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PanoMito: mapping mitochondrial heterogeneity from morphofunctional profiling to single-mitochondrion spatial transcriptomics

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Abstract

The complexity of the relationship between mitochondrial morphology and function has been widely recognized, yet systematic investigation of this relationship has been hindered by the lack of accurate segmentation tools and comprehensive benchmarks. Here we present PanoMito, an integrated toolbox built upon PanoMitoAtlas, to our knowledge the largest multi-modal fluorescence mitochondrial dataset comprising >130,000 annotated instances across 16 species; and PanoMitoNet, a deep-learning framework unifying instance segmentation and unsupervised classification. PanoMitoNet achieves superior precision (AP₅₀ of 65.0%) compared to existing methods. At single-mitochondrion resolution, PanoMito reveals extensive mitochondrial heterogeneity across species and cell types, providing a quantitative approach to dissect the complex morphology-function relationship without relying on morphological inference alone. Furthermore, integration with *in situ* sequencing enables single-mitochondrion spatial transcriptomics. By providing single-organelle resolution across morphology, function, and transcriptomics, PanoMito establishes a comprehensive, universally applicable framework for dissecting mitochondrial biology from model systems to non-model organisms.

Mitochondria serve as central hubs of cellular metabolism and bioenergetics and govern a wide array of cellular processes including cell fate determination¹, immune response², neural function^{3,4}, and organismal longevity⁵. The execution of these functions is intrinsically linked to the electron transport chain (ETC), which drives ATP synthesis, establishes the mitochondrial membrane potential ($\Delta\Psi_m$), and generates reactive oxygen species (ROS) as a byproduct. Moreover, it gives rise to the proton motive force essential for mtDNA copy number maintenance⁶. As highly dynamic organelles, mitochondria exhibit remarkable morphological heterogeneity across different tissues, cell types, and cellular states. Moreover, even within a single cell, mitochondria are not homogeneous; their morphology and function vary across subcellular regions, reflecting localized metabolic demands and dynamic regulation. For instance, within a single cell, distinct mitochondrial subpopulations specialize in either ATP production or biosynthesis⁷. These structural dynamics fundamentally shape the spatial distribution and assembly patterns of ETC complexes, thereby modulating electron transfer efficiency and ultimately fine-tuning a spectrum of key mitochondrial functional outputs to match cellular demands⁸⁻¹¹.

The relationship between mitochondrial morphology and function is complex and context dependent rather than linear. For instance, in mammalian cells, rescuing antioxidant capacity reversed ACBD5 knockdown-induced mitochondrial elongation, yet this morphological change did not restore the concomitant loss of membrane potential¹². Similarly, H₂O₂-induced fragmentation in yeast led to a disrupted mitochondrial network but left $\Delta\Psi_m$ and electron transfer uncompromised¹³. In *Drosophila* flight muscle, which has an exceptionally high ATP demand, enhancing mitochondrial fusion paradoxically impaired proper muscle development and resulted in a leg-like muscle type, rather than supporting flight muscle formation as might be expected¹⁴. These observations demonstrate that the link between mitochondrial morphology and core functional parameters is highly context-dependent and exhibits considerable variability and complexity. However, existing analytical frameworks have largely relied on morphology alone to infer functional states or on population-averaged measurements that mask single-organelle heterogeneity^{15,16}, leaving this complexity insufficiently addressed. To bridge this gap, research tools that integrate quantitative morphological analysis with direct measurements of multiple functional readouts (e.g., $\Delta\Psi_m$, ROS, molecular profiles) at high throughput and single-organelle resolution are urgently needed to dissect this complex relationship.

A major barrier to such integrated, quantitative analysis is the lack of computational tools for accurately segmenting individual mitochondria from fluorescent images. Both morphological and functional quantification depend on precise delineation of the abundant, densely packed mitochondria. Conventional thresholding-based segmentation algorithms, including MitoMeter¹⁷, which was developed for automated segmentation and tracking of mitochondria in live-cell 2D and 3D time-lapse images, often struggle to resolve the intricate and densely entangled architecture of mitochondrial networks against complex background signals. Deep learning approaches using semantic segmentation followed by morphological postprocessing have achieved success in electron microscopy. More recently, MoDL^{15,16}, a mitochondria-specific segmentation method, extended this strategy to fluorescence microscopy, yet its instance-level generalizability across modalities remains to be evaluated. Moreover, established instance segmentation tools such as StarDist¹⁸, CellPose¹⁹⁻²², and OmniPose²³ are designed primarily for convex or elongated cellular shapes. These methods often yield fragmented segmentation of branched structures or incorrectly merge adjacent instances when segmenting highly entangled mitochondrial networks. Other mitochondria-specific tools, including MitoHacker²⁴, Momito²⁵, and MitoSegNet²⁶, perform binary or semantic segmentation and provide valuable post-segmentation analysis platforms, yet they do not directly output individual mitochondrial instance masks required for systematic morphological and functional profiling at single-organelle resolution.

Equally limiting is the scarcity of large-scale, multi-modal datasets for training and benchmarking. While electron microscopy datasets such as Mito-EM^{27,28} and OpenOrganelle²⁹ have provided high-resolution structural atlases, they are not suitable for live cell studies and lack functional and molecular readouts at the single-organelle level. The MoDL^{15,16} dataset is publicly available only as semantic segmentation outputs rather than instance-level annotations. To our knowledge, no publicly available large-scale fluorescence datasets annotated for mitochondrial instance segmentation currently exist. Additionally, the existing classification of mitochondria predominantly relies on manual morphological categorization³⁰⁻³³, which struggles to resolve fine-grained subtypes and inevitably introduces subjective bias. Likewise, the lack of methods to systematically

87 correlate morphology with functional and molecular states limits integrative understanding of mitochondrial
88 biology. These gaps necessitate tools capable of accurate segmentation, objective classification, and multi-
89 dimensional analysis at single-mitochondrion resolution.

90 To overcome these limitations, we developed PanoMito, a comprehensive toolbox built on two core resources.
91 First, PanoMitoNet is a deep learning framework that unifies the state-of-the-art supervised instance
92 segmentation with an unsupervised deep clustering module for objective morphological classification. The
93 segmentation component overcomes the limited precision of existing methods in delineating individual
94 organelles in entangled networks, enabling accurate segmentation of filamentous and densely packed
95 mitochondria. Second, to train and benchmark this model, we curated PanoMitoAtlas, a large-scale, multi-modal
96 benchmark dataset containing over 130,000 annotated mitochondrial instances across 16 species and 14 tissue
97 types with different imaging modalities. On this challenging benchmark, PanoMitoNet achieves an AP₅₀ of 65.0%
98 on the entire dataset, significantly outperforming established methods such as MaskDINO, CellPose, OmniPose,
99 etc. Using this framework, we first integrate morphological profiling with functional imaging to study single-
100 mitochondrion heterogeneity across species and cell types. Beyond morphofunctional profiling, we address the
101 unresolved challenge of assigning mitochondrial transcripts to individual organelles by combining PanoMito
102 with in situ sequencing, achieving single-mitochondrion spatial transcriptomics and providing a robust
103 framework to dissect the complex interplay between mitochondrial architecture and molecular identity. The
104 PanoMito toolbox is available at <http://www.panomito.com>.

Results

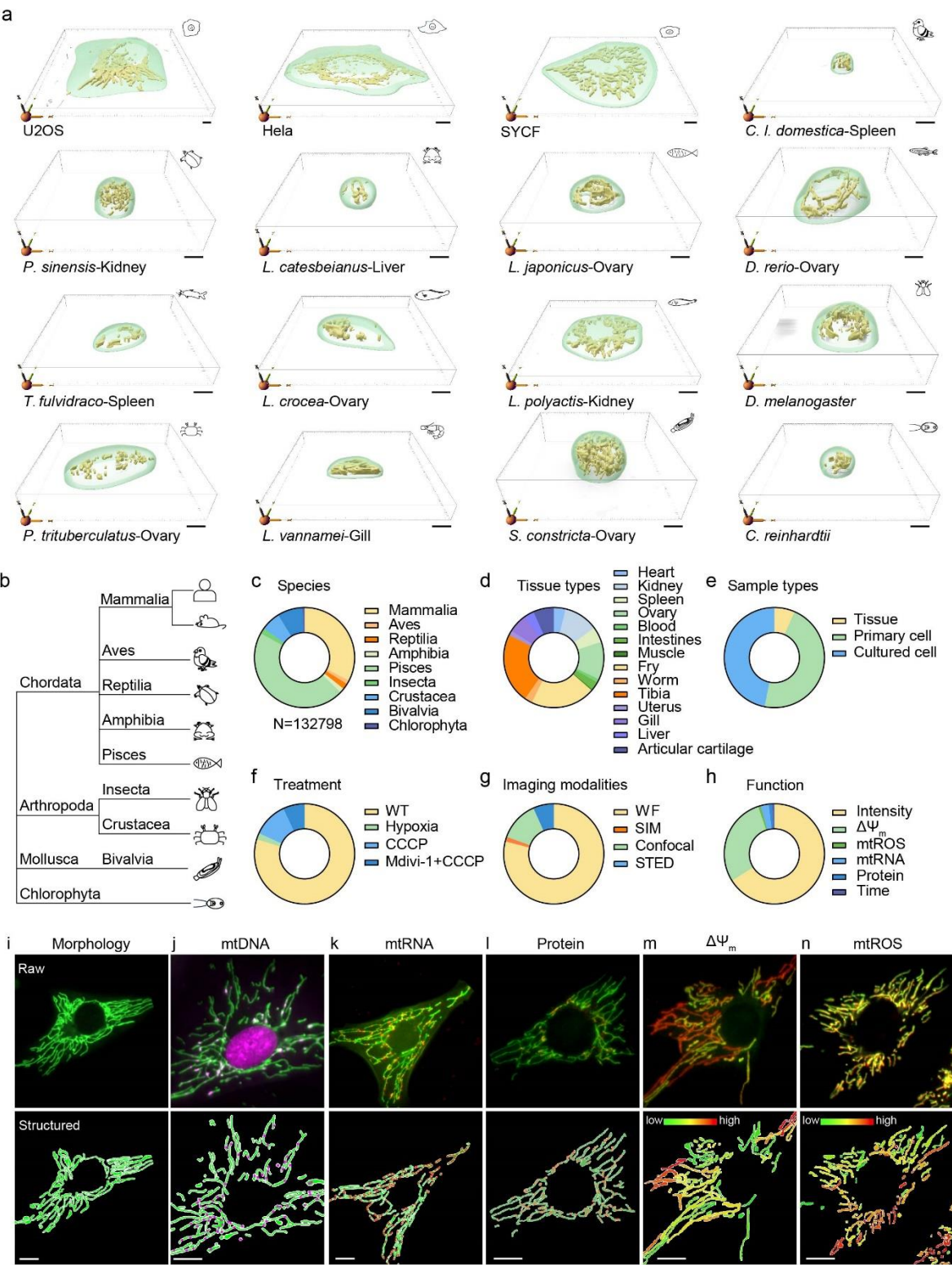


Fig. 1 – PanoMitoAtlas: A large-scale, cross-species, multimodal dataset for mitochondrial segmentation and morphofunctional studies. (a) Three-dimensional surface renderings of mitochondrial networks across

diverse species, visualized within the cellular volume (species indicated by icons). Scale bars: 5 μm . **(b)** Phylogenetic classification of species included in the dataset, spanning Mammalia, Aves, Reptilia, Amphibia, Pisces, Insecta, Crustacea, Bivalvia, and Chlorophyta. **(c–h)** Distributions of annotated mitochondrial instances across the dataset, categorized by (c) species, (d) tissue type, (e) sample type, (f) treatment, (g) imaging modality, and (h) function. Total number of annotated instances is $N = 132,798$. **(i–n)** Representative examples showing raw fluorescence images (top row) and corresponding processed digital structured images of mitochondrial representations (bottom row). (i) Structured image depicts mitochondrial instances with white outlines and green fill. (j–l) Dual-color raw fluorescence images (top) paired with structured images (bottom) of mitochondrial instances overlaid with magenta mtDNA puncta (j), red mtRNA puncta (k) or orange mitochondrial protein puncta (l). (m, n) Dual-color raw images (top) and structured image with pseudocolored ratios (bottom) quantifying average mitochondrial membrane potential ($\Delta\Psi_m$: TMRM/MitoTracker fluorescence ratio; m) and mitochondrial reactive oxygen species (mtROS: MitoSOX/MitoTracker fluorescence ratio; n) at the single-organelle level. Scale bar: 5 μm .

122 PanoMitoAtlas: A large-scale, cross-species, and multimodal benchmark for 123 mitochondrial segmentation and morphofunctional studies

To address the pressing need for robust segmentation tools and to provide a systematic resource for interrogating mitochondrial morphology–function relationships, we present PanoMitoAtlas, a foundational community resource for mitochondrial biology and the largest multi-modal mitochondrial dataset currently available. It comprises 132,798 manually annotated mitochondria (Fig. 1, Table S1), each representing a spatially continuous 2D mitochondrial object in a single image plane without redundant sampling across z-sections or time. The full annotation criteria, sampling strategy, and dataset composition are detailed in the Methods and Table S1. The dataset captures a remarkable morphological diversity of mitochondrial networks across species (Fig. 1a) and was deliberately designed to incorporate multidimensional biological and technical variation along several key axes: species, tissue types, sample types, experimental perturbations, and imaging modalities, thereby ensuring broad biological relevance and mitigating model overfitting or limited generalizability caused by homogeneous sampling.

First, in terms of phylogenetic breadth, the dataset includes samples from 16 evolutionarily diverse species spanning vertebrates to invertebrates and plants, including *Homo sapiens* (*H. sapiens*), *Columba livia domestica* (*C. l. domestica*), *Pelodiscus sinensis* (*P. sinensis*), *Lithobates catesbeianus* (*L. catesbeianus*), *Danio rerio* (*D. rerio*), *Portunus trituberculatus* (*P. trituberculatus*), *Drosophila melanogaster* (*D. melanogaster*), and *Chlamydomonas reinhardtii* (*C. reinhardtii*) (Fig. 1b, c; full list in Table S1). The dataset covers 14 tissue types, such as heart, liver, kidney, and muscle (Fig. 1d), thereby reflecting the physiological heterogeneity of mitochondrial morphology across organ systems. This phylogenetic and tissue breadth ensures trained models to capture evolutionarily conserved mitochondrial features while remaining robust across diverse biological scenarios.

Second, PanoMitoAtlas integrates multiple sample types, including tissue sections, primary cells, and cultured cell lines (Fig. 1e). Notably, it contains a substantial number of primary cell samples isolated from the native tissues using a standardized protocol (Fig. S1). This efficient, compatible, and reproducible workflow enables rapid cell isolation, staining, and imaging across diverse species and tissue types, supporting simultaneous acquisition of mitochondrial morphology with organelle-level functional or molecular readouts. By preserving cells in a near-native state, it provides a biologically relevant alternative for studying non-model organisms, where establishing immortalized cell lines is difficult. Thus, it serves as a practical, transferable toolkit to accelerate research progress in these fields.

Third, to link morphology with function, the dataset introduces controlled experimental perturbations to cultured cells, which modulate mitochondria dynamics and homeostasis, including treatment with depolarization, fission inhibition, and hypoxic exposure (Fig. 1f). These interventions expand the morphological diversity captured in the atlas and provide functional context for observed structural changes. Fourth, PanoMitoAtlas contains multimodal images acquired by wide-field, spinning-disk confocal³⁴, structured illumination microscopy (SIM)^{35,36}, and Stimulated emission depletion (STED)³⁷ (Fig. 1g).

158 Critically, PanoMitoAtlas integrates functional and molecular measurements across approximately 50000
159 mitochondrial instances (Fig. 1h), establishing an unprecedented resource for structure-function analyses. These
160 include mitochondrial DNA (mtDNA, Fig. 1j), mitochondrial mRNA (mtRNA) distribution (Fig. 1k), protein
161 expression (Fig. 1l), $\Delta\Psi_m$ (TMRM/MitoTracker fluorescence ratio, Fig. 1m), and mtROS levels
162 (MitoSOX/MitoTracker fluorescence ratio, Fig. 1n). Collectively, this multimodal dataset allows for the direct
163 correlation of structural changes with functional and molecular readouts, thus offering a comprehensive resource
164 for investigating mitochondrial morphofunctional relationships.

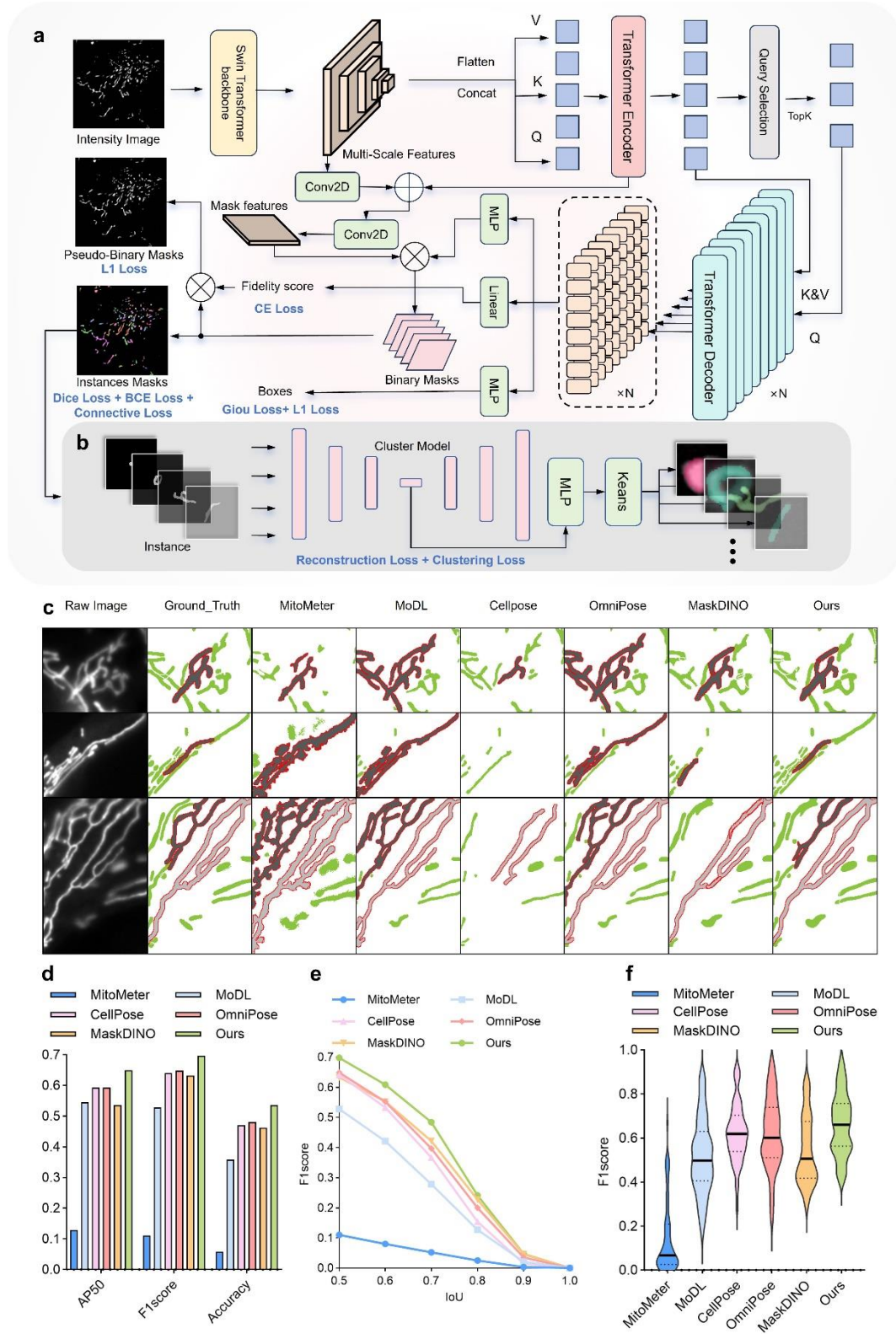


Fig. 2 – PanoMitoNet: a unified framework for mitochondrial instance segmentation and classification. (a) Overview of the PanoMitoSeg architecture. The segmentation framework employs a Swin Transformer backbone and an encoder-decoder architecture. Following the decoder, three task-specific heads are applied: a multilayer perceptron (MLP) head outputs bounding boxes, a linear head predicts the instance fidelity score, and a separate MLP head generates weight queries to select features for instance mask generation. The instance masks are combined via a fidelity-score-weighted summation to form a pseudo-semantic map which serves as

global guidance. **(b)** Overview of the PanoMitoCluster architecture. The instance masks generated by the segmentation network are resized to 128x128 pixels and then serve as input to the subsequent clustering network. The clustering network employs an encoder-decoder architecture and is optimized through a self-reconstruction objective. The bottleneck features are processed through two fully connected layers, with a Kullback–Leibler (KL)-divergence loss applied to the output distribution to enhance cluster separability. The final 128-dimensional feature vectors are used for k-means clustering of morphological phenotypes. **(c)** Visualization of mitochondrial segmentation results. The leftmost panel displays the raw image of the selected region. The green areas on the right show the predictions of each method. The region highlighted as an example is emphasized with a red outline and gray fill. **(d)** Benchmark of our method against MitoMeter, MoDL, CellPose-SAM, OmniPose and MaskDINO on average precision at 50% (AP_{50}) intersection over union (IoU), F1score and accuracy ($\frac{TP}{TP+FP+FN}$) metrics at 50% IoU across entire PanoMitoAtlas dataset. AP_{50} : 65.0% (ours), 53.6% (MaskDINO), 59.4% (OmniPose), 59.4% (CellPose-SAM), 54.5% (MoDL), 12.9% (MitoMeter). F1score: 69.8% (ours), 63.3% (MaskDINO), 64.9% (OmniPose), 64.1% (CellPose-SAM), 52.8% (MoDL), and 11.0% (MitoMeter). Accuracy: 53.6% (ours), 46.3% (MaskDINO), 48.1% (OmniPose), 47.1% (CellPose-SAM), 35.9% (MoDL), and 5.8% (MitoMeter). **(e)** F1score comparison on the entire PanoMitoAtlas dataset across various IoU thresholds that range from 0.5 to 1.0. **(f)** Image wise F1score comparison on the mitochondria-dense subset, which consists of images containing ≥ 100 instances.

PanoMitoNet achieves accurate instance segmentation of complex mitochondria

To enable the precise segmentation and objective classification of mitochondria across our diverse dataset, we developed PanoMitoNet, a deep learning framework that unifies a supervised instance segmentation network and an unsupervised deep clustering module (Fig. 2a, b). Conventional threshold-based segmentation methods such as MitoMeter are highly susceptible to image noise. As shown in the last two rows of Fig. 2c, it fails to accurately delineate fine mitochondrial structures under noisy conditions. Two-stage deep learning methods derive individual instances from intermediate segmentation outputs through post-processing. Among these, MoDL is the most closely related mitochondria-specific segmentation method to our work in terms of biological application; it generates binary mitochondrial masks using a U-Net-style model and then obtains individual mitochondrial objects through connected-component analysis. However, when adjacent mitochondria are connected in the predicted binary mask, they are prone to being assigned to a single component—a failure mode particularly evident in densely packed, adherent, or intertwined networks (Fig. 2c). CellPose and OmniPose reconstruct object masks from intermediate geometric representations (flow fields or distance-field gradients, respectively); CellPose tends to over-segment intricate morphologies and miss detections, while OmniPose struggles to separate adherent instances (Fig. 2c). End-to-end architectures such as MaskDINO^{38–40} avoid post-processing, but even after dataset-specific training they frequently suffer from missed detections and duplicate predictions for the same instance (Fig. 2c, bottom row).

To overcome these limitations, the segmentation core, PanoMitoSeg, adopts an end-to-end Detection Transformer (DETR)⁴¹. This architecture leverages self-attention mechanisms to capture global context without relying on shape-specific post-processing (Fig. 2a), thereby offering superior topological flexibility for handling the complex structural variations of mitochondria. To address the missed-detection and duplicate-prediction problems that persist in general-purpose end-to-end models, PanoMitoSeg implements a pseudo-semantic segmentation map (Fig. 2a, S2) and a connective loss function (Methods) to establish a robust framework capable of high-fidelity instance prediction without the need for manual parameter tuning. We next benchmarked PanoMitoSeg against established segmentation methods on PanoMitoAtlas to quantitatively validate its performance. To ensure a fair comparison, all benchmark methods were retrained or fine-tuned on PanoMitoAtlas under the same unified framework with identical training and testing splits. (Methods) For MoDL and CellPose-SAM, multiple parameter settings were tested and the best-performing configurations were selected; for OmniPose, the authors' recommended settings were used; for MaskDINO, the backbone and training configuration were matched to PanoMitoSeg as closely as possible. MitoMeter, being a non-trainable tool, was applied using its original implementation. The training implementation details and parameter-tuning procedures for each method are summarized in Table S2 and Table S3.

We evaluated all the algorithms with the average precision metric at 50% intersection over union (IoU)

thresholds (AP_{50}), F1score and accuracy ($\frac{TP}{TP+FP+FN}$) in instance level. On the complete PanoMitoAtlas benchmark, PanoMitoNet achieved AP_{50} of 65.0%, substantially outperforming established methods including MaskDINO (53.6%), OmniPose (59.4%), CellPose-SAM (59.4%), MoDL (54.5%), and MitoMeter (12.9%) (Fig. 2d). We evaluated the F1score metric across various IoU thresholds and our method outperformed all the other approaches over the entire spectrum of IoU thresholds (Fig. 2e). Furthermore, we selected a challenging subset characterized by densely packed and structurally complex mitochondria to further evaluate the effectiveness of each method. Results on this subset demonstrate that both MitoMeter and MoDL nearly fail in such dense regions. MaskDINO exhibits degraded performance, while CellPose and OmniPose remain viable but our approach shows best performance (Fig. 2f). Representative images of densely packed mitochondria and the corresponding segmentation examples are shown in Fig. S5.

To assess the generalizability and cross-modality transferability of PanoMitoSeg, we further evaluated all benchmarked methods on species- and cell-line-specific subsets and performed modality-stratified validation to examine the influence of imaging modality and the cross-modality generalization of PanoMitoSeg across SIM, wide-field fluorescence microscopy, and confocal microscopy. The corresponding results are provided in Fig. S3 and Fig. S4. We note that several mitochondria-specific tools, including MitoHacker²⁴, Momito²⁵, and MitoSegNet²⁶, were not included in this benchmark because they perform binary or semantic segmentation rather than instance-level mask prediction, which is incompatible with our evaluation criteria. Table S4 provides a comprehensive comparison of mitochondria-specific and generalist segmentation tools, summarizing their instance-segmentation capability, segmentation strategy, downstream analysis, and key limitations.

Following segmentation, PanoMitoNet employs an unsupervised deep learning clustering module (PanoMitoCluster) to process the extracted instances (Fig. 2b, S6). The clustering module is constructed as an encoder-decoder network and trained with a self-reconstruction objective. The bottleneck features are passed through two fully connected layers, where a Kullback-Leibler (KL) divergence loss regularizes the output distribution to enhance cluster separability. The resulting 128-dimensional feature embeddings are then clustered using k-means to categorize mitochondrial morphological phenotypes. Unlike manual classification methods that rely on subjective morphological criteria, PanoMitoCluster first clusters mitochondrial instances in an unsupervised, data-driven manner and then assigns biological labels using a deterministic hierarchical rule based on cluster-level morphological features, including branch points, circularity, and aspect ratio. This strategy provides a reproducible classification into Branched, Loop, Sphere, Short tubule, Middle tubule, and Long tubule classes, supporting downstream morphofunctional analyses.

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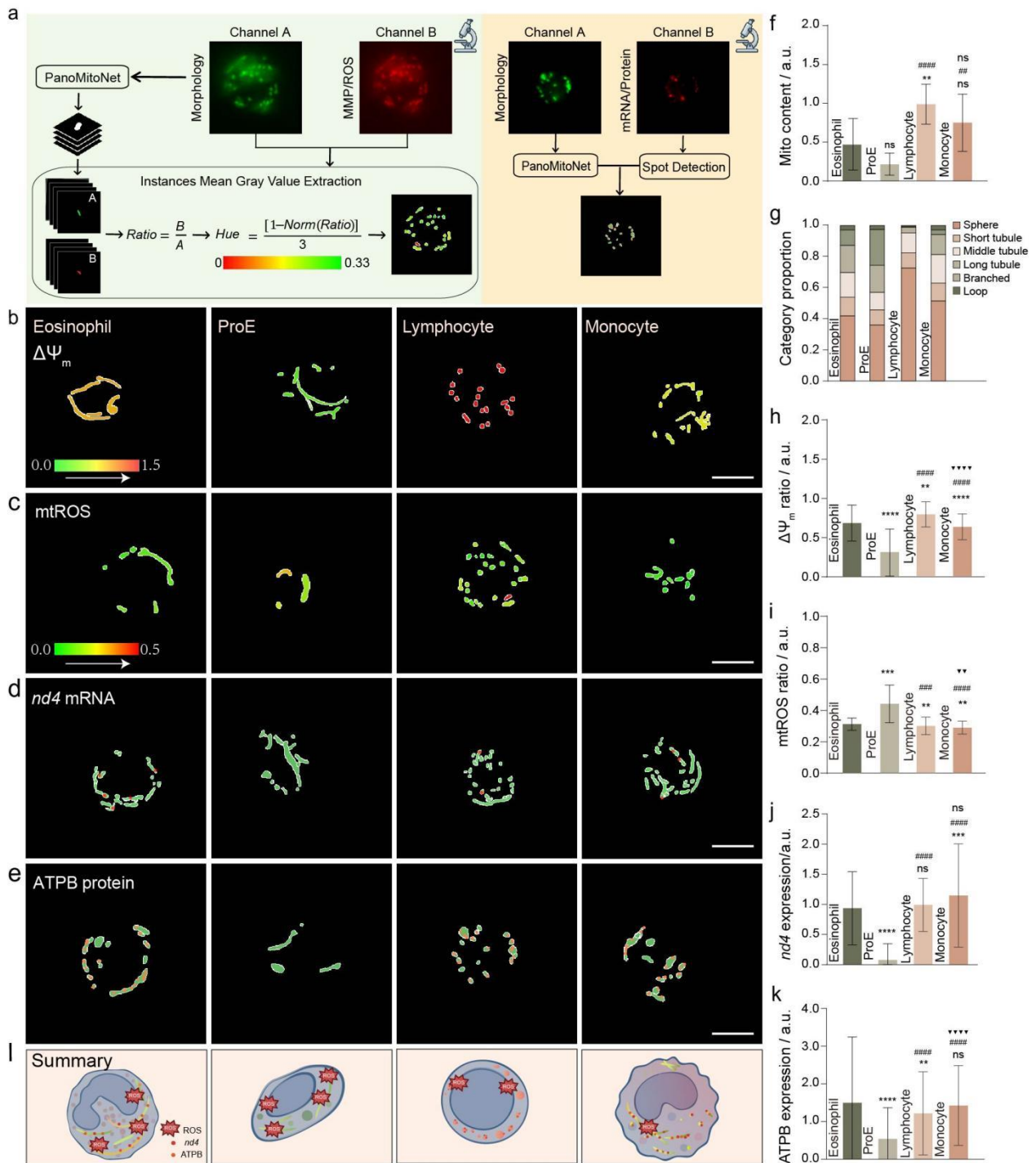


Fig. 3 – Intracellular mitochondrial heterogeneity across cell types in zebrafish kidneys. (a) Schematic of the processing pipeline to generate the final structured images. Morphofunctional images consist of two channels: a morphology channel and a function channel. PanoMitoNet segments individual mitochondria in the morphology channel. For $\Delta\Psi_m$ /mtROS measurements, the intensity ratio per mitochondrion is visualized as a red-to-green pseudocolor (high-to-low activity). For mRNA/protein measurements, spot-detection algorithms identify punctate signals within each mitochondrial boundary. **(b–e)** Representative structured images of mitochondrial morphology and function in *D. rerio* kidney immune cells: (b) $\Delta\Psi_m$, (c) mtROS, (d) *nd4* mtRNA, (e) ATPB protein. Corresponding raw fluorescence images are in Fig. S11. Scale bar: 5 μ m. **(f, g)** Quantitative morphological analysis in immune cells (n $\geq$ 10 per cell type): (f) relative mitochondria counts per cell, (g) proportion of six morphological categories. **(h–k)** Quantitative function/expression in immune cells: (h) $\Delta\Psi_m$, (i) mtROS, (j) *nd4* mtRNA, (k) ATPB protein. **(l)** Schematic summary. Data: mean $\pm$ s.d. * vs the first group; #

265 vs the second group; ▲ vs the third group ($p < 0.05$).

266 Single-mitochondrion morphofunctional profiling across species and cell types

267 To systematically investigate mitochondrial heterogeneity across different biological scales, we established a
268 multi-dimensional analytical workflow based on the PanoMito platform (Fig. 3a). This workflow integrates
269 morphological segmentation with functional or molecular measurements at single-mitochondrion resolution.
270 We employed modality-specific extraction strategies in functional profiling: for $\Delta\Psi_m$ and mtROS measurements,
271 we extracted average fluorescence intensity from each segmented mitochondrion in both channels, calculating
272 the ratio of function-to-morphology signal and mapping this to a red-to-green HSV color gradient for
273 visualization (red: high activity, green: low activity); for mRNA and protein signals, we applied spot-detection
274 algorithms to identify punctate functional signals within each mitochondrial mask. This integration enables
275 simultaneous quantification of morphology (six categories) and function (four modalities) at individual
276 organelle resolution.

277 We first examined oogonia, the most stable cell in the germ cell lineage that is characterized by a large nucleus
278 and the smaller spherical shape⁴², from four fish species: freshwater *D. rerio* and *L. japonicus*, and marine *L.*
279 *crocea* and *L. polyactis* (Fig. S7a). Benchmarking against manual annotations confirmed that PanoMitoNet
280 accurately quantified key morphometrics, including average mitochondrial count (Fig. S7b), area (Fig. S7c),
281 and length (Fig. S7d), yielding results that closely approximated the ground truth. Based on automated
282 segmentation and clustering, we classified mitochondrial morphology into six categories: sphere, branched,
283 short-tubule, middle-tubule, long-tubule, and loop ($n \geq 20$ cells per species) (Fig. S8, Fig. S7e). Subsequent
284 quantification revealed that mitochondrial counts were significantly higher in *D. rerio* and *L. japonicus* ($p <$
285 0.05 , Fig. S7f). In contrast, *L. crocea* and *L. polyactis* exhibited larger sphere area (Fig. S7g) and longer short
286 tubule length (Fig. S7h). These differences may reflect adaptive responses to different aquatic environments,
287 but they could also arise simply from inherent species-specific variation. Disentangling these possibilities is
288 beyond the scope of this study.

289 Building upon this analytical foundation, we applied single-mitochondrion morphofunctional profiling
290 workflow to dissect mitochondrial heterogeneity across immune cell populations in zebrafish kidney. To ensure
291 reliable comparison, all immune cells were derived from the same batch of primary zebrafish kidney cells and
292 were plated, stained, and imaged under identical conditions using exactly the same imaging channels. We
293 aligned the Giemsa-stained brightfield images with the fluorescence images by coordinate registration and
294 segmented individual cells using a fine-tuned CellPose model (Fig. S9). Cell identity was determined solely by
295 Giemsa staining based on established morphological criteria. Distinct morphologically defined immune cell
296 types, including eosinophils, primitive erythrocytes (ProE), lymphocytes, and monocytes were identified by
297 Giemsa staining (Fig. S10) and subjected to multi-dimensional morphofunctional profiling using PanoMito (n
298 ≥ 10 per cell type). Data were analyzed at the cell level with mitochondrial features aggregated per cell and
299 each cell contributed one data point. The fluorescence images (Fig. S11), corresponding structured images (Fig.
300 3b-e), and quantitative analysis (Fig. 3f-l) all demonstrated significant differences in their mitochondrial
301 morphologies, functional levels, and molecular expressions. Verapamil controls confirmed that dye efflux did
302 not significantly affect TMRM measurements across immune cell types (Fig. S12).

303 Single-mitochondrion morphofunctional profiling revealed distinct bioenergetic strategies across these
304 populations among those cell types. Eosinophils displayed a predominantly tubular mitochondrial network
305 (45.4%) with high $\Delta\Psi_m$, consistent with their elevated expression of *nd4* mtRNA and ATPB protein. These
306 results support findings that eosinophils possess an enhanced mitochondrial respiratory capacity, which is often
307 associated with high $\Delta\Psi_m$ under steady-state conditions⁴³. ProE, while also tubular, exhibited the lowest $\Delta\Psi_m$,
308 highest ROS level, and lowest expression of *nd4* mRNA and ATPB protein, aligning with the metabolic
309 remodeling and oxidative stress characteristic of early erythrocyte differentiation⁴⁴. Intriguingly, lymphocytes
310 employed a distinct strategy: they contained numerous but highly fragmented mitochondria showing the highest
311 sphere proportion (72.7%) and smallest sphere area, yet maintaining a relatively high $\Delta\Psi_m$. This observation
312 suggests a distinct bioenergetic configuration, although $\Delta\Psi_m$ alone does not directly measure ATP production
313 efficiency, and the underlying mechanism requires further investigation⁴⁵. Finally, monocytes presented a mixed
314 morphology (spheres: 51.5%, tubules: 42.7%) with moderate $\Delta\Psi_m$, lowest mtROS level, and

P
A
G
E

315 highest *nd4* expression, reflecting the preparatory metabolic state of a plastic precursor cell⁴⁶.

316 To validate whether 2D-based segmentation yields biologically meaningful conclusions for comparative
317 analysis, we performed manual 3D annotation on a subset of zebrafish kidney immune cells and repeated the
318 mitochondria contents and TMRM quantification. The statistical comparisons based on 3D-annotated ground
319 truth closely matched those obtained from 2D segmentation (Fig. S13), confirming that 2D-based analysis was
320 consistent with 3D annotation under the chosen cell sample and imaging setup. Furthermore, to test whether
321 these findings are zebrafish-specific, we performed the same analysis in *L. japonicus* kidney immune cells,
322 which revealed broadly similar trends but also subtle interspecies differences (Fig. S14). Together, these cross-
323 validation and cross-species analyses establish that PanoMito resolves mitochondrial heterogeneity at single-
324 organelle resolution across species and cell types.

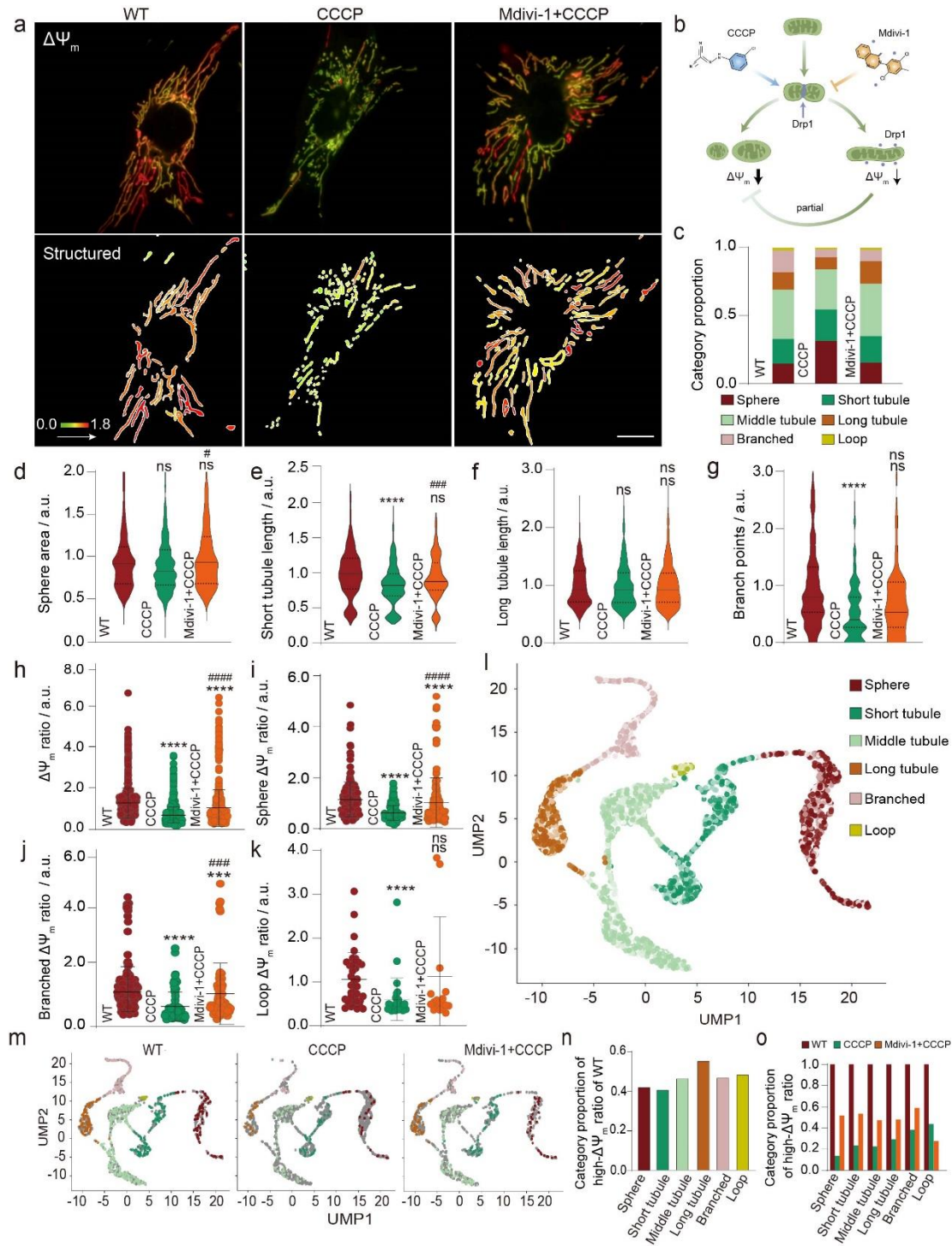


Fig. 4 – Intracellular mitochondrial heterogeneity under pharmacological perturbation. (a) Representative $\Delta\Psi_m$ images and digital structured images of SYCF cells that were treated with CCCP or Mdivi-1 + CCCP. Scale bar=10 μm . (b) Schematic illustration of mitochondria network remodeling following CCCP treatment alone and in combination with Mdivi-1. (c–g) Quantitative analysis of mitochondrial morphology across pharmacological perturbations: (c) Category proportion of the six morphological types, (d) relative sphere area, (e) relative short tubule length, (f) relative long tubule length. And (g) relative branch points. (h–k) Quantitative comparison of $\Delta\Psi_m$ across treatments: (h) $\Delta\Psi_m$ was compared at the overall mitochondrial level, and within the subtypes of (i) sphere, (j) branched and (k) loop. (l) UMAP projection of mitochondrial morphological clusters across treatment groups. Points denote single mitochondria, colored by their $\Delta\Psi_m$ value (more saturated color indicates higher $\Delta\Psi_m$). (m) UMAP projections for each treatment group, depicting the distribution of high- $\Delta\Psi_m$ mitochondria. Mitochondria with a $\Delta\Psi_m \geq Q3$ (the third quartile) are colored by morphological categories; all others are shown in gray. (n, o) Proportion of high- $\Delta\Psi_m$ mitochondria within each morphological cluster in the (n) WT group and (o) across treatment groups. Quantitative data are presented as the mean $\pm$ s.d. Significance ($p < 0.05$) for pairwise comparisons is denoted as follows: * vs the first group; # vs the second group; $\blacktriangle$ vs the third group.

Intracellular mitochondrial heterogeneity and remodeling in non-model organism

We use the fish *L. polyactis* as a non-model system to investigate dynamic mitochondrial remodeling at single-organellar resolution. First, we compared mitochondrial morphology between primary cells isolated from different tissues and cultured cells (SYCF) derived from this species. Quantitative analysis using the PanoMito platform revealed significant differences in mitochondrial morphology among these cells (Fig. S15), confirming the platform's capacity to capture mitochondrial phenotypic variations across different sample origins. We next investigated whether this platform could capture intracellular heterogeneity and dynamic remodeling under pharmacological stresses in SYCF cells. SYCF cells were subjected to mitochondrial depolarization (Carbonyl Cyanide m-Chlorophenylhydrazone, CCCP) or fission inhibition (mitochondrial division DRP inhibitor, Mdivi-1)^{47–49} followed by depolarization (Fig. 4). CCCP treatment induced mitochondrial fragmentation and decreased $\Delta\Psi_m$; pre-treatment with Mdivi-1 largely suppressed CCCP-induced fragmentation and partially attenuated the decrease in $\Delta\Psi_m$ (Fig. 4b). PanoMitoNet maintained robust segmentation accuracy across these perturbations (Fig. 4a) and enabled unsupervised clustering of mitochondria into six distinct morphological categories (Fig. 4c). Verapamil controls confirmed that dye efflux did not significantly affect TMRM measurements in SYCF cells under these experimental conditions (Fig. S12). CCCP titration experiments confirmed the concentration-dependent responsiveness of TMRM to $\Delta\Psi_m$ changes in SYCF cells (Fig. S16), supporting the use of this probe to assess depolarization-induced mitochondrial heterogeneity.

Quantitative profiling revealed that CCCP treatment, consistent with previous reports⁵⁰, triggered extensive fragmentation that is characterized by specific shifts in morphological populations (Fig. 4c). For instance, the proportion of sphere mitochondria more than doubled from 14.7% to 31.5% without a significant change in individual mitochondria area (Fig. 4d). Concurrently, the proportion of short tubular mitochondria increased, accompanied by a decrease in average length (Fig. 4e), whereas the proportion of long tubular mitochondria declined without a significant change in length (Fig. 4f). In parallel, branched mitochondrial networks collapsed, as reflected by a significant reduction in branch points (Fig. 4g). Mdivi-1 intervention effectively reversed these alterations, restoring the morphological landscape to near WT levels (Fig. 4d–g). These dynamic morphological changes confirm that PanoMito is capable of sensitively capturing mitochondrial structural remodeling under different metabolic states. In addition, a control group treated with Mdivi-1 alone showed only minor, statistically non-significant changes in morphology and $\Delta\Psi_m$ compared to the WT (Fig. S17), indicating that the observed effects of Mdivi-1 in the presence of CCCP are primarily due to its modulation of CCCP induced stress rather than any baseline changes in these parameters. For pharmacological perturbation experiments, statistical comparisons were performed at the field-of-view level to account for the nested structure of mitochondria within cells and treatment wells.

We further integrated morphological features with $\Delta\Psi_m$ at the single organelle level. Overall quantification showed that CCCP treatment significantly reduced the average $\Delta\Psi_m$, which was only partially restored by Mdivi-1 (Fig. 4h), consistent with previous reports⁵¹. However, the structured images (Fig. 4a) revealed that even within the same cell or morphological category, individual mitochondria exhibited considerable

heterogeneity in $\Delta\Psi_m$. To further investigate this functional heterogeneity, we performed an analysis across morphological categories. The results showed that specific types of mitochondria, such as sphere (Fig. 4i), branched (Fig. 4j), and loop mitochondria (Fig. 4k), responded to CCCP and Mdivi-1 in a manner consistent with the overall trend. Nevertheless, within each morphological cluster, the broad dispersion of data points also indicated significant variability in membrane potential across individual mitochondria. Consistently, UMAP visualization further confirmed a broad distribution of $\Delta\Psi_m$ values within each morphological cluster (Fig. 4l). Together with the intercellular measurements across cell types (Fig. 3g-k), these analyses indicate a decoupling between morphology and functional state, challenging the conventional simplified structure-function assumption^{12,13}.

However, analyses based solely on population averages may obscure the important highly functional organelles. We therefore dissected the high- $\Delta\Psi_m$ mitochondria ($\Delta\Psi_m \geq$ the third quartile (Q3) across all treatment groups, representing the metabolically robust subpopulation) within each morphological category (Fig. 4m-o). The results showed that high- $\Delta\Psi_m$ mitochondria were evenly distributed across all morphological types in the WT group (Fig. 4m, n). Under depolarization treatment, high- $\Delta\Psi_m$ branched mitochondria exhibited the highest retention of the high- $\Delta\Psi_m$ status, retaining approximately 38.0% of their functional population compared to WT. Long tubular mitochondria followed with 29.3% retention, while short and middle tubule retained only 23.5% and 22.6%, respectively. Sphere mitochondria were the most vulnerable, preserving merely 13.8% of their high- $\Delta\Psi_m$ subpopulation (Fig. 4m, o). Following Mdivi-1 treatment, functional restoration also varied by morphology: branched mitochondria demonstrated the largest increase in the proportion of high- $\Delta\Psi_m$ mitochondria (reaching 58.8% of WT levels), followed by sphere and short tubular subtypes (reaching 51.7% and 53.3% of WT levels), whereas middle and long tubular mitochondria showed lower recovery (reaching 47.2% and 47.8% of WT levels). Notably, under CCCP stimulation, loop mitochondria showed the highest relative retention (43.6%), but this dropped sharply to the lowest level among all forms (27.6%) after Mdivi-1 intervention, indicating that this state represents an unsustainable compensatory response⁵² (Fig. 4m, o). Corresponding results for U2OS cells are provided in Fig. S18, which showed broadly similar morphological and functional trends, supporting the cross-cell-type robustness of these observations.

Additionally, representative structured images of mtROS levels and mtRNA (*cox1* and *cytb*) under pharmacological perturbations are shown in Figs. S19 and S20, demonstrating the morphological and functional heterogeneity captured by PanoMito at single-organelle resolution. These results suggest that mitochondria morphology and metabolic state ($\Delta\Psi_m$) are not strictly coupled, yet specific architectures are associated with different levels of high- $\Delta\Psi_m$ retention under stress, highlighting a complex, non-linear morphofunctional interplay.

Given that the above analyses revealed drug-induced static heterogeneity yet underscored that mitochondria, as dynamic organelles, rely on real-time remodeling for functional regulation, we next sought to capture this dynamic dimension. Although the present analysis is cross-sectional and cannot establish causal relationships or track individual mitochondria over time, time-series data were analyzed at the field-of-view level, tracking average dynamic changes rather than treating individual time points as independent replicates. We validated PanoMito's capacity to track mitochondrial dynamic processes, including fission, fusion, and motility⁵³. Using manually annotated time-series images of SYCF cells (30 frames, 1-second intervals), images were first segmented by PanoMitoNet or alternative methods and followed by the temporal analysis used in MitoMeter¹⁷. PanoMito achieved higher accuracy in quantifying relative speed (Fig. S21a), fission/fusion event ratios (Fig. S21b), and morphological dimensions (Fig. S21c-d), with measurements closely approximating ground truth.

Building on this validation, we employed the platform to dissect the real-time kinetics driving remodeling during pharmacological perturbations. CCCP treatment induced a sharp increase in mitochondrial speed accompanied by rapid size reduction, driven by synchronous elevation of both fission and fusion frequencies. Conversely, Mdivi-1 partially blocked CCCP-induced morphological changes and suppressed fission/fusion rates below baseline, while motility remained elevated (Fig. S21e-l). Additionally, PanoMito tracked rapid functional fluctuations, capturing progressive $\Delta\Psi_m$ decay over 120 seconds in an acute phototoxicity model (Fig. S21m).

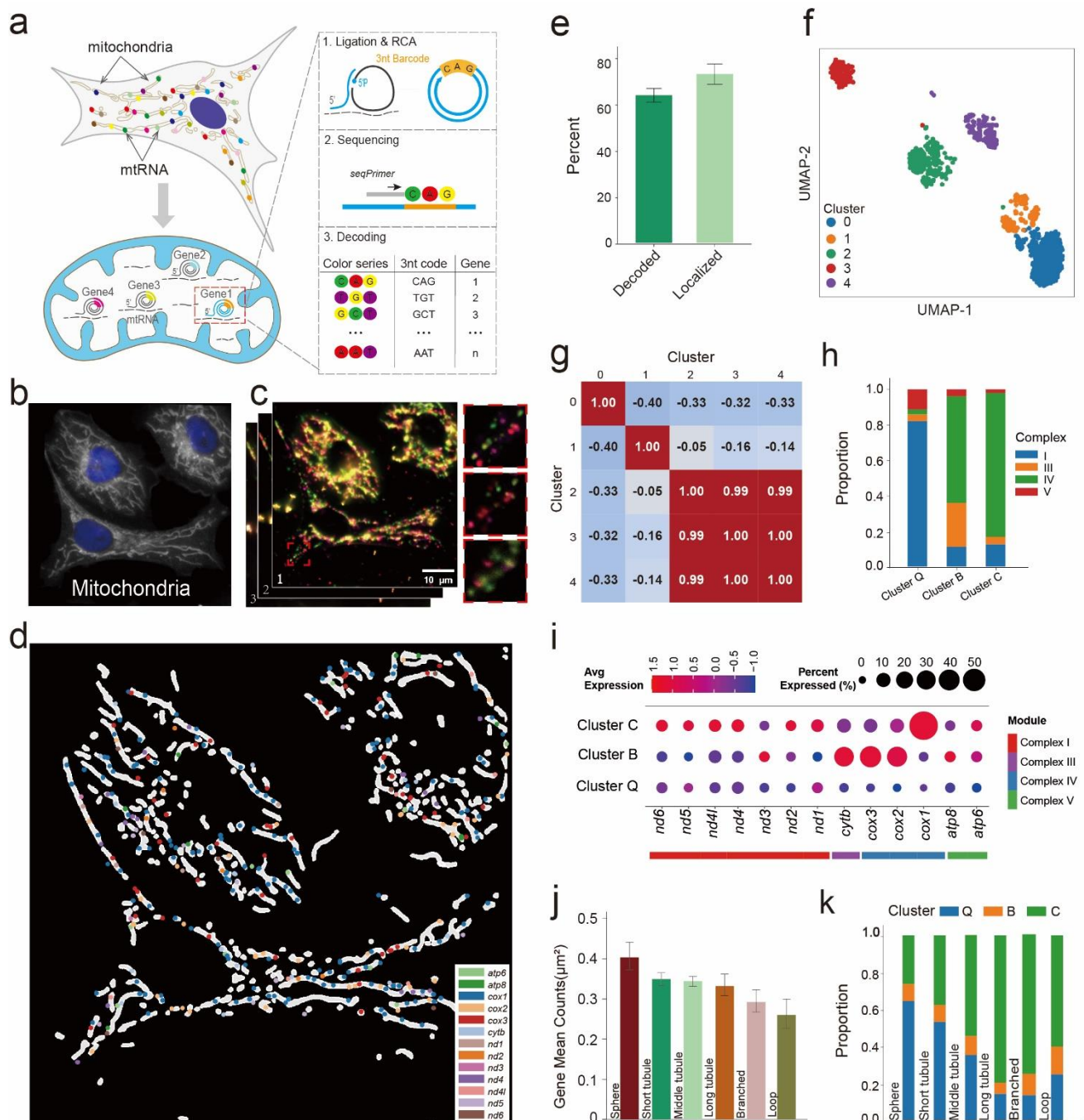


Fig. 5 – PanoMito-enabled single-mitochondrion spatial transcriptomics. (a) Schematic of the PanoMito-integrated *in situ* sequencing workflow. Padlock probes hybridize to mitochondrial transcripts (mtRNAs), followed by ligation and rolling-circle amplification (RCA) to generate DNA nanoballs. The 3-nt ACTG barcodes are sequenced to identify all 13 mtRNA species. (b, c) Representative images of SYCF cells showing (b) mitochondrial (gray) and nucleus (blue), and (c) three cycles of four-color fluorescence imaging for ACGT base decoding. Insets show magnified mtRNA puncta. (d) Reconstructed structured images with grayscale mitochondrial instances overlaid with spatially mapped mtRNAs indicated by 13 pseudocolors. (e) Quantification of the *in situ* sequencing fidelity. Of all detected puncta, 63.6% were correctly decoded by matching to the codebook, among which 72.74% colocalized with mitochondrial instances. (f) UMAP projection of single-mitochondrion transcriptomics revealing five primary clusters. (g) Pearson correlation matrix of mean gene expression across the five clusters. Clusters 2-4 exhibit highly correlated profiles, whereas cluster 0 and cluster 1 represent distinct transcriptional states. (h, i) Consolidation of five clusters into three molecular states based on gene expression signatures: Quiescent (Cluster Q, uniformly low expression),

Balanced (Cluster B, co-elevated *cytb* and *cox2/cox3*), and *cox1*-biased (Cluster C, elevated *cox1*). (h) The proportion of different oxidative phosphorylation (OXPHOS) complexes across three states. (i) Dot plot of differentially expressed mitochondrial genes across consolidated molecular states. Color scale indicates mean expression level, and dot size represents percentage of mitochondria expressing each gene. (j) Mean mitochondrial gene expression normalized by area across morphological categories. Mean detected transcripts per μm^2 are shown for each mitochondrial morphology category. No statistically significant differences were observed among groups. (k) Distribution of molecular states across morphological categories. Long tubules and brancheds are dominated by Cluster C (79.5% and 75.0%), whereas sphere and short tubule mitochondria are enriched for Cluster Q (64.5% and 54.0%). Loops display a distinct composition, with the highest proportion of Cluster B despite low overall expression.

Integrating PanoMito with *in situ* sequencing enables single-mitochondrion spatial transcriptomic profiling

To further bridge the gap between morphological phenotypes and their molecular drivers, we integrated PanoMito with *in situ* sequencing to profile gene expression at the single-mitochondrion level. As a proof-of-concept in SYCF cells, we employed a targeted spatial transcriptomic approach^{54–57} based on padlock probe hybridization, ligation, rolling-circle amplification (RCA), and sequencing (Fig. 5a, S22a). We designed padlock probes (Table S5) using a strategy similar to STARmap⁵⁶, containing hybridization region and 3-nt gene-unique barcode. The sequencing workflow was as follows: the first cycle captured the mitochondrial and DAPI images (Fig. 5b), while the subsequent three cycles recorded four-channel fluorescence signals to decode the ACGT bases (Fig. 5c, S22b). Then the molecular identities of all localized puncta were determined by mapping the decoded 3-nt sequences against our pre-defined barcode codebook. The mapped mtRNAs were assigned to the PanoMito-segmented instances to generate per-mitochondrion expression profiles, which were defined as the molecule counts per instance for downstream analysis (Fig. 5d, S23). After excluding non-cellular background puncta, approximately 63.6% of detected puncta were successfully decoded to a valid barcode, of which 72.74% localized to mitochondrial structures (Fig. 5e, S22c), consistent with published decoding rate published *in situ* sequencing methods^{54–56} ranging from 50% - 90% (Fig. S24f). In single-gene control experiments, 91.9–96.6% of decoded puncta matched the expected barcode (Fig. S24a–e), further confirming high decoding fidelity (Fig. S24a–e).

Having validated the fidelity of single-mitochondrion profiling, we classified the mitochondrial population into five clusters with unsupervised clustering (Fig. 5f). Examination of the Pearson correlation matrix (Fig. 5g) and the mean expression heatmap (Fig. S25c) revealed that three of these clusters (original clusters 2, 3, and 4) exhibited near-identical expression profiles. This strong concordance suggested that these clusters represented over-splitting rather than biologically distinct populations. We therefore merged these three clusters into a single group, yielding three biologically distinct clusters (Fig. 5h,i): (1) The Quiescent state (Cluster Q, original cluster 0), characterized by uniformly low transcript abundance; (2) The *cox1* (Cytochrome c oxidase 1)-biased state (Cluster C, original cluster 2–4), exhibiting specifically elevated *cox1* (Complex-IV) levels relative to other subunits (81.2%); (3) The Balanced state (Cluster B, original cluster 1), characterized by co-elevated expression of Complex-III (*cytb*) and Complex-IV (*cox2*, *cox3*) subunits (24.8% and 60.1%, respectively). The distinct transcriptional signatures of Clusters C and B correspond to the core *cox1* catalytic module and the integrating Complex-III and Complex-IV modules, respectively. This also aligns with the central role of the Complex IV modular assembly model^{58,59}, suggesting that Complex-IV assembly and regulation represent an important molecular feature of mitochondrial heterogeneity. Co-localization analysis between mtDNA and *cox1* transcripts revealed both mtDNA-proximal and spatially distributed transcript puncta (Fig. S25a, b), suggesting that the detected signals are not exclusively restricted to nascent transcription sites.

To investigate the correspondence between molecular identity and morphology, we mapped the transcriptional phenotypes onto the morphological landscape (Fig. 5j, k, S26). Although long tubules and brancheds contained higher absolute transcript counts than spheres and short tubules (Fig. S25d), area-normalized transcript densities were comparable across morphologies (Fig. 5j), indicating that these differences reflect organelle size rather than transcriptional activity per unit area. Despite comparable transcript densities, the proportional composition of molecular states differed markedly across morphologies (Fig. 5k): long tubules and brancheds were

dominated by the *cox1*-biased cluster (79.5% and 75.0%, respectively), while spheres and short tubules were overwhelmingly populated by the Quiescent cluster (64.5% and 54.0%, respectively). Notably, loop mitochondria displayed a unique molecular signature: despite low overall expression level, they were primarily composed of the *cox1*-biased subpopulation and exhibited the highest proportion of the Balanced cluster among all morphologies. As *cox1* is the core catalytic subunit of COX in the OXPHOS pathway⁶⁰, elevated *cox1* transcript levels indicate a transcriptional state associated with enhanced OXPHOS capacity, while the enrichment of the Quiescent cluster in fragmentation morphologies correlated with reduced transcriptional activity. Collectively, PanoMito provides single-organelle evidence that mitochondrial morphology is tightly coupled to molecular states, with distinct morphological subpopulations achieving functional specialization through unique transcriptional signatures.

Discussion

In this study, we developed and validated PanoMito, an integrated framework that enables precise, automated quantification of mitochondrial morphofunction. Validated against PanoMitoAtlas, the largest cross-species mitochondrial dataset to date comprising > 130,000 annotated instances across 16 species, PanoMitoNet achieves an AP₅₀ of 65.0%, substantially outperforming established tools such as MaskDINO (53.6%) and CellPose (59.4%). Beyond segmentation, PanoMito unifies unsupervised morphological classification, multiparametric morphofunctional profiling, temporal tracking of organelle dynamics, and single-mitochondrion spatial transcriptomics to enable comprehensive analysis of mitochondrial heterogeneity. By making this standardized resource freely available at <http://www.panomito.com>, we move beyond qualitative description to enable the systematic dissection of mitochondrial heterogeneity across evolutionary scales, tissue environments, and physiological states.

The complexity of the relationship between mitochondrial morphology and function is highlighted by our multi-scale analysis. For instance, we find that lymphocytes maintain the highest $\Delta\Psi_m$ despite a fragmented mitochondrial network. Furthermore, subcellular analysis shows that $\Delta\Psi_m$ is broadly distributed even within the same morphological category. These observations demonstrate the complexity of the relationship between mitochondrial morphology and function and underscore the need for direct functional readouts rather than inference from morphology alone. Furthermore, we observed that under pharmacological stress, branched mitochondria showed a relatively higher retention of high- $\Delta\Psi_m$ status compared to other morphological types, indicating an association between morphology and $\Delta\Psi_m$ status under stress conditions. Such nuanced, complex mitochondrial heterogeneity, invisible to conventional averaging, is precisely resolved by PanoMito's instance-level analysis, which directly measures functional parameters ($\Delta\Psi_m$, ROS, mtDNA, mtRNA, protein) at the single mitochondrion resolution. This strategy differs fundamentally from methods such as MoDL, which predict average mitochondrial function from morphological features under the assumption of a strong morphology-function correlation. In contrast, PanoMito acknowledges the complex, context dependent coupling between morphology and function and therefore relies on direct functional measurements rather than morphological inference.

Finally, we demonstrate that PanoMito bridges the gap between organelle phenotype and molecular genotype. By integrating our segmentation framework with *in situ* sequencing, we achieved spatial transcriptomics at single-mitochondrion resolution, successfully mapping 13 mRNA distributions to specific morphological instances. This single-mitochondrion spatial transcriptomics allows researchers to ask not just how mitochondria look or function, but which specific molecular signatures drive the formation of resilient subpopulations like the branched networks identified here. The core advancement of PanoMito is based on assigning transcripts to segmented mitochondria rather than the sequencing chemistry itself, making it readily compatible with image-based spatial transcriptomic platforms that preserve intracellular spatial coordinates, such as Xenium, CosMx, and MERFISH.

Limitations of the study

A fundamental limitation of this study is the use of 2D instances to describe the three-dimensional structures of mitochondria. Consequently, a single continuous mitochondrion may be split into multiple instances across

adjacent z-planes, and the apparent morphology of the same organelle may vary with imaging angle and sectioning plane, potentially leading to underrepresentation or misclassification of certain 3D mitochondrial structures. While this discrepancy is substantially mitigated in our cell samples, it becomes pronounced in tissues where overlap of 3D-oriented mitochondrion and strong background signals limit segmentation precision. Furthermore, while PanoMito facilitates dynamic analysis, the current tracking module relies on conventional matching algorithms applied to segmentation outputs from consecutive frames; future development employing deep neural networks will be essential to further enhance tracking fidelity in highly dynamic scenarios.

Beyond image analysis, functional measurements carry specific interpretive constraints. $\Delta\Psi_m$ serves as an indirect indicator of ETC activity rather than a direct measure of ATP output, and the TMRM/MitoTracker ratio provides a relative rather than absolute measure of $\Delta\Psi_m$. Although absolute calibration (e.g., FCCP titration) was not performed, we validated the graded responsiveness of TMRM via CCCP titration (Fig. S16). Regarding dye efflux, verapamil controls confirmed no significant effect in the tested immune cell types and SYCF cells, although this control has not yet been extended to all cell types and perturbation conditions. For the comparisons among different immune cell types, the mtRNA and protein assays are semi-quantitative, and we therefore interpret these results as relative rather than absolute quantifications. Furthermore, cristae architecture—the most indicative structural feature of bioenergetically active mitochondria—is inherently challenging to assess and was therefore not included as a functional metric in this study. However, we have already obtained clear super-resolution images of mitochondrial cristae (Fig. S28), and further optimization is ongoing to enable its future integration into the PanoMito platform.

Finally, the current in situ sequencing implementation does not distinguish mature mitochondrial transcripts from precursor polycistronic RNAs, and thus the molecular signatures reported here should be interpreted as steady-state transcript abundance rather than direct functional readouts. In addition, because mitochondrial transcripts are densely packed within individual organelles, the spatial resolution required for unambiguous transcript assignment is higher than that for nuclear transcripts. Consequently, the decoding rate and spatial precision for mtRNA are moderately lower than those typically achieved for nuclear genes in comparable in situ sequencing experiments. Importantly, the current implementation does not resolve mitochondrial DNA heteroplasmy or sequence-level mtDNA variation. It is worth noting that capture-based approaches, including Stereo-seq, Slide-seq, and Open-ST, are less readily integrated with PanoMito because they do not preserve organelle-level positional information. Nevertheless, this limitation does not affect the core utility of PanoMito for single-mitochondrion spatial transcriptomics.

Methods

Animals

The *L. crocea* and *L. polyactis* used in this study were self-bred and reared at the Ningbo Marine and Fisheries Technology Innovation Base. The other species, obtained from a local market in Ningbo City, Zhejiang Province, included the following: Aves (e.g., *Columba livia domestica*); Reptiles and Amphibian (e.g., *Pelodiscus sinensis*, *Lithobates catesbeianus*); Pisces (e.g., *Danio rerio*, *Lateolabrax japonicus*, *Tachysurus fulvidraco*); Crustacea (e.g., *Portunus trituberculatus*, *Sinonovacula constricta*); as well as the model organisms *Drosophila melanogaster* and *Chlamydomonas reinhardtii*.

All animal care and experimental protocols complied with the Experimental Animal Management Law of China (GB/T 35823–2018), the Zhejiang Provincial Regulations on the Administration of Experimental Animals (Order No. 263, 2009), and standard aquaculture research practices. To minimize suffering, fish were euthanized with eugenol (100 mg/L, Macklin) after anesthesia, and all sampling procedures adhered to the aforementioned regulations.

Primary cells

Primary cell extraction and culture: Following anesthesia and surface disinfection of the animals, target tissues were aseptically collected. The tissues were washed with 1×PBS containing antibiotics, minced into small fragments, and centrifuged at 50 g for 5 min. The resulting pellet was digested using 0.25% trypsin (Gibco,

15050065) until tissue fragments dissociated into a flocculent suspension. The digestion was terminated by adding complete medium supplemented with 20% fetal bovine serum (FBS; Gibco, 10099141C). The digestate was filtered through a 100 µm cell strainer, centrifuged, and the cell pellet was resuspended in complete medium for subsequent culture at an appropriate density (Fig. S1).

Giemsa staining. To identify distinct immune cell types in primary cultures derived from zebrafish kidney⁶¹, cells were subjected to Giemsa staining after fluorescence imaging. Briefly, cells cultured on coverslips or glass-bottom dishes were fixed with methanol for 5 min at room temperature. The fixed cells were then immersed in freshly prepared Giemsa working solution and incubated for 15 min at room temperature. After staining, the cells were rinsed with PBS. Stained cells were examined under a brightfield microscope, and immune cell types were identified based on standard morphological criteria. It should be noted that this classification is based solely on morphological criteria (Giemsa staining). Thus, these results should be interpreted as morphological subtype-associated findings.

Cell Lines

The cell lines used in this study included: HeLa (human cervical carcinoma; ATCC CCL-2), U2OS (human osteosarcoma), COS-7 (African green monkey kidney) and SYCF (the small yellow croaker fry). SYCF cell was established and maintained by the authors, while all other lines were commercially sourced. All cells were cultured in base medium supplemented with 10% FBS, 2% Penicillin-Streptomycin (Thermo Fisher, 15140122). HeLa, U2OS, and COS-7 cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) at 37°C under 5% CO₂. SYCF cells were cultured in Leibovitz's L-15 medium at 27°C without CO₂ supplementation.

Cell drug treatments

Wild-type U2OS and SYCF cells were maintained in standard culture conditions. For mitochondrial stress experiments, cells were seeded in confocal culture dishes and grown to 60–70% confluence. To induce mitochondrial depolarization, cells were treated with 10 µM CCCP (carbonyl cyanide m-chlorophenyl hydrazone; MedChemExpress, 555-60-2) in L-15 medium supplemented with 10% adult bovine serum (5A/L-15) at 27°C for 15 min. Another parallel group was pre-treated with 10 µM Mdivi-1 (mitochondrial division inhibitor 1; MedChemExpress, 338967-87-6) for 2 h prior to 10 µM CCCP exposure. A separate group of cells was treated with Mdivi-1 alone as a control for baseline effects. Following treatment, cells were washed three times with PBS and immediately processed for imaging or fixation.

Live-cell mitochondria staining and functional assays

Mitochondrial morphology labeling. Following two washes with PBS, cells were incubated with either PK Mito Red (1:4,000; Genvivo Biotech, PKMO-1) or MitoTracker Green (1:5,000; Thermo Fisher, M7514) in culture medium for 15 min at culture-specific temperatures (27°C for SYCF, 37°C for U2OS) under light-protected conditions. After staining, cells were washed twice with PBS and maintained in fresh medium for imaging.

Mitochondrial membrane potential. Cells were loaded with 0.5 µM tetramethylrhodamine methyl ester (TMRM; MedChemExpress, HY-D0984), a potentiometric dye that accumulates in mitochondria in a membrane potential-dependent manner. Co-staining with MitoView™Green (1:5,000; Biotium, 70054-T) was performed to visualize mitochondrial morphology independent of membrane potential. Staining was conducted in L-15 medium at culture-specific temperatures (27°C for SYCF, 37°C for U2OS) for 15 min under light-protected conditions. Following staining, cells were washed twice with PBS and imaged immediately in fresh medium to preserve physiological conditions.

Mitochondrial ROS detection. Mitochondrial reactive oxygen species (mtROS) were detected using MitoSOX Red (MedChemExpress, HY-D1055). Cells were incubated with 1 µM MitoSOX Red and MitoTracker Green in culture medium at culture-specific temperatures (27°C for SYCF, 37°C for U2OS) for 15 min under light-protected conditions. Following staining, cells were washed twice with PBS and imaged immediately in fresh medium to preserve physiological conditions.

CCCP titration. To validate the responsiveness of TMRM to graded depolarization, SYCF cells were treated with increasing concentrations of CCCP (0, 1, 10, 20, 30, and 50 μ M) for 5 min. Prior to CCCP treatment, cells were stained with 0.5 μ M TMRM and MitoView™ Green (1:5,000) for 15 min at 27°C in the dark. TMRM and MitoView™ Green images were acquired under identical settings, and the TMRM/MitoTracker ratio (R) was calculated for each mitochondrion. Relative $\Delta\Psi_m$ (%) was calculated as $(R - F_{\min}) / (F_{\max} - F_{\min}) \times 100\%$, where $F_{\max}$ is the mean ratio at 0 μ M (baseline) and $F_{\min}$ is the mean ratio at 50 μ M (complete depolarization). At least 5 cells were analyzed per concentration.

Dye-efflux control experiments. Cells were pretreated with verapamil (5, 10, 20 μ M) for 15 min prior to TMRM staining. TMRM fluorescence was then measured under identical imaging conditions, and the signals were compared with those from untreated control cells.

Batch effect control. To minimize batch effects, all experiments within each figure panel were conducted under strictly standardized conditions. For ratiometric imaging (TMRM/MitoTracker and MitoSOX/MitoTracker), both channels were acquired simultaneously using a QuadImage splitter to eliminate temporal offsets and excitation fluctuations. All key comparisons (Figs. 3-5) used samples processed in parallel from the same batch, with identical imaging settings (laser power, exposure time, gain) and staining conditions. The PanoMitoAtlas dataset is intended as a public resource for mitochondrial instance segmentation. For between-group statistical analysis, only the SYCF and U2OS subdatasets were employed, for which cross-condition comparisons are valid; cross-species comparisons of mitochondrial networks are also feasible for primary cell datasets. The hierarchical nesting structure was explicitly addressed as described above. For the remaining datasets, no quantitative conclusions in the manuscript depend on cross-channel or cross-modality comparisons.

Immunofluorescence staining and imaging

Primary zebrafish kidney cells were stained with 200 nM MitoTracker Red at 27 °C for 15 min to label mitochondria. After fixation in 4% paraformaldehyde for 15 min, cells were permeabilized with 0.3% Triton X-100 in PBS for 15 min and then blocked with 5% BSA for 2 h at room temperature. Subsequently, samples were incubated with a rabbit anti-ATPB primary antibody (1:70 dilution; Proteintech, 17247-1-AP) overnight at 4 °C, followed by an Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (1:1,000; Beyotime, A0423) for 1 h at 37 °C. A no-primary-antibody control was included to evaluate nonspecific binding. Finally, cells were washed twice with PBS and imaged immediately.

Single-mitochondrion mtRNA detection

We employed two complementary approaches for detecting mitochondrial transcripts at single-organelle resolution: single-molecule fluorescence *in situ* hybridization for individual gene detection in primary cells, and sequencing-based single-molecule nucleic acid amplification and *in situ* localization for multiplexed gene detection in cultured cells.

Probe design. Hybridization probes were designed following the STARmap strategy⁵⁶ and consisted of a 5' targeting sequence (20–32 nucleotides) complementary to mitochondrial mRNA targets, and a 3' padlock ligation template containing a 3-nucleotide barcode sequence for identification. Probe sequences are provided in Table S5.

In situ amplification procedure. Cells were fixed in 3% paraformaldehyde/1% glutaraldehyde for 10 min at room temperature, washed with PBS, and hybridized with target-specific probes at 40°C for 2 h. Following stringent washes, samples underwent T4 DNA ligase-mediated (GCATbio) padlock ligation at 37°C for 1 h, followed by RCA at 37 °C for 2 h, and subsequent hybridization with FAM/AF647-labeled detection probes or sequencing primers at 55°C for 5 min. Final washes were performed sequentially with 5× SSC, wash buffer, and 0.1× SSC.

Sequencing-based localization. Sequencing was performed in a custom-assembled microfluidic flow cell constructed by reversibly bonding a polydimethylsiloxane (PDMS) slab containing prefabricated channels to a glass slide. Each sequencing-by-synthesis (SBS) cycle comprised: (i) two washes with wash buffer (50 mM

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677 Tris-HCl pH 9.0, 50 mM NaCl, 0.05% Tween-20); (ii) incubation with sequencing mix (1× reaction buffer, 1
678 μM fluorescently labeled dNTPs, 2% Bst 3.0 DNA polymerase) for 10 min at room temperature; (iii) three
679 washes with wash buffer; (iv) three rinses with imaging buffer (0.1× SSC); and (v) image acquisition.
680 Fluorophores and blocking groups were cleaved by incubation with TCEP-containing cleavage mix (75 mM
681 Tris-HCl pH 9.0, 1 M NaCl, 0.05% Tween-20, 10 mM TCEP) for 10 min at room temperature, followed by
682 brief washes with 5× SSC before proceeding to the next cycle.

683 **Microscopy systems**

684 **Widefield fluorescence microscopy.** Fluorescence images were acquired on an inverted Nikon Eclipse Ti-E
685 microscope (Nikon Instruments) equipped with high-numerical-aperture objectives (Apo Plan 60X, NA 1.4).
686 For dual-channel ratio imaging, a QuadImage image splitter (Beijing Airy Technologies) was mounted on the
687 side port, enabling simultaneous acquisition of ROX and FITC channels using a dichroic beamsplitter and
688 emission filters. This configuration generated split-view images with TMRM/mtROS and mitochondria
689 subfields, eliminating temporal offsets and excitation intensity fluctuations that compromise ratiometric
690 measurements. Images were registered using the Chromagnon Python package to ensure precise spatial
691 alignment, with Channel 1 assigned to TMRM/mtROS and Channel 2 to mitochondria for downstream single-
692 mitochondrion analysis. For spatial transcriptomics experiments, sequential imaging was performed using filter
693 cubes specific to DAPI (nuclei), GFP (mitochondria), and Alexa Fluor 532, ROX, Cy5, and Alexa Fluor 700
694 (fluorescently labeled nucleotides A, C, G, and T, respectively). The microscope is automatically controlled by
695 Micro-Manager 2.0. The acquired images were further deconvolved using the ZS-deconvnet⁶².

696 **Spinning disk confocal microscopy.** High-speed volumetric imaging was performed on a Nikon Ti-E inverted
697 microscope equipped with a CFI Apochromat TIRF 100× oil objective (NA 1.49) and a CSU-W1 spinning disk
698 confocal unit (Yokogawa). The system incorporated an extended-resolution module (Live SR, Gataca) for
699 enhanced spatial resolution. Excitation was provided by a 488 nm laser (iLAS 3), and emission was collected
700 through bandpass filters (green: 525/50 nm; yellow: 617/73 nm; red: 685/40 nm; Chroma Technology) onto a
701 Prime 95B sCMOS camera (Photometrics).

702 **Structured illumination microscopy.** Super-resolution imaging was performed on a custom-built
703 pyramidalSIM system³⁶ constructed on an Olympus IX83 inverted microscope. The system utilized an oil-
704 immersion objective (PlanApo N 60×/1.42 Oil, Olympus) and a multiband dichroic mirror
705 (ZT405/488/561/640-phase R, Chroma). Illumination was provided by 488 nm and 561 nm lasers (OBIS LS-
706 100, Coherent) coupled through polarization-maintaining single-mode fibers. The beam path incorporated a
707 ferroelectric liquid crystal spatial light modulator (SXGA-3DM, Forth Dimension Displays) for pattern
708 generation, relay optics, a hexa-pyramidal lens (prism angle 18), and a custom azimuthally segmented half-
709 wave plate (PizzaHWP, Lbtek) for polarization control. The ±1 diffraction orders were selectively transmitted
710 through a spatial filter mask at the intermediate pupil plane and focused onto the sample to generate structured
711 illumination patterns. Fluorescence was detected by a Dhyana 400BSI sCMOS camera (Tuscon) after passing
712 through appropriate emission filters. The microscope is automatically controlled by Micro-Manager 2.0.

713 **ISO-STED nanoscopy.** Isotropic stimulated emission depletion (ISO-STED) microscopy was performed on a
714 custom-built system. A 775 nm nanosecond pulsed laser served as the depletion beam and provided
715 synchronization triggers for a 635 nm picosecond excitation laser to ensure precise temporal alignment. The
716 depletion beam passed through a half-wave plate and polarizing beam splitter for power and polarization control,
717 followed by phase modulation via a spatial light modulator (SLM). The phase-modulated beam was relayed
718 through a 4f system to a 30th-order vortex retarder plate, then to galvanometric scanning mirrors. The excitation
719 beam was expanded and combined with the depletion beam at a dichroic mirror. Both beams were scanned
720 through a high-NA objective using a piezo-driven objective scanner for axial positioning. Fluorescence was
721 descanned through the same optical path, separated by a second dichroic mirror, filtered to remove background,
722 and detected by an avalanche photodiode (APD).

723 **PanoMitoSeg architecture**

724 PanoMitoSeg is a transformer-based instance segmentation network for precise mitochondrial boundary
725 detection built on MaskDINO (<https://github.com/IDEA-Research/MaskDINO>), Detectron2 (<https://github.com/facebookresearch/detectron2>)

b.com/facebookresearch/detectron2) and Deformable-DETR (<https://github.com/fundamentalvision/Deformable-DETR>).

Backbone and encoder design. PanoMitoSeg employs a Swin Transformer Large backbone for hierarchical multi-scale feature extraction. The encoder processes these features through convolutional normalization layers followed by a 6-layer deformable transformer block, which enhances representation learning for objects with irregular geometries such as mitochondria. Multi-scale encoder outputs are resized and aligned to generate normalized feature maps for subsequent mask decoding.

Query-based instance decoding. The decoder implements a dynamic query selection strategy, selecting the top-100 highest-confidence queries from learned object queries. These queries interact with encoder features via mixed query attention (MQA), where queries serve as Q and encoder features as K and V. The decoder comprises 9 stacked deformable transformer layers with progressive refinement supervision—each layer outputs are independently supervised, enabling iterative improvement of predictions.

Multi-task prediction heads. Each decoder layer feeds three parallel prediction heads: (i) a mask head generating instance-level segmentation masks; (ii) a bounding box head (MLP-based) predicting object localization; and (iii) a fidelity score head estimating prediction confidence. Mask features are generated by integrating highest-resolution backbone and encoder features through a convolutional fusion layer. Final instance masks are produced by Einstein summation between decoder queries and mask features.

Regularization strategies for mitochondrial segmentation

Pseudo-semantic regularization for duplicate suppression. Dense instance segmentation tends to generate overlapping duplicate predictions for elongated, intertwined mitochondria. We address this by introducing a pseudo-semantic segmentation task that constrains instance density per pixel. From ground-truth instance annotations, we generate pseudo-semantic labels where each pixel value equals the number of overlapping mitochondrial instances at that location (background = 0). Model predictions are aggregated into a pseudo-semantic map by summing instance masks weighted by their fidelity scores. An L1 loss is applied between predicted and target pseudo-semantic maps at each decoder layer, explicitly regularizing the allowable instance overlap and effectively suppressing large-area duplicate detections.

Connective loss for morphology preservation. To prevent fragmentation artifacts where single mitochondria are erroneously split into disconnected regions, we designed a connective loss enforcing topological continuity. This loss function encourages morphological continuity by penalizing small isolated regions beyond the primary connected component of each instance mask. For each instance mask probability map $M \in R^{h \times w}$ we apply sigmoid activation and thresholding (0.5) to obtain binary mask B . We identify all connected components in B . Connected component analysis identifies the primary region R_p (largest component) and fragment regions R_f . We compute the magnitude of spatial gradients of M in horizontal (G_x) and vertical (G_y) directions. The fragment regions R_f are dilated by a kernel of radius 3 to include boundary uncertainty, yielding a dilated region $\widetilde{R}_f$. The loss is defined as the mean gradient magnitude within $\widetilde{R}_f$.

$$\mathcal{L}_{con} = \sum_{instances} \frac{1}{\widetilde{R}_f} \left(\sum_{(i,j) \in \widetilde{R}_f} G_x(i,j) + G_y(i,j) \right)$$

Minimizing $\mathcal{L}_{con}$ forces the model to either merge fragments into R_p by smoothing probability transitions or suppress them by reducing confidence scores, thereby preserving biologically accurate mitochondrial morphology.

Loss Functions

Cross-Entropy Loss: Cross-entropy loss measures the performance of a classification model whose output is a probability value between 0 and 1, penalizing larger deviations between predictions and labels. $\mathcal{L}_{CE} = -\sum_{i=1}^C y_i \log \hat{y}_i$ where C denotes the total number of classes, y_i is the one-hot encoded label (where $y_i \in \{0,1\}$), and $\hat{y}_i$ denotes the predicted label for class i .

L1 Loss: L1 loss calculates the absolute difference between predicted values and ground-truth values. $\mathcal{L}_{L1} = \sum_{i=1}^D |\hat{y}_i - y_i|$ where D denotes the dimension of the ground-truth vector, y_i represents the ground-truth label for the i -th dimension (or class i), and $\hat{y}_i$ denotes the predicted value for the i -th dimension (or class i).

Generalized Intersection over Union Loss: GIoU loss is an improved variant of IoU loss for object detection tasks. $\mathcal{L}_{GIoU} = 1 - \left(\frac{|\hat{y} \cap y|}{\hat{y} \cup y} - \frac{|B^U - (\hat{y} \cup y)|}{|B^U|} \right)$ where y denotes the ground-truth bounding box, $\hat{y}$ represents the predicted bounding box, B^U denotes the smallest enclosing bounding box that can simultaneously contain both the predicted box $\hat{y}$ and the ground-truth box y , and its area is denoted by $|B^U|$.

Dice Coefficient Loss: Dice Coefficient loss measures the overlap between two sets, predicted segmentation mask and ground-truth mask. $\mathcal{L}_{Dice} = 1 - \frac{2|\hat{y} \cap y|}{|\hat{y}| + |y|}$ where y represents the ground-truth segmentation mask, $\hat{y}$ denotes the predicted segmentation mask.

Binary Cross-Entropy Loss: Binary cross-entropy loss is a specialized form of cross-entropy loss for binary classification tasks. $\mathcal{L}_{BCE} = -[y \log \hat{y} + (1 - y) \log(1 - \hat{y})]$, where y is the ground truth label, and $\hat{y}$ denotes the prediction.

Connective Loss: as defined above.

Our total loss: $\mathcal{L}_{total} = \lambda_1 \mathcal{L}_{Fidelity} + \lambda_2 \mathcal{L}_{Box} + \lambda_3 \mathcal{L}_{GIoU} + \lambda_4 \mathcal{L}_{Dice} + \lambda_5 \mathcal{L}_{Mask} + \lambda_6 \mathcal{L}_{Connective} + \lambda_7 \mathcal{L}_{Semantic}$ where $\lambda_1 = 4, \lambda_2 = 5, \lambda_3 = 5, \lambda_4 = 8, \lambda_5 = 5, \lambda_6 = 0.1, \lambda_7 = 1$

Implementation and training details

Training protocol. PanoMitoSeg was pre-trained on the TissueNet dataset (nuclear and cell segmentation) and fine-tuned on PanoMitoAtlas for 20,000 iterations with batch size 16, base learning rate 1×10^{-5} (cosine decay), and AdamW optimizer (weight decay 0.05). The dataset was split into training and test sets at a 7:3 ratio, ensuring that all imaging modalities were represented in both splits. The same training and test sets were subsequently used for all benchmarked methods to avoid dataset-level bias. Data augmentation included random flipping, rotation (90° increments), intensity scaling ($0.8-1.2\times$), and elastic deformation. For the modality-stratified experiments, models were trained using only the corresponding modality-specific subset from the training set, while all other training parameters were kept unchanged. These models were then evaluated across different imaging modalities to assess cross-modality generalization. PanoMitoCluster was trained from scratch on PanoMitoSeg outputs for 5,000 epochs with learning rate 1×10^{-4} .

Patch-based scalable inference. For images exceeding 512×512 pixels, we implement a sliding-window inference strategy. Input images are tiled into overlapping patches (stride = 256 pixels, 50% overlap). Post-inference, instances detected in overlapping regions are merged using IoU-based non-maximum suppression (threshold = 0.5). This approach reduces GPU memory requirements by $4-8\times$ while preserving subpixel structural details critical for mitochondrial analysis.

Benchmark comparisons. We evaluated PanoMitoNet against established methods using standardized training protocols on PanoMitoAtlas. All trainable methods were trained, retrained, or fine-tuned using the same training/testing split to ensure a fair comparison. The benchmarked methods included MitoMeter (parameter-free); MoDL (trained 100 epochs, $lr=1 \times 10^{-5}$, batch=2, from scratch); CellPose-SAM (CPSAM pre-trained weights, 500 epochs, $lr=1 \times 10^{-5}$, batch=1); OmniPose (4,000 epochs, $lr=0.1$, batch=16, from scratch); and MaskDINO (TissueNet pre-training, 20,000 iterations, $lr=1 \times 10^{-5}$, batch=16). More details are shown in [Table S2](#).

Evaluation metrics

For each predicted instance, compute the Intersection over Union (IoU) with all ground truth instances: $IoU = \frac{\text{Area of Overlap}}{\text{Area of Union}}$. A prediction is considered a true positive (TP) if its IoU with any ground truth instance exceeds a predefined IoU threshold; otherwise, it is a false positive (FP). Unmatched ground truth instances are counted as false negatives (FN). Precision and recall are then computed as: $Precision = \frac{TP}{TP+FP}$ $Recall = \frac{TP}{TP+FN}$.

AP50. Average Precision at 50% Intersection over Union (AP_{50}) quantifies the precision-recall trade-off at a fixed IoU threshold of 0.5. Plot the precision-recall curve by sorting predictions by descending confidence score. AP_{50} is the area under the precision-recall curve. $AP_{50} = \int_0^1 p(r)dr$ where $p(r)$ is the precision at recall r . Since some methods lack a confidence score, making it infeasible to compute the average precision, we use the IoU between predictions and ground truth as the confidence metric. This approach yields the theoretical upper bound of the AP metric.

F1score. The F1score is the harmonic mean of precision and recall.

$$F1score = \frac{2 \times Precision \times Recall}{Precision + Recall} = \frac{2 \times TP}{2 \times TP + FP + FN}$$

Accuracy. We define instance detection accuracy as the ratio of correctly segmented instances to all instances involved in the evaluation. $Accuracy = \frac{TP}{TP+FP+FN}$.

PanoMitoCluster for phenotypic profiling

Self-supervised feature learning. Following instance segmentation by PanoMitoSeg, each mitochondrial object is extracted as a 128×128 pixel binary image (zero-padded to standardize dimensions) and processed by PanoMitoCluster. This network adopts a CNN encoder-decoder architecture with a self-reconstruction objective: the encoder compresses input patches to low-dimensional latent representations, which the decoder reconstructs to original inputs. The first-stage training minimizes reconstruction loss, forcing the encoder to learn biologically meaningful features without manual annotation.

Clustering. To guide optimization and ensure cluster separability, the second-stage finetuning incorporates Kullback-Leibler (KL) divergence loss between feature distributions and target cluster assignments. Feature vectors from the encoder bottleneck are extracted for each mitochondrial instance and further processed through two fully connected layers. K-means clustering was performed on 128-dimensional feature vectors to initially identify 12 clusters; adjacent clusters were subsequently assigned using a deterministic hierarchical rule based on cluster-level morphological features to yield 6 final morphological classes for biological interpretation.

Class definitions. The six morphological classes were designated as sphere, branched, short tubule, middle tubule, long tubule, and loop. Since mitochondrial morphology is continuous, the biological names of the PanoMitoCluster outputs were assigned using a deterministic hierarchical rule based on cluster-level morphological features. Specifically, clusters with mean branch points ≥ 0.8 and circularity ≤ 0.35 were labeled branched; clusters with mean branch points ≥ 0.8 and circularity > 0.35 were labeled loop; remaining clusters with mean aspect ratio < 1.5 were labeled sphere; and the remaining tubule clusters were split into long, middle, and short by normalizing mean aspect ratio to the maximum value and labeling normalized ratios ≥ 0.7 as long, between 0.3 and 0.7 as middle, and < 0.3 as short. The rules were applied sequentially, with earlier steps assigned higher priority; therefore, clusters assigned as branched, loop, or sphere were not further reclassified by the length-based criteria. The same thresholds and priority order were applied uniformly to all clusters and datasets.

PanoMitoAtlas dataset

Mitochondrial instance definition and annotation criteria. In PanoMitoAtlas, a single mitochondrial instance is defined as a spatially continuous mitochondrial object visible in a given 2D image plane. Adjacent mitochondria were annotated as separate instances when a discernible boundary or intensity separation was present, whereas continuous tubular or branched structures were annotated as a single instance. All annotations were performed by trained researchers following standardized criteria established to ensure consistency.

Sampling strategy and dataset composition. The 132,798 annotated instances were derived from two primary imaging configurations: (i) static 2D images of adherent cultured cells and primary cells, in which each field of view represents an independent sampling location without overlapping tiles or repeated views of the same cell; and (ii) 3D image stacks of primary cells, in which annotation was performed on sparsely sampled optical sections (original z-step 500 nm, every other section annotated, yielding an effective axial spacing of $\sim 1 \mu\text{m}$). This spacing is comparable to or larger than typical mitochondrial diameters ($\sim 0.5\text{--}1 \mu\text{m}$), substantially reducing

859 the probability that the same organelle appears in multiple consecutive annotated planes. Time-lapse sequences
860 were acquired for dynamic analyses, but temporal redundant instances are excluded from the 132,798 count to
861 avoid temporal redundancy. We additionally generated 3D instance annotations in which each mitochondrion
862 spanning multiple z-sections is counted as a single object.

863 **Human-in-the-loop annotation strategy.** Annotation of PanoMitoAtlas employed an iterative human-in-the-
864 loop workflow (Fig. S27). An initial segmentation model was trained on a small set of manually annotated images
865 to generate preliminary annotations. These pre-annotations were then manually corrected in CVAT by trained
866 annotators. The annotator group consisted of trained researchers following standardized criteria established to
867 ensure consistency. After manual correction, AI-assisted quality control was performed to identify potential
868 annotation errors. Specifically, annotated mitochondrial objects were cropped and zoomed to 128×128 binary
869 masks to normalize for size variation, then classified by a pretrained lightweight model. Certain categories
870 directly indicated obvious errors—such as merged adjacent mitochondria, fragmented masks, or spurious small
871 masks outside mitochondrial structures—which were flagged by AI-generated prompts for targeted expert
872 correction to produce final ground-truth annotations. These high-quality annotations were incorporated into the
873 training set to improve the segmentation model. The iterative process continued until dataset completion.

874 **Cross-frame annotation.** We extended the 2D annotation workflow to time-lapse or z-section dataset. Each
875 individual frame or z-section was first annotated independently following the procedure described above.
876 Afterward, annotators compared neighboring frames or z-sections and assigned the same cross-frame instance
877 ID to 2D annotations that belonged to the same mitochondrion across different frames. Thus, a single
878 mitochondrion spanning consecutive time-lapse frames or z-sections was linked and counted as a single instance,
879 rather than as multiple independent observations.

880 **Data format and composition.** Images are stored in Tiff format and converted to PNG format for annotation.
881 Annotations are stored in COCO JSON format preserving overlapping instance labels, accurately reflecting the
882 physically interwoven nature of mitochondrial networks. Instance masks support partial overlap, enabling
883 realistic representation of tubular and reticular morphologies. Dataset statistics and download information are
884 provided in Table S1 and Data Availability.

885 Image-based quantification

886 **Single-channel analysis.** Mitochondrial morphology was quantified from PanoMitoNet segmentation masks,
887 extracting for each instance: mitochondria type, length (major axis), area (number of mask pixels), perimeter
888 (number of boundary pixels), aspect ratio (ratio of major to minor axis), circularity ($4\pi \cdot \text{area}/\text{perimeter}^2$), and
889 branched point count (number of skeleton branch points).

890 **Dual-channel ratiometric analysis.** Following channel registration and background subtraction (100 intensity
891 units), ratio maps were calculated by dividing the functional channel (TMRM or MitoSOX) by the morphology
892 channel (MitoTracker). Morphology images were segmented using PanoMitoNet, and for each mitochondrion,
893 we extracted instance level mean ratio values as functional readouts.

894 Spatial omics data processing

895 **Single-gene detection.** Three-dimensional image stacks underwent maximum intensity projection (MIP) along
896 the Z-axis to generate 2D multi-channel images. Channel registration was performed using Chromagnon⁶³ to
897 correct chromatic aberration. Multi-scale background subtraction was applied, followed by spot detection and
898 centroid extraction. Four-channel intensity vectors were normalized, and a linear crosstalk matrix was estimated
899 from single-gene sequencing data to correct spectral bleed-through.

900 **Multi-gene decoding.** Each sequencing cycle was processed using the single-gene pipeline. The crosstalk
901 matrix from single-gene calibration was applied for spectral unmixing. Difference-of-Gaussians (DoG) filtering
902 enhanced punctate signals, and spots were detected to extract coordinates and intensity vectors. ACGT decoding
903 was performed by assigning the nucleotide corresponding to the maximum normalized channel intensity.

904 **Cross-cycle registration and decoding.** Spot correspondence across three sequencing cycles was established
905 using Cycle 1 as reference. Coarse alignment using grid searching optimized rotation and translation parameters,

P
A
G
E

906 followed by affine transformation estimation from high-confidence nearest-neighbor matches of puncta within
907 a distance threshold. Spots detected were sequentially decoded in all cycles. Three-cycle nucleotide sequences
908 were concatenated and matched against 13 target gene codebook; non-matching spots were labeled “Unknown”.

909 **Transcript assignment and ambiguity assessment.** Successfully decoded transcript spots were assigned to
910 segmented mitochondrial objects based on spatial overlap with the PanoMito segmentation masks. Each
911 transcript spot was assigned exclusively to a single mitochondrial object and was never counted multiple times.
912 To assess assignment ambiguity in densely packed mitochondrial regions, we quantified decoded spots
913 overlapping more than one segmented mitochondrion. These potentially ambiguous spots were identified and
914 their proportion relative to all decoded transcript spots was calculated. The robustness of downstream analyses
915 was further evaluated by excluding all potentially ambiguous spots and repeating the analyses.

916 **Single-mitochondrion transcriptomic data preprocessing.** Data were transformed into AnnData format, with
917 gene expression matrices (X) and mitochondrial morphometric features or functional measurements (obs).
918 Principal component analysis (PCA) was performed on the gene expression matrix for dimensionality reduction,
919 followed by construction of a k-nearest neighbor (KNN) graph and Leiden clustering to identify molecular
920 subpopulations. Uniform manifold approximation and projection (UMAP) was used for visualization of
921 molecular clusters. Cluster-average z-score-normalized gene expression was visualized as a heatmap. The
922 composition of molecular clusters within each morphology-defined cluster was quantified by calculating the
923 proportions of individual molecular subpopulations.

924 Statistical analysis

925 Statistical analyses were performed using GraphPad Prism. Data are presented as mean $\pm$ standard deviation
926 (s.d.). Normality (Shapiro-Wilk test) and homogeneity of variances were assessed before selecting appropriate
927 tests. Parametric tests (one-way/two-way ANOVA with Dunnett's post-hoc test for multiple comparisons; two-
928 tailed Student's t-test for two groups) were used for normally distributed data. Non-parametric alternatives
929 (Kruskal-Wallis with Dunn's test; Mann-Whitney U test) were applied for non-normal distributions.
930 Significance levels: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

931 Resource availability

932 Materials availability

933 The study did not generate new unique reagents.

934 Data availability

935 The dataset and pretrained models have been uploaded to Zenodo
936 (<https://zenodo.org/records/21129338>; <https://zenodo.org/records/20959653>). The dataset includes all
937 2D images, time-lapse images, and their corresponding annotations in COCO format, as well as the
938 training and test splits used in this study, enabling researchers to reproduce our experiments and reuse
939 the dataset for future studies. The model resources include the pretrained weights and trained models
940 used for PanoMitoSeg, PanoMitoCluster, and benchmark comparison.

941 Code availability

942 The complete PanoMito toolbox is available on <https://github.com/DCILab2024/PanoMito>. The
943 repository includes source code, a detailed README, dependency files, and demo workflows for
944 benchmark evaluation and segmentation-based morphological clustering.

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948 **Author contributions**

949 K.Z. and J.Q.W. conceived the project. K.Z., J.L.Q., D.Y.J., X.P.G. supervised the research. J.Q.W., K.Q., R.L.Z.,
950 X.Y.C. and Y.N.D. conducted mitochondrial morphofunctional experiments. K.Z. and S.F.G developed the
951 PanoMitoNet algorithm. Y.C., J.Q.W. and K.Q. conducted *in situ* sequencing experiments. All authors
952 contributed to the PanoMitoAtlas dataset. K.Z., J.Q.W., S.F.G., Y.C. and J.Y.H. analyzed the data. K.Z., J.Q.W.,
953 S.F.G. and Y.C. wrote the manuscript with input from all authors. All authors reviewed and provided feedback
954 on the manuscript.

955 **Ethics declarations**

956 **Competing interests**

957 The authors declare no competing interests.

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