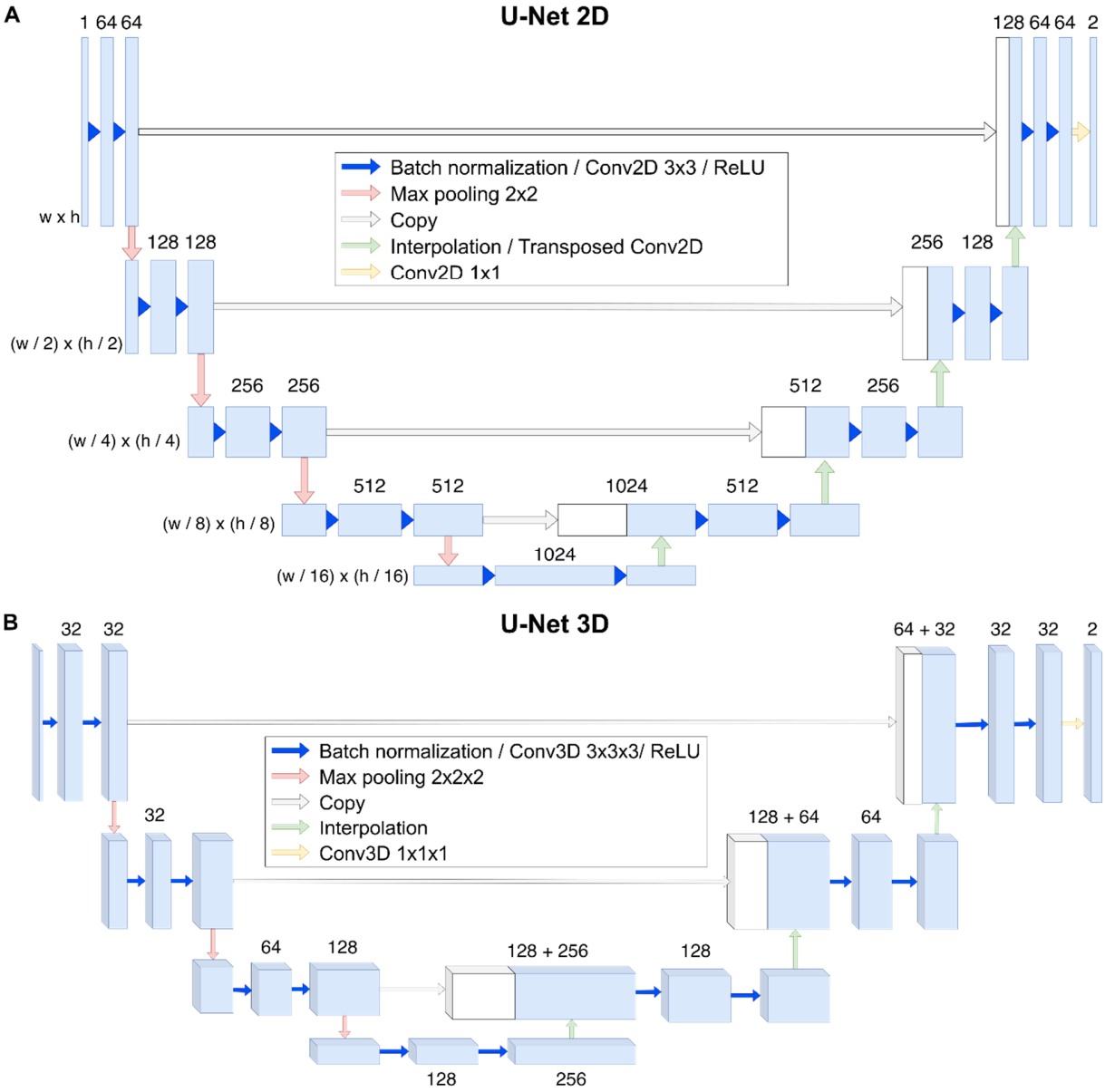
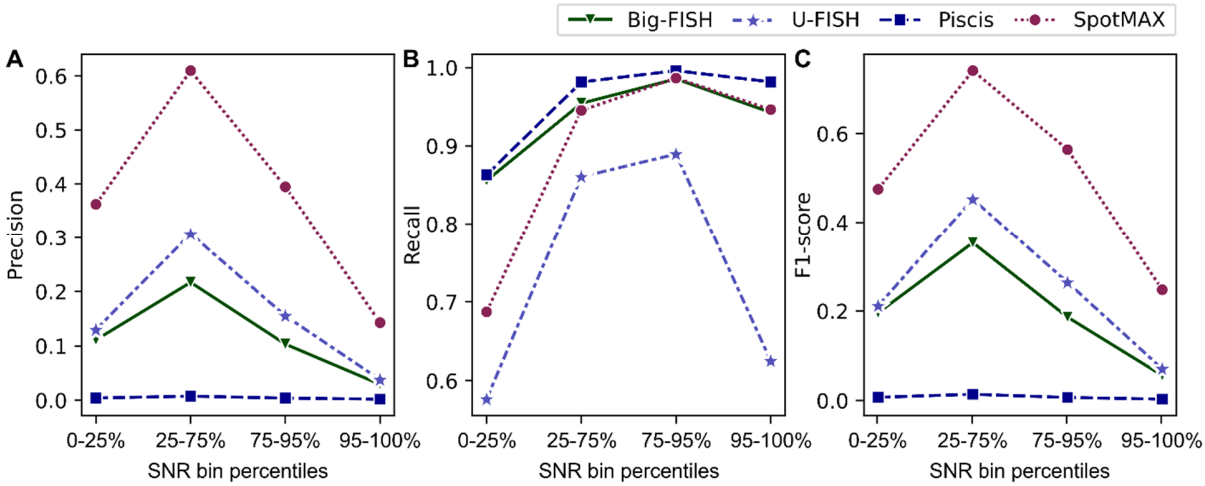
This PDF file includes:

Fig. S1.



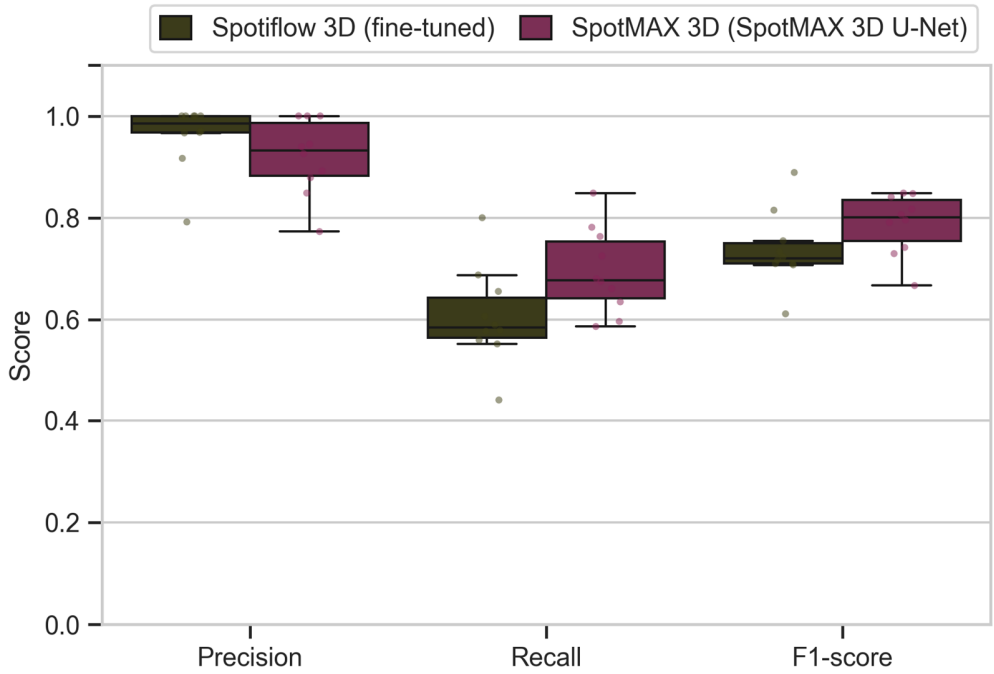
Model architectures of the SpotMAX U-Net models. Blocks represent feature maps, while the number on top is the number of filters. a, U-Net 2D. Compared to the original U-Net, we use padding during convolution to avoid decreasing the image size. b, U-Net 3D based on the library pytorch3d-unet.

Fig. S2.



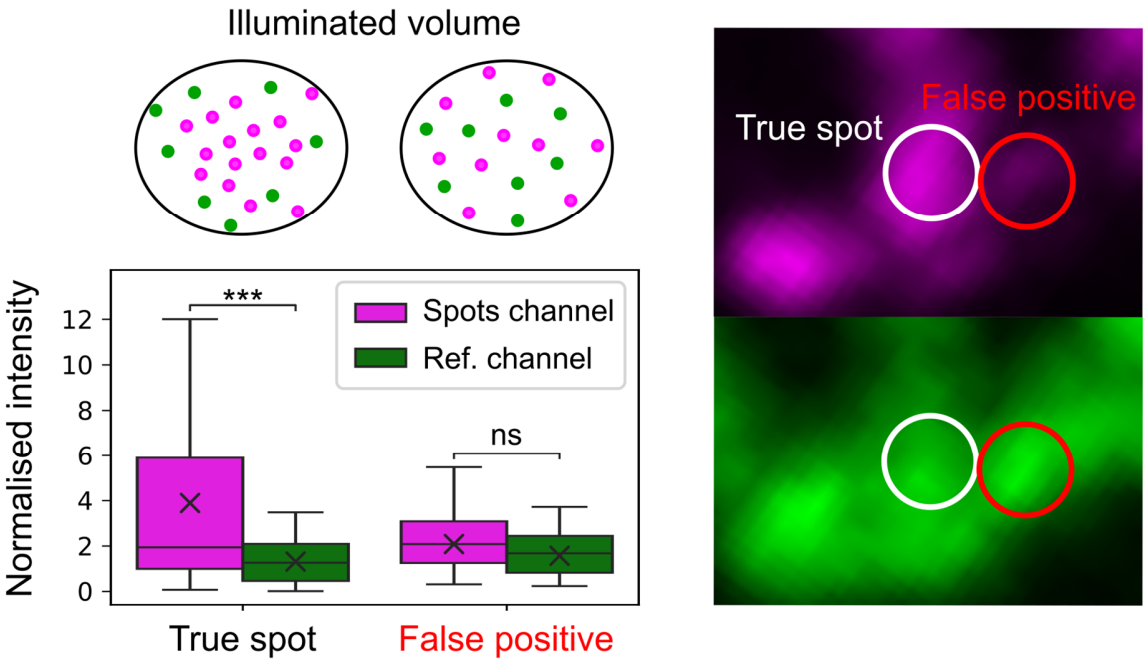
Benchmarking detectors on the entire dataset stratified by spot signal-to-noise ratio (SNR). Precision (a), Recall (b), and F1-score (c) of Big-FISH, U-FISH, Piscis, and SpotMAX as a function of spot signal-to-noise ratio (SNR). Ground-truth spots were assigned to four SNR bins (0–25%, SNR=0.08-2.90; 25–75%, SNR=2.90-5.74; 75–95%, SNR=5.74-9.29; and 95–100%, SNR=9.29-20.21) based on their relative SNR ranking within the dataset. SNR for each ground-truth spot was computed as the difference between the mean intensity inside a spheroidal spot mask centred at the spot coordinates with z, y, x radii = (3, 4, 4) and the mean of the in-cell background divided by the background standard deviation. The background pixels were the pixels inside the cell mask and outside of the spot masks. To calculate precision, the total number of false positives was used in every bin. The F1-score was computed as the harmonic mean of precision and recall. Stratifying performance by SNR reveals method-specific differences in detection sensitivity and robustness across low- to high-contrast scenarios. Notably, this analysis enables direct comparison of method performance as a function of detection difficulty, where different imaging modalities are mixed.

Fig. S3.



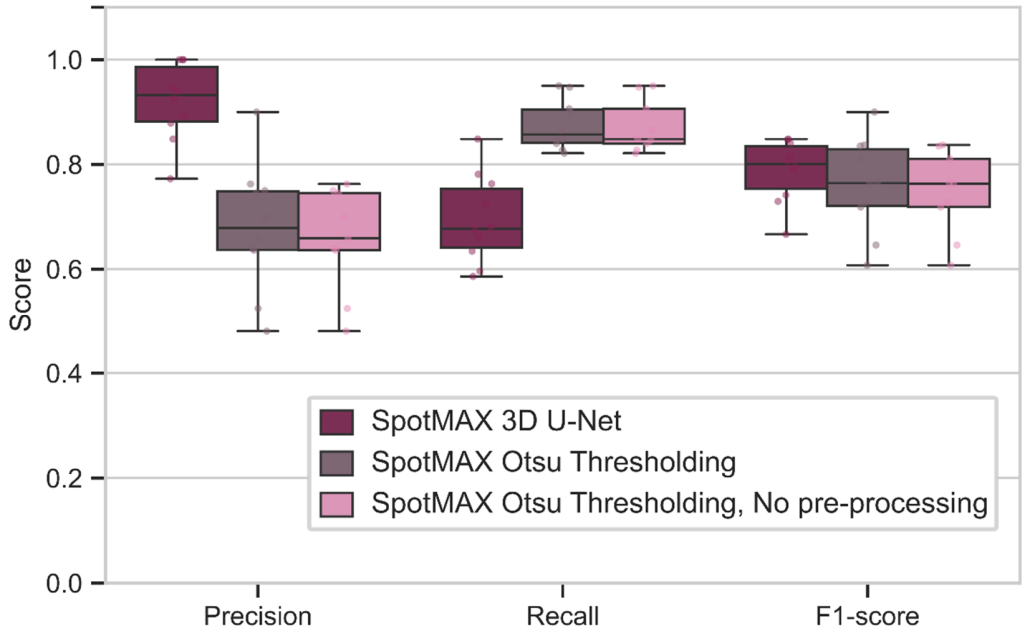
Benchmarking SpotMAX and Spotiflow fine-tuned on the same dataset. To benchmark the performance of the SpotMAX 3D U-Net, we fine-tuned both SpotMAX and Spotiflow on the same dataset and computed precision, recall, and F1 score on a test dataset that was not seen during training. The dataset is the same as in Fig. 2 (190 z-stacks), and it was split into 170 z-stacks for training, 10 for validation, and 10 for testing. Spotiflow was fine-tuned from the pre-trained "Synthetic 3D" dataset following the instructions provided by the Spotiflow documentation. SpotMAX was fine-tuned by visually inspecting a single image from the validation set and manually determining a threshold value of 0.2 for the minimum Glass effect size of the true spots (compared to the background). This numerical feature was then passed to SpotMAX to further refine the analysis presented in Fig 2. The Glass effect size is one of the standard numerical features computed by SpotMAX, and it is calculated as the difference between the mean intensity of each spot and the mean of the background outside of the segmented spots (and inside the segmented cells) divided by the standard deviation of the background pixels.

Fig. S4.



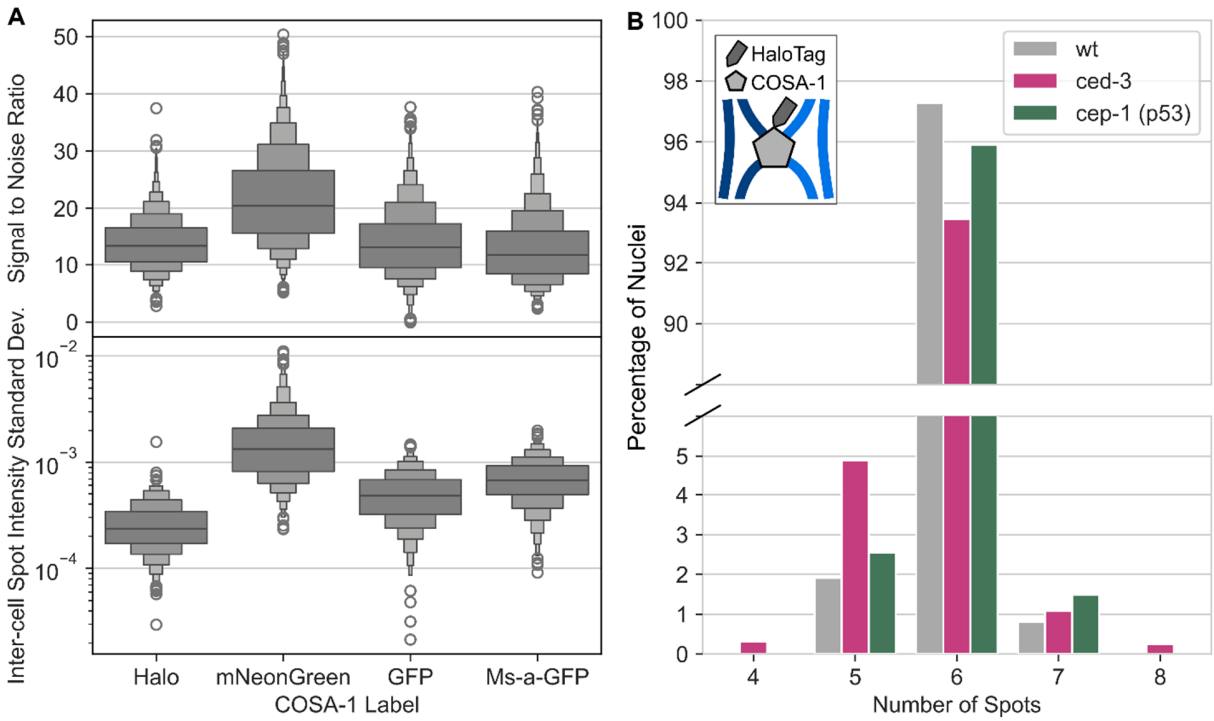
Validation of spot detections using a reference channel. Representative images of the spot channel and reference channel are shown, with example true and false-positive detections highlighted. For each candidate spot, intensities within the spot mask are compared between channels after normalization by the median intensity inside the reference channel mask and outside spot regions. Box plots show the intensity distributions for true and false positive detections in both channels. The horizontal line is the median, while the "X" marker indicates the mean. In the S. cerevisiae mtDNA datasets, spots are retained if the normalised signal in the spot channel (mtDNA) is significantly higher (Welch's t-test, $p < 0.025$) than in the reference channel (mitochondria network). p-values: True spot, $p < 1.0x10\text{-}10$; False positive, $p = 0.2$.

Fig. S5.



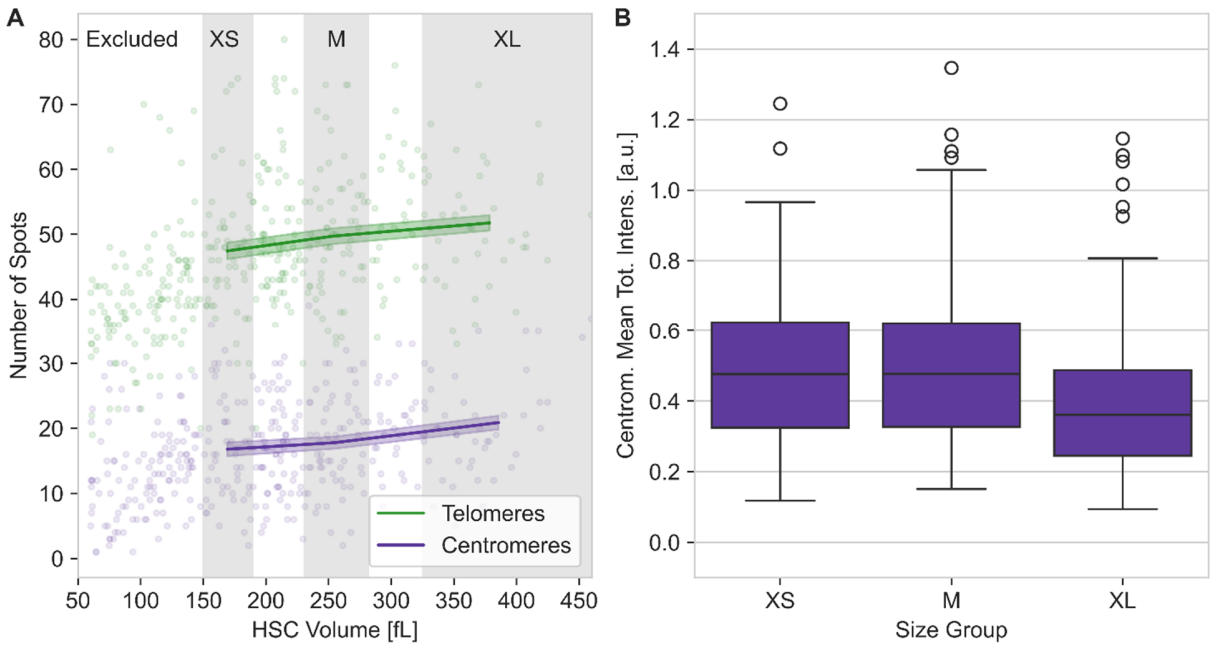
Ablation analysis of SpotMAX pipeline components. To assess the contribution of individual components, we performed a stepwise ablation of the SpotMAX pipeline. First, we used the Otsu thresholding algorithm instead of the 3D U-Net, and then we disabled pre-processing as well. Median detection performance decreased progressively with each ablation.

Fig. S6.



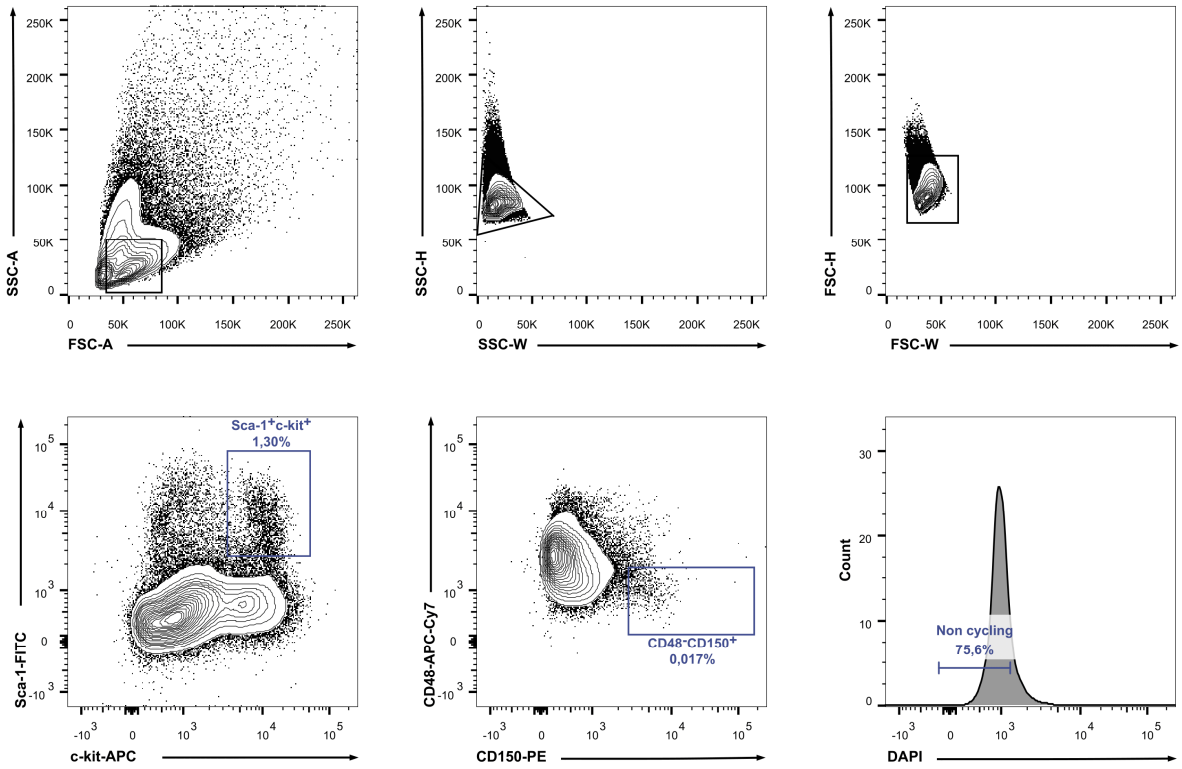
SpotMAX analysis of COSA-1 labels and crossover counting in *C. elegans* apoptosis mutants. a, Signal-to-Noise Ratio (SNR) of each spot (top) and standard deviation of the spot intensity distribution in each nucleus (bottom) for the 4 visualisation methods tested (see main text). SpotMAX calculates the SNR as the difference between the mean of the spot intensities within the resolution-limited volume and the mean of the background, all divided by the standard deviation of the background (i.e. Glass effect size). The background was defined as those pixels outside the spot masks and inside the nuclear masks. **b**, Number of chromosomal crossovers in all the segmented nuclei without excluding nuclei positive for RAD-51 (i.e., without unresolved DNA breaks).

Fig. S7.



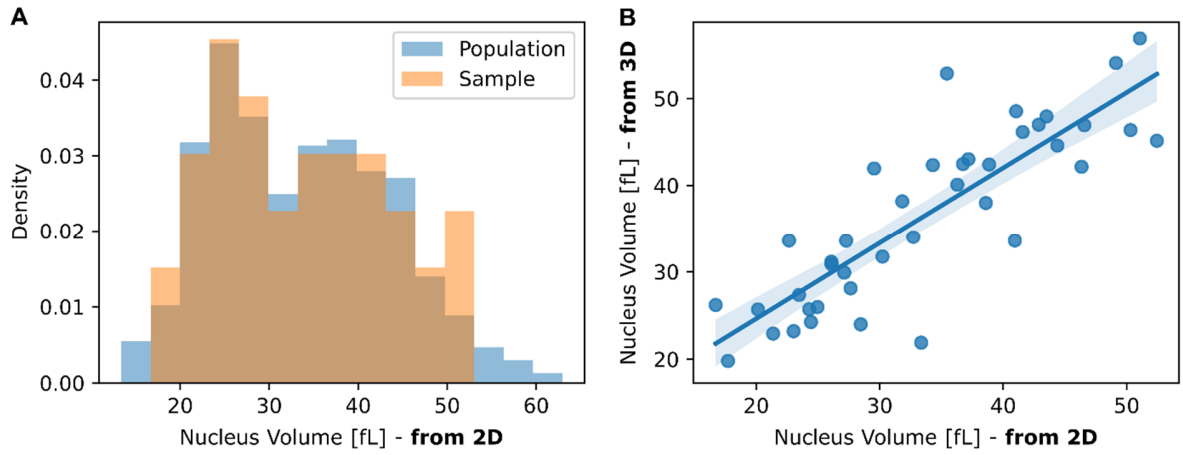
SpotMAX analysis of telomeres and centromeres from DNA-FISH data in hematopoietic stem cells. a, Number of spots for both telomeres and centromeres as a function of the entire range of analysed hematopoietic stem cells. In the main analysis, cells below 150 fL were excluded because the number of spots was increasing with cell volume, indicating a higher degree of telomeres and centromeres clustering in the smaller cells. **b**, Centromeres mean total intensity in each cell for each size category group. Number of cells per category: Excluded = 123, XS = 55, M = 65, XL = 55, Total = 175.

Fig. S8.



Example of gating strategy. Sorting protocol used to isolate BM-derived live G0/1 (Hoechst) HSCs (Lin-, Sca1/Ly6+, CD117/cKit+, CD150/Slamf1+, CD48/Slamf2-) from an 8-12 week-old mouse in representative FACS plots.

Fig. S9.



Validation of 2D-based nuclear volume estimation using a representative subset of 3D segmentations. a, Distribution of 2D-estimated nuclear volumes of intestine nuclei in L1 *C.elegans* larvae for the full population and the stratified sample selected for 3D segmentation. The sample was obtained by proportional stratified sampling across 20 quantiles of the volume distribution (estimated from 2D), ensuring coverage of the full dynamic range. Both distributions are shown as normalized histograms (density) using identical binning. **b**, Comparison of 2D-estimated and 3D-measured nuclear volumes for the sampled subset. Each point represents a single nucleus. The solid line shows the linear fit, with the shaded region denoting the 95% confidence interval obtained via bootstrap resampling. The strong agreement between the two measurements is reflected in high Pearson and Spearman correlation coefficients (0.851 and 0.850, respectively), indicating both linear correspondence and good preservation of rank across the full range of volumes.

Table S1.

Dataset	Organism	Method	Number of volumes	Number of cells	Gene	Pixel size (pixel/µm)	z-space (µm)	Figure
Yeast_ smFISH	*S. cerevisiae*	Single-molecule RNA FISH	51	373	*MDN1*	0.0721	0.24	2a,b
			109	1616	*SUT509*			
Yeast_mito	*S. cerevisiae*	LacO-LacI-mNeonGreen	190	357	mtDNA (LacO)	0.0672	0.35	2c,d
C_elegans_CO	*C. elegans*	(Endogenous) tagging or antibody staining	19	548	*cosa-1*	0.0339	0.15	2e,f

Experimental datasets used to benchmark SpotMAX. "mtDNA" stands for mitochondrial DNA, and LacO are the Lactose Operon repeats stably integrated into mtDNA. More details about the mtDNA visualisation system are available in Ref. (*16, 30*).

Table S2.

Dataset	Organism	Method	Number of volumes	Number of cells	Gene	Pixel size (pixel/µm)	z-space (µm)	Figure
C_elegans_CO_labels	*C. elegans*	(Endogenous) tagging or antibody staining	58	1687	*cosa-1*	0.0339	0.15	3b,c,d
C_elegans_CO_apopt	*C. elegans*	Endogenous tagging	120	3399	*cosa-1*	0.0339	0.15	3e,f,g
Yeast_mito_timelapse	*S. cerevisiae*	LacO-LacI-mNeonGreen	223	280	mtDNA (LacO)	0.0792	0.35	4
HSCs_telo_centromere	Murine Hematopoietic stem cells	DNA FISH	292	298	Telomeres and centromeres repeat	0.0547	0.75	5
C_elegans_Raptor	*C. elegans*	Endogenous tagging	41	715	*fib-1*	0.1346	0.2	6

Experimental datasets used to test SpotMAX's ability to obtain new biological insights. "mtDNA" stands for mitochondrial DNA, and LacO are the Lactose Operon repeats stably integrated into mtDNA. More details about the mtDNA visualisation system are available in Ref. (*16, 32*).

Table S3.

Name	Genotype	Description	Origin	Figure
FPY004-1	***Mat α/a;*** *ura3/URA3, TRP1/TRP1, mt-LacO, ADE2/ADE2, HO/HO::CUP1prom-SU9-2xmNeonGreen-LacI-PGK1prom-SU9-mKate2-KanMX4*	Diploid microscopy strain with LacO-LacI system	This study	4

Yeast strain used in this work. All strains are based on W303.