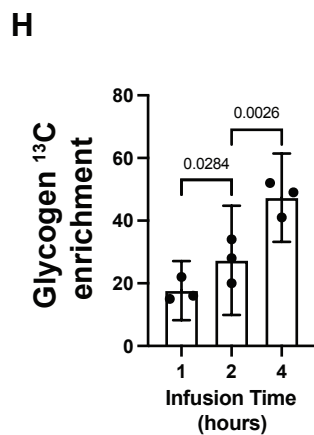
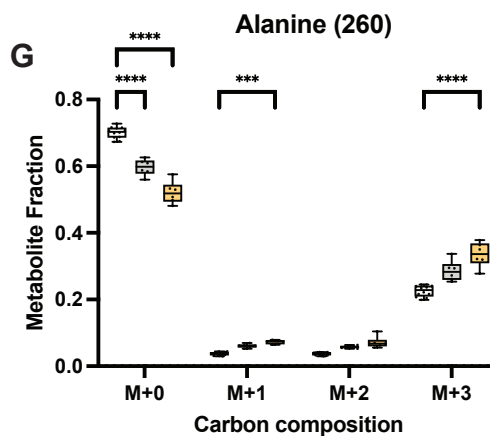
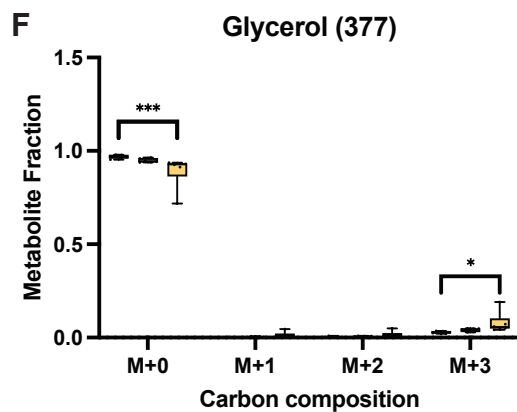
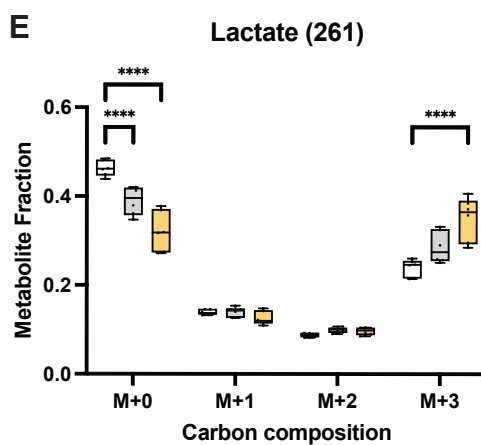
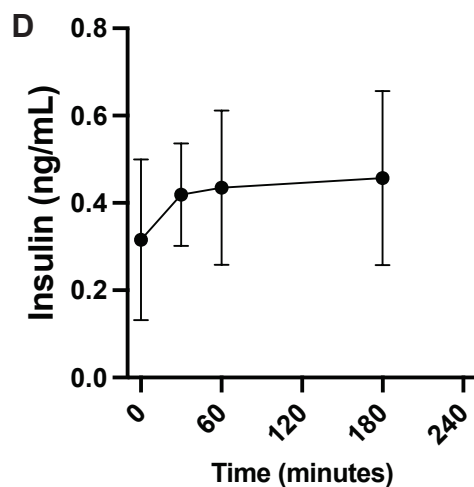
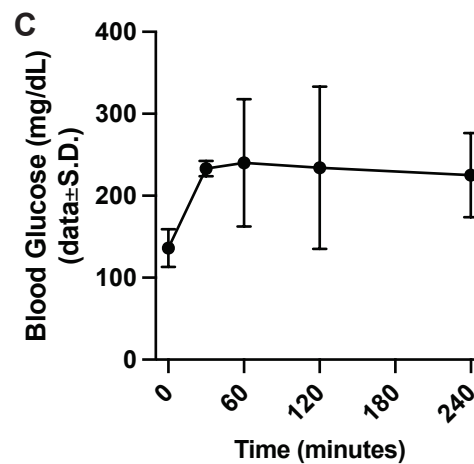
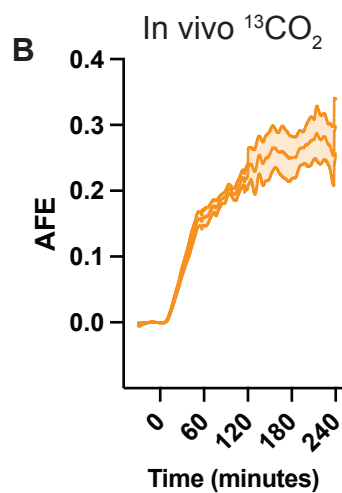
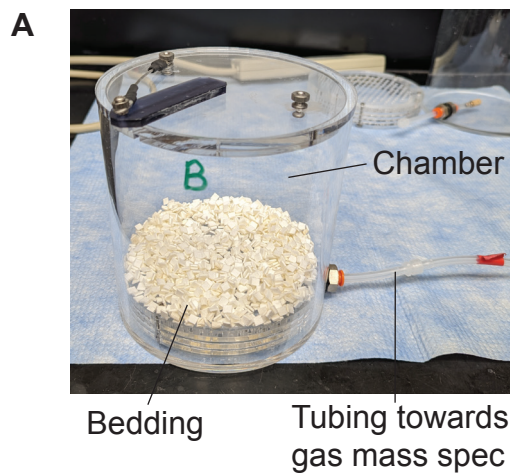
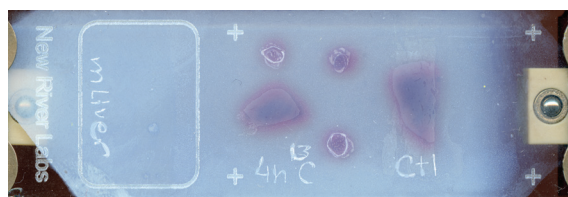




Supplementary Figure 1. In vivo measurements of ^{13}C -glucose metabolism (A) Photo of a custom made cylindrical metabolic cage for *in vivo* glucose oxidation measurements. (B) Expelled $^{13}\text{CO}_2$ (in parts per million (ppm)) in mice continuously infused with 40mg/min/kg for up to 4 hours. (C-D) Circulating glucose and insulin levels in mice infused with 40mg/min/kg for up to 4 hours. (E-G) GC-MS analysis to determine the fractional ^{13}C enrichment of lactate, glycerol, and alanine. Each dot represents an animal. (H) ^{13}C enrichment accessed using GC-MS of isolated glycogen extracted from [U- ^{13}C]-glucose-labelled mice after 1, 2, or 4 hours of continuous infusion with 40min/mg/kg of total body mass. P values are shown. In (E-G), **** p<0.001. p values are shown in (H).

A

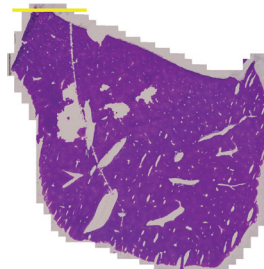
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B

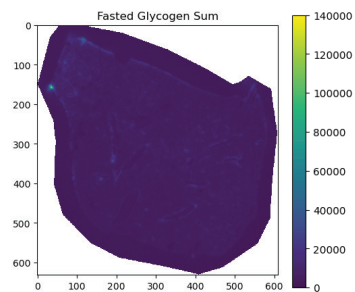
H&E

Control - Fasted

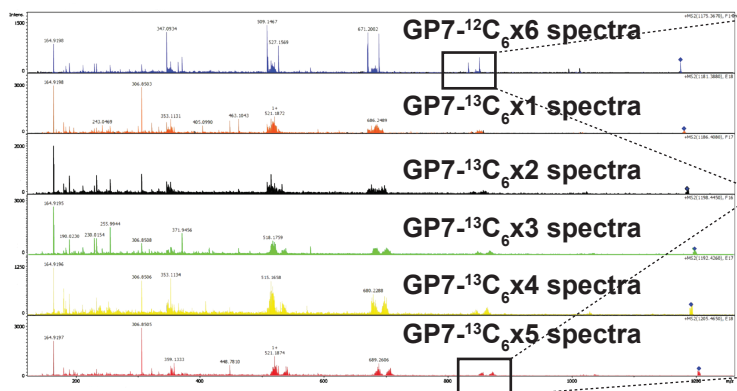
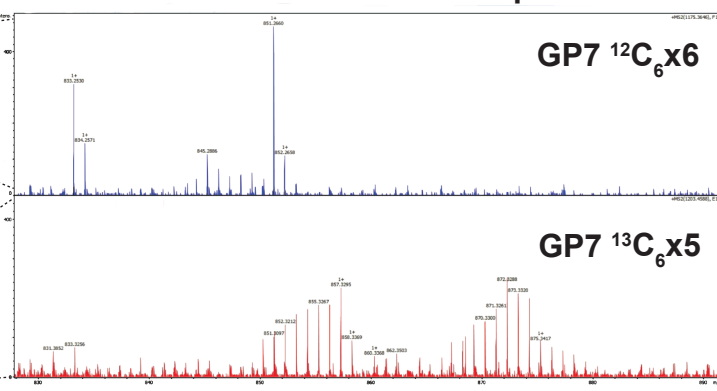


MALDI

Fasted Glycogen Sum



C

Full GP7 ^{13}C spectraZoom in GP7 ^{12}C vs ^{13}C spectra

D

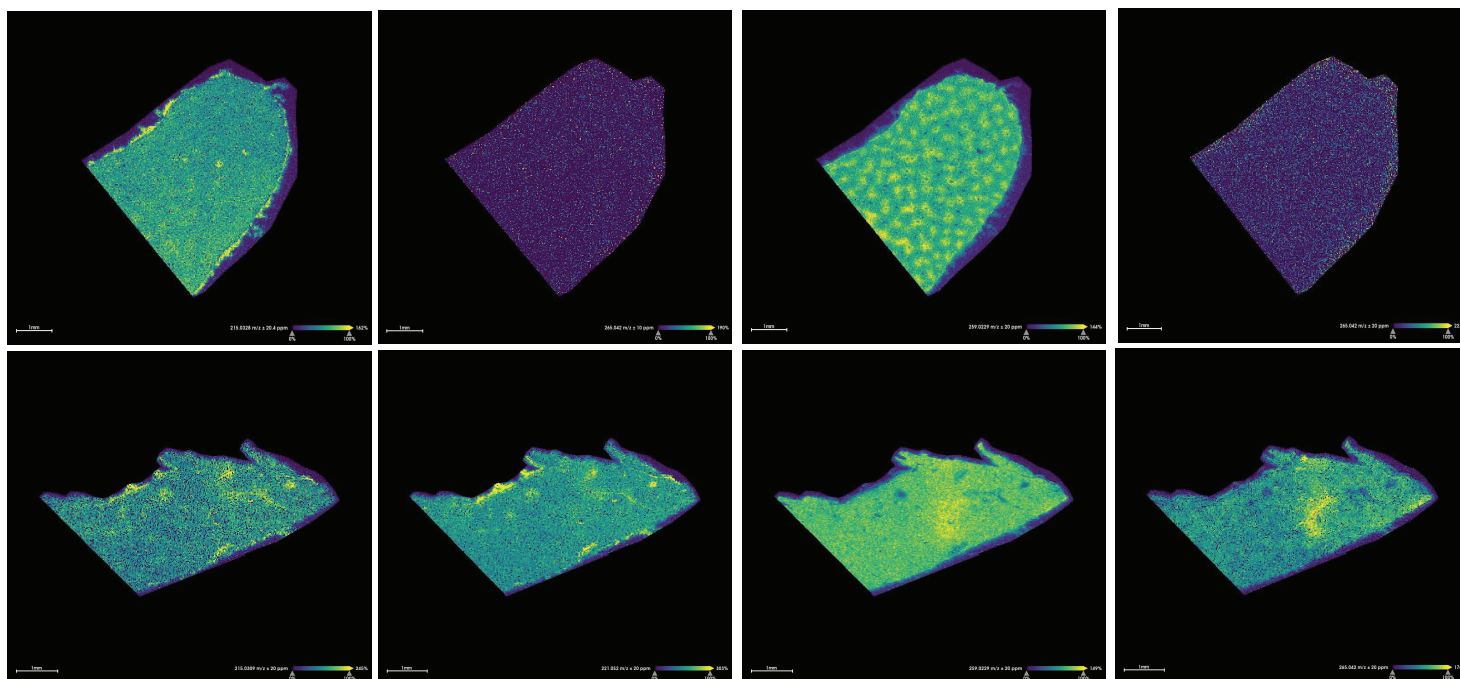
 $^{12}\text{C}_6$ -glucose $^{13}\text{C}_6$ -glucose

E

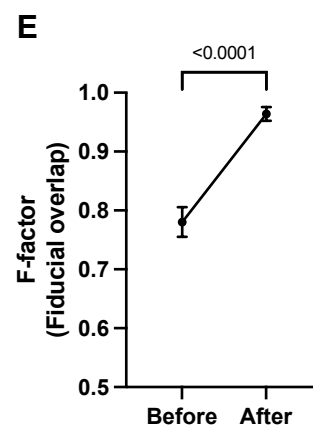
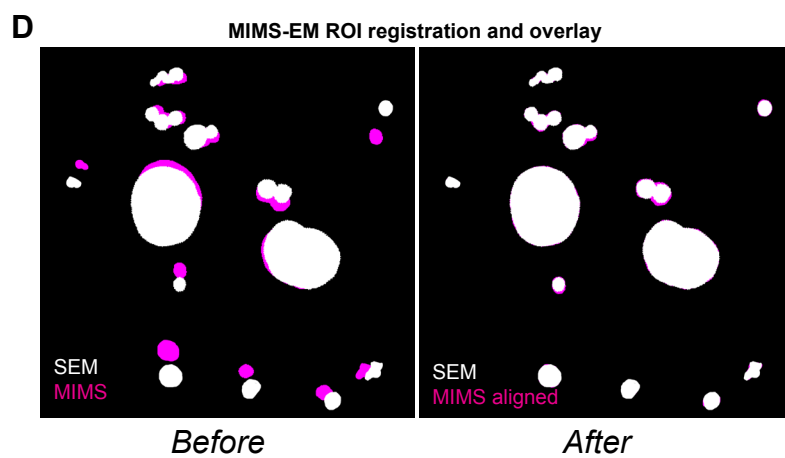
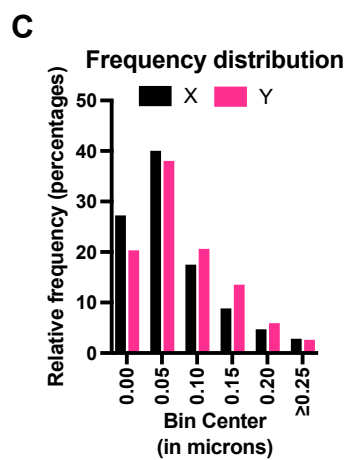
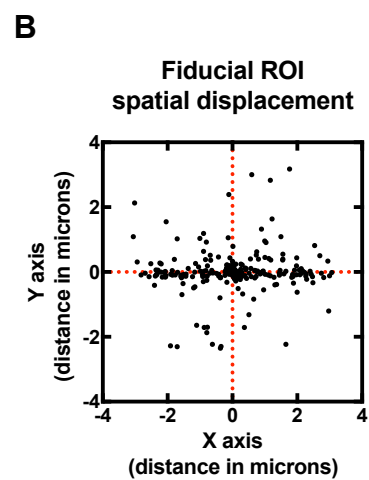
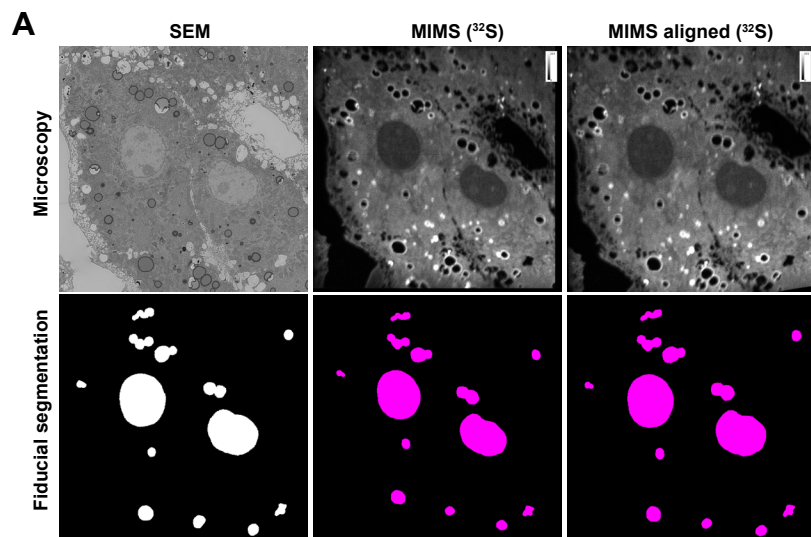
 $^{12}\text{C}_6$ -H6P $^{13}\text{C}_6$ -H6P

Fasted

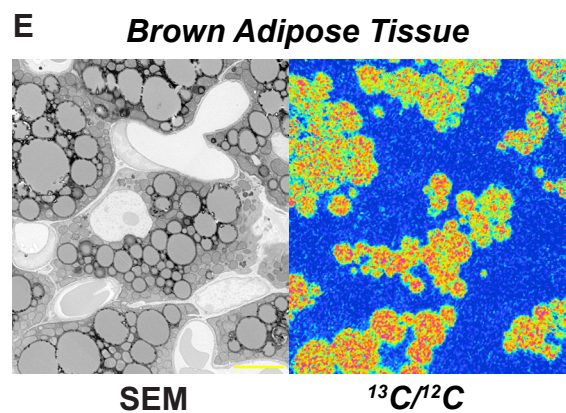
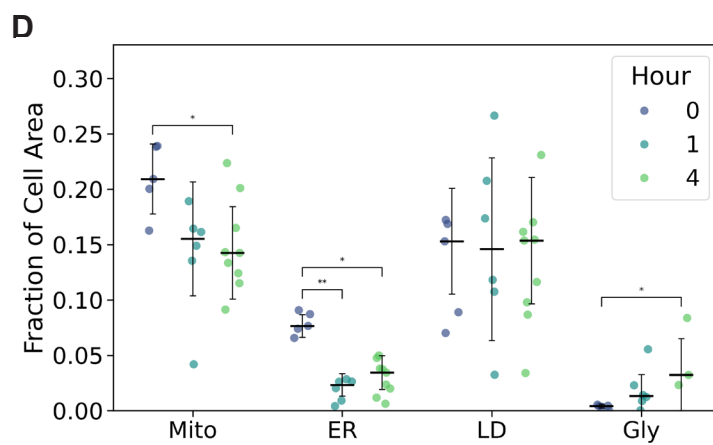
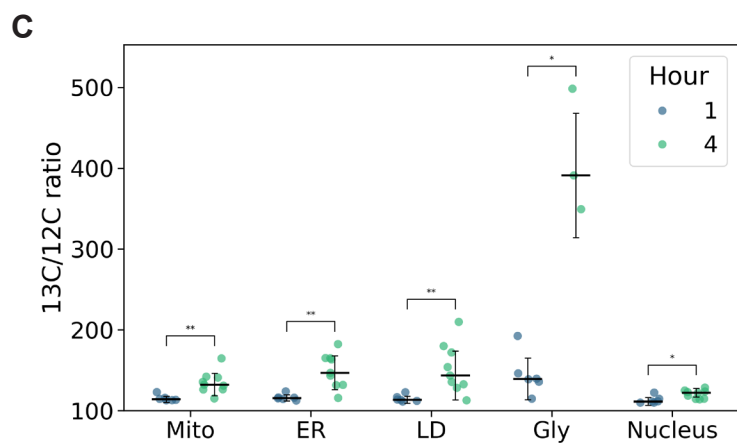
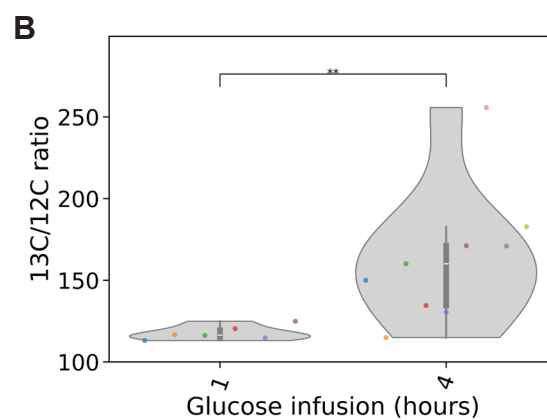
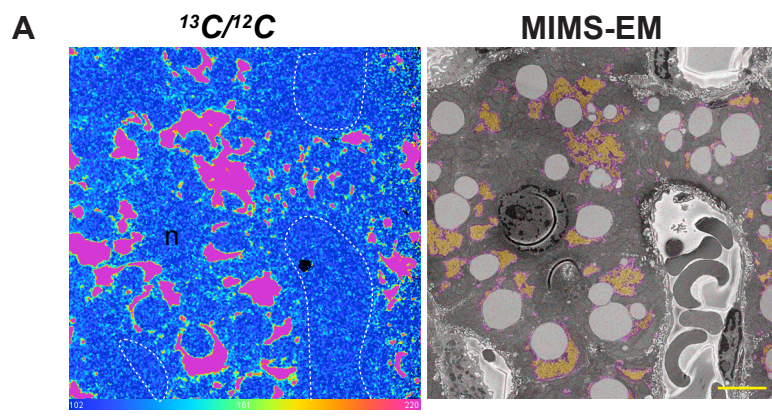
4 hour infusion



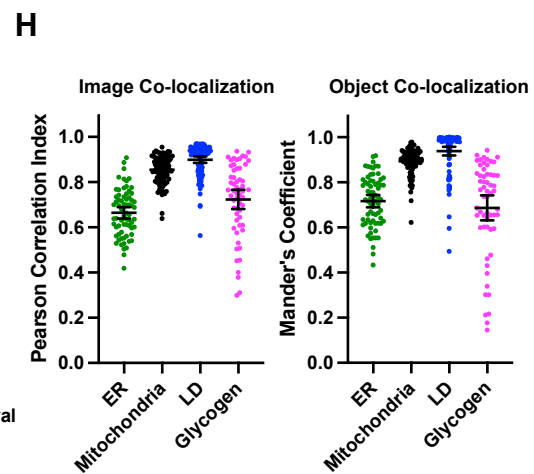
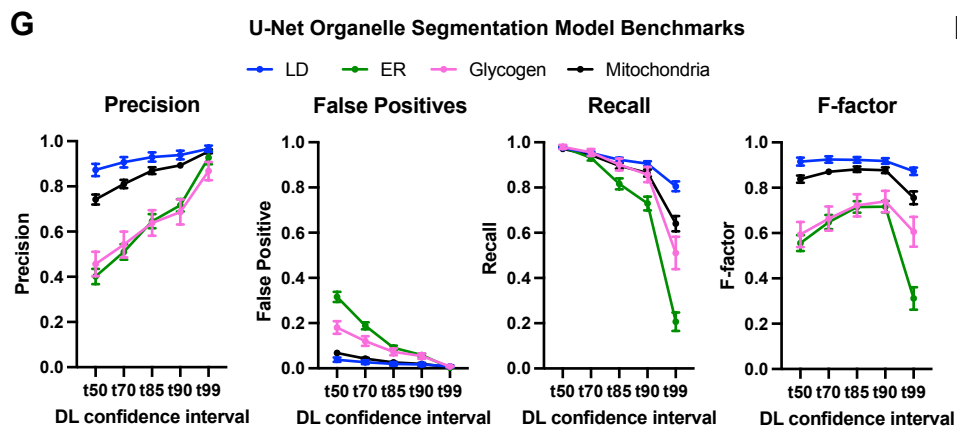
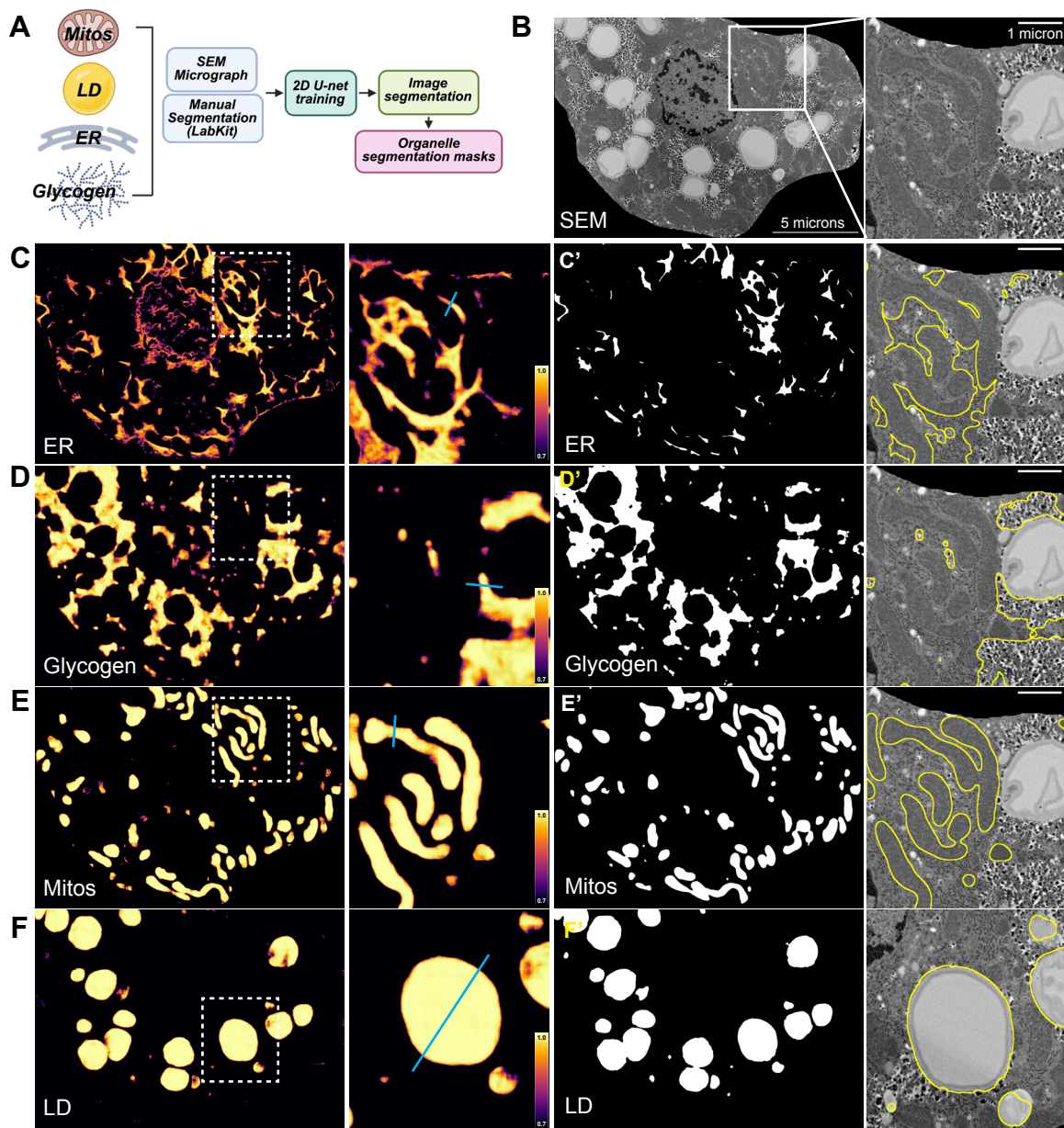
Supplementary Figure 2. Imaging mass spectrometry of ^{13}C -labelled liver sections. (A) Representative brightfield microscopy image of liver tissue sections mounted on a slide for MALDI-MS imaging. **(B)** Representative images of hematoxylin and eosin (H&E) staining and total glycogen content map measured using MALDI-MS of a mouse liver section fasted overnight and without infusion of $[\text{U-}^{13}\text{C}_6]$ -glucose. **(C)** Snapshot of glycogen mass spectrometry (MS) fragmentation spectra for glucose polymer (GP) with 7 glucose molecules (GP7). Zoom inset shows the spectra of ^{12}C and ^{13}C -labelled GP7. **(D-E)** Liver MALDI-MS images from fasted or $[\text{U-}^{13}\text{C}]$ -glucose-infused (40mg/min/kg) mice and detecting ^{12}C or ^{13}C -labelled glucose and hexose-6-phosphate (H6P) molecules.



Supplementary Figure 3. MIMS-EM imaging of ^{13}C -labelled hepatocytes. (A) MIMS-EM registration panels. Raw or aligned MIMS, and SEM data are shown on the top row. MIMS images of the stable isotope ^{32}S are shown. Bottom row, segmentation of fiducial markers in SEM and MIMS datasets both before and after MIMS-EM registration. (B) Grid graph displaying the displacement of fiducial markers after the image registration process. (C) Histogram distribution showing the relative frequency of points in X and Y (plot in the y-axis) and their total spatial displacement in both X and Y axis (bins, in microns, plotted in the x-axis). (D) Overlay image of segmented fiducial regions of interest (ROIs) in a representative SEM (white) and MIMS (magenta) dataset before and after registration. (E) *F-score* calculated to quantify the overlap of individual SEM and MIMS ROI objects before and after the MIMS-EM image registration process.

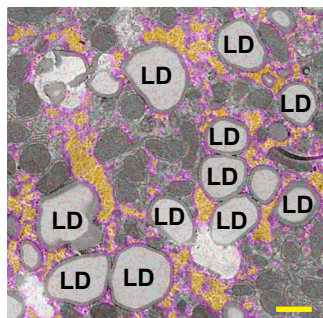
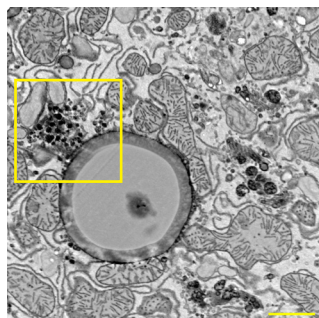
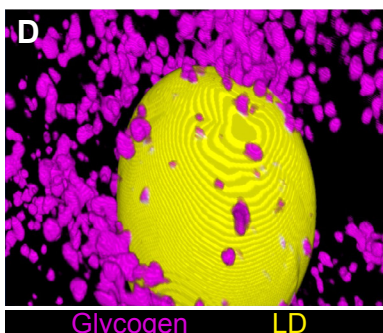
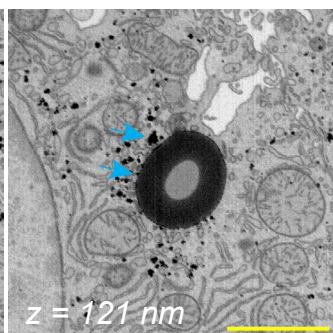
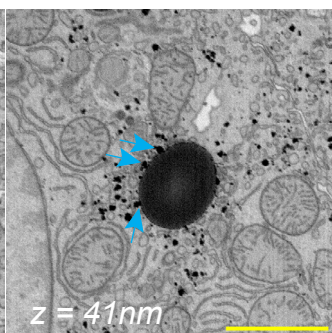
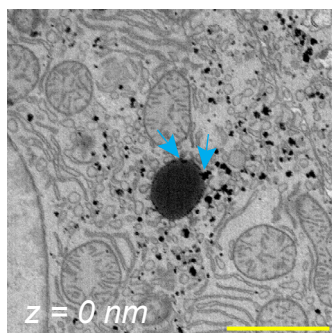


Supplementary Figure 4. MIMS-EM imaging of mouse hepatocytes. (A) Correlated ^{13}C -to- ^{12}C ($^{13}\text{C}/^{12}\text{C}$) and SEM images acquired using MIMS-EM of hepatocytes from mice continuously infused with 40mg/min/kg of [U- ^{13}C]-glucose for 2 hours. Scale bar, 5 microns. (B) Mouse averages for hepatocyte $^{13}\text{C}/^{12}\text{C}$ ratios. (C) Mouse averages for hepatocyte organelle type $^{13}\text{C}/^{12}\text{C}$ ratios. (D) Mouse averages for hepatocyte organelle cell fraction. (E) Representative MIMS-EM images displaying the $^{13}\text{C}/^{12}\text{C}$ levels in brown adipocytes from mice infused with 40mg/min/kg of [U- ^{13}C]-glucose for 4 hours. In (C-D), ** $p < 0.01$ and * $p < 0.05$ using One-way ANOVA with Dunns test.



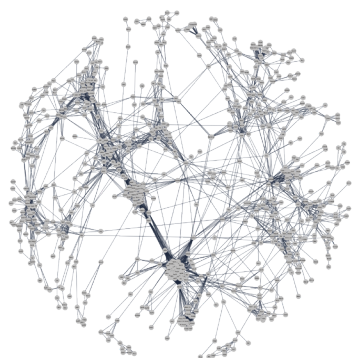
Supplementary Figure 5. Machine learning-based segmentation of hepatocyte organelles.

(A) Illustration of our organelle segmentation pipeline. A sub-set of SEM images are manually segmented to and used to train a 2D U-net model. This organelle-specific model is applied to SEM micrographs to create organelle segmentation masks that are used for downstream data analysis. Figure created using biorender.com. **(B)** SEM micrograph of a single hepatocyte. Right, zoomed-in region of the cell marked by the white dashed square in (B) to highlight clustered mitochondria, lipid droplet, and glycogen. Cell from a mouse infused with 40mg/min/kg of [U-¹³C]-glucose for 4 hours. **(C-F and C'-F')** Pixel classification confidence maps created by 2D U-nets trained to segment endoplasmic reticulum (ER), glycogen, mitochondria (Mitos), and lipid droplets (LDs), respectively. Here, each pixel receives a score of 0 to 1, where 1 indicates 100% confidence that pixel is correctly classified by a given DL model. In (C-F), raw data from DL-segmentation of the cell shown in (B), including zoomed-in region. Color scale bar represents the segmentation confidence interval from 70-to-100% model confidence. In (C'-F'), binarized organelle segmentation masks after 2D U-net segmentation and image processing and overlay on SEM data. Overlays are shown for the zoomed in quadrant highlighted in (B). **(G)** Performance benchmark indexes for our trained deep-learning image segmentation models. Precision, rate of false positives, object recall, and f-score are shown for different levels of confidence thresholding (t) intervals, from 50-to-95% confidence (t50 to t95). For all our analyzes, we chose a t value of 90%. **(H)** Pearson's and Mander's co-localization indexes quantifying the overlap between manual and 2D U-net segmentation pipelines of the same image or object, respectively.

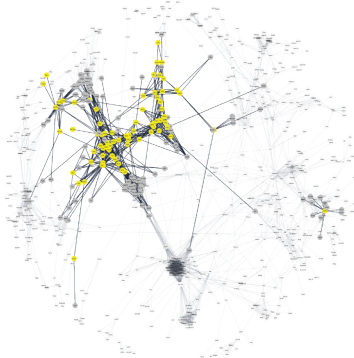
A**B*****Fasted liver*****C**

Supplementary Figure 6. MIMS-EM imaging of glucose ^{13}C enrichment around lipid droplets (LDs). (A) Representative zoomed in MIMS-EM image of the hepatocyte shown in Supplemental Figure 3B. Lipid droplets (LD) are annotated. Scale bar, 1 micron. (B) Representative scanning electron microscopy (SEM) image of a mouse hepatocyte after overnight fasting. Yellow bounding box highlights small glycogen clusters next to a lipid droplet (LD). (C) Serial slices extracted from a previously published study with a volumetric EM dataset centered in a lipid droplet. Relative distance in the z-axis from the first section are shown in white. Blue arrows indicate glycogen depots in the periphery of the lipid droplet. (D) 3D reconstruction rendering of the volume shown in (C).

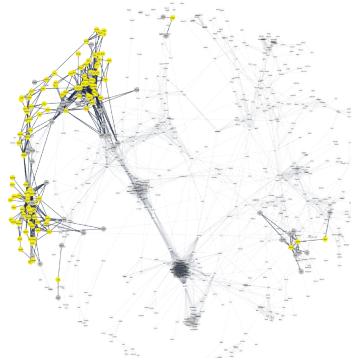
A Glycogen Protein Interaction Network



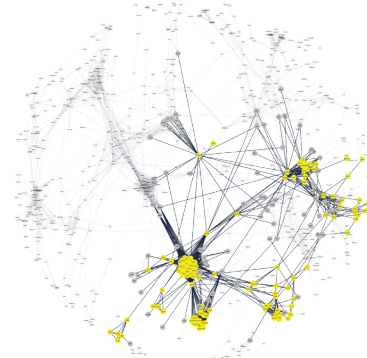
B Carbon Metabolism



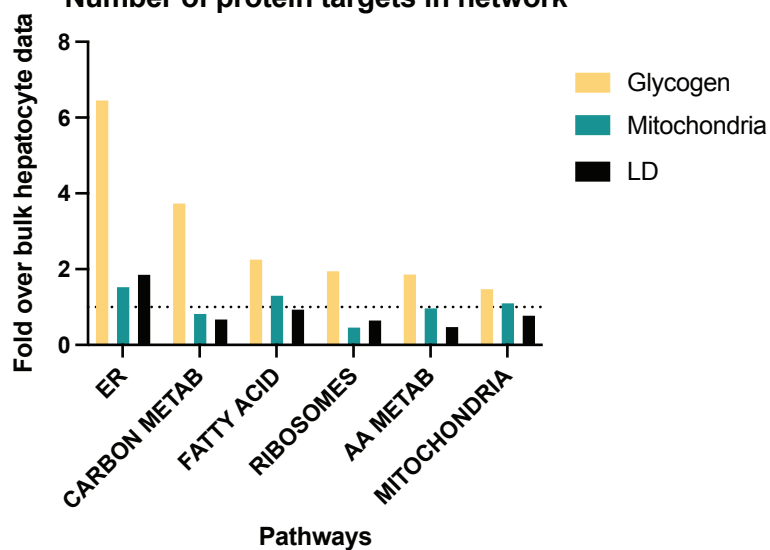
Fatty Acid Metabolism



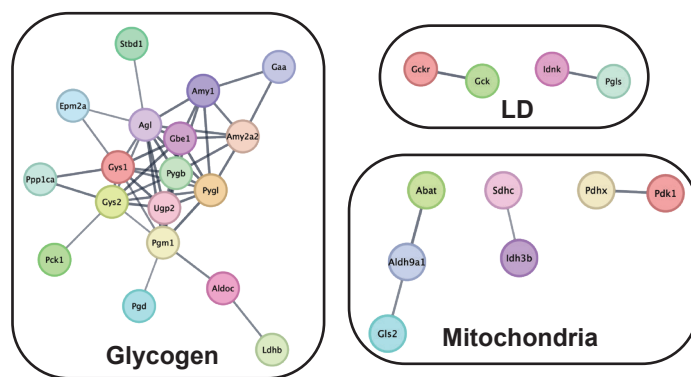
ER



C Number of protein targets in network

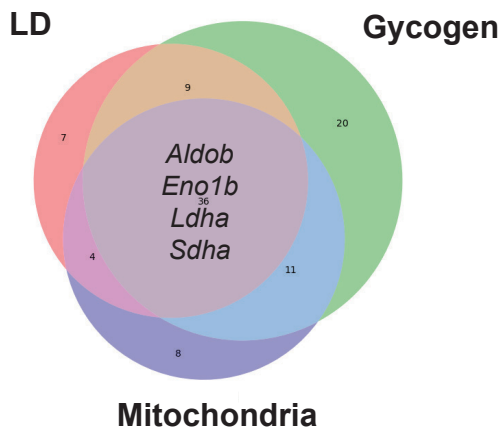
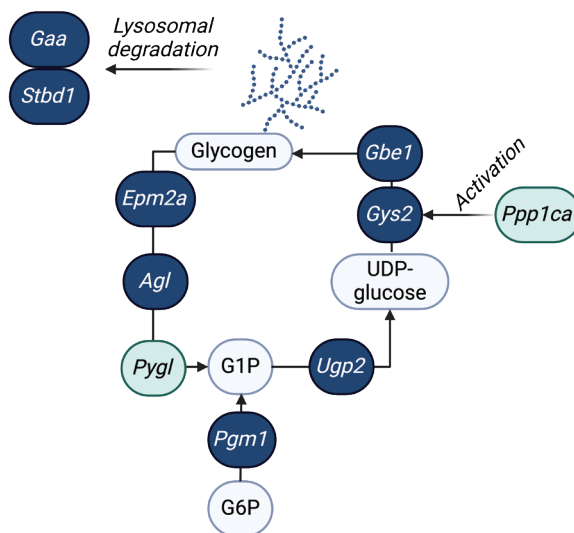


D Carbon Metabolism PPI Networks

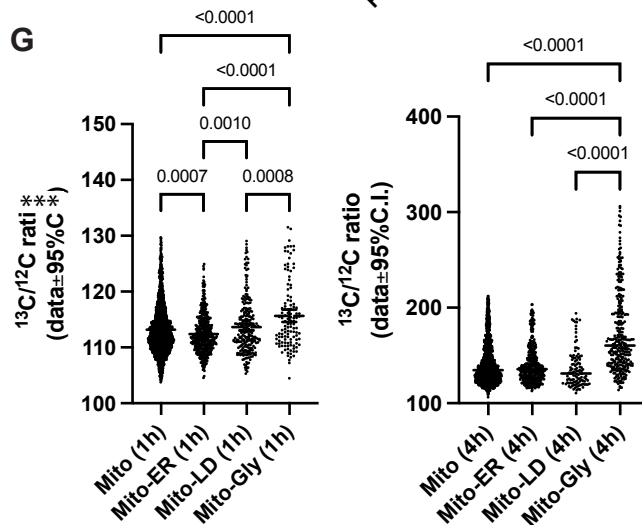
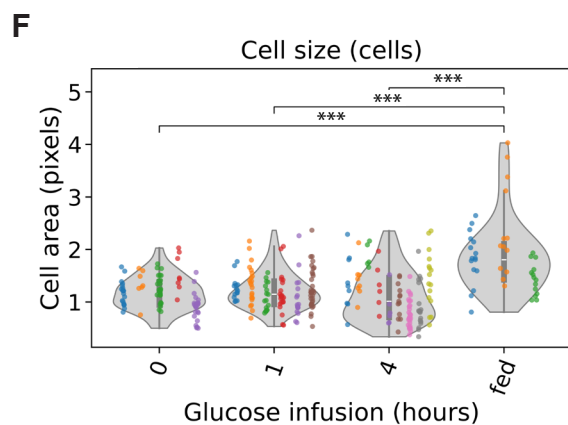
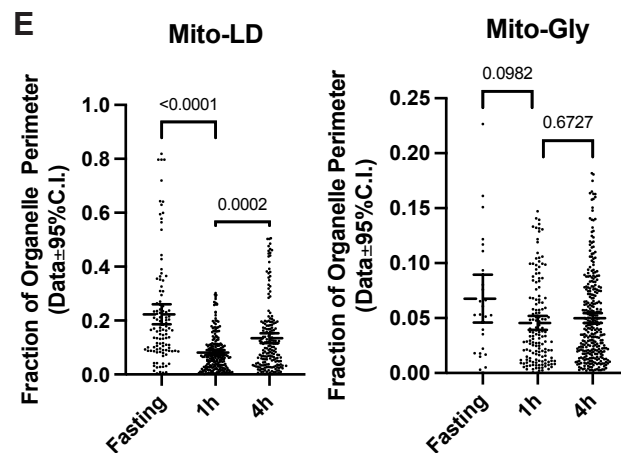
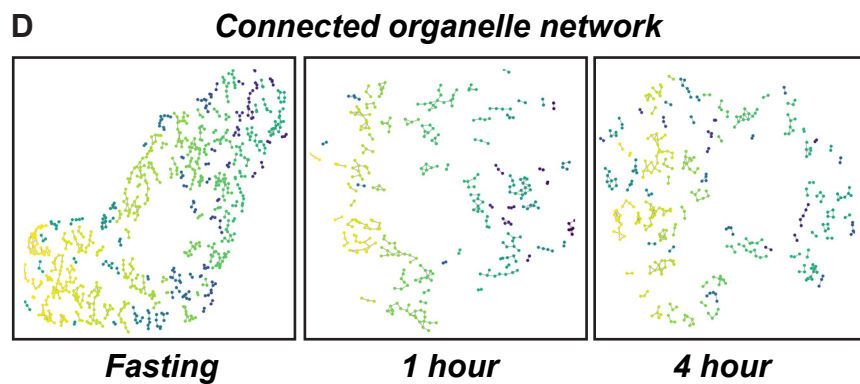
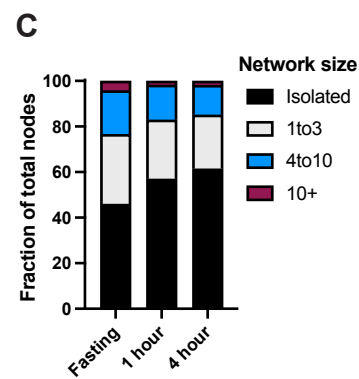
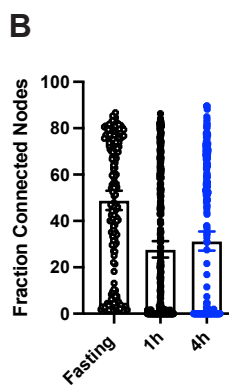
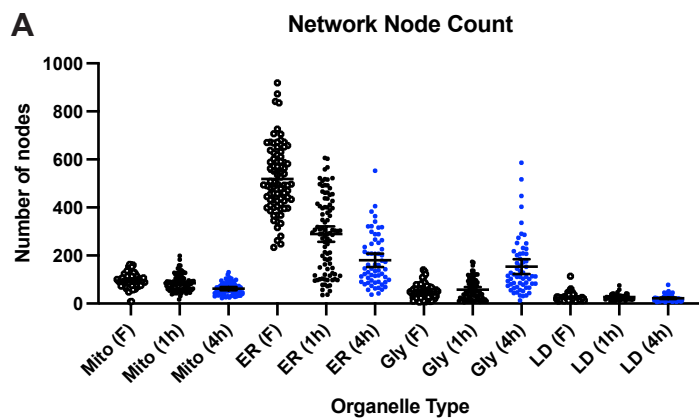


E Carbon Metabolism Pathway

LD Glycerol

**F**

Supplementary Figure 7. STRING DB analysis of hepatocyte proteins. **(A)** Reconstructed protein-protein interaction network of glycogen-enriched proteins detected using mass spectrometry of isolated glycogen analyzed from ⁴⁷. Networks were built using the top 2000 most abundant proteins and queried using StringDB and visualized using Cytoscape. Each node represents a protein in the network. **(B)** Yellow nodes represent proteins significantly enriched in carbon metabolism, fatty acid metabolism, or ER-associated pathways. **(C)** Relative enrichment of proteins found in the isolated glycogen, mitochondria, or lipid droplet (LD) mass spectrometry datasets. Proteins were clustered using StringDB clustering to identify proteins linked to ER, carbon metabolism, fatty acid metabolism, ribosomes, amino acid (AA) metabolism, and mitochondria-related pathways. Data reflects a fold change in enrichment for each pathway using data acquired from bulk hepatocyte proteomics as a benchmark. Dotted line marks a fold enrichment of 1. **(D)** Reconstructed protein-protein interaction network of proteins enriched in LD, glycogen, and mitochondria datasets. Protein names are shown inside nodes. **(E)** Venn diagram illustrating the overlap of proteins identified in glycogen, mitochondria, or LD-isolated fractions. Common proteins are highlighted in the middle. Numbers represent number of proteins inside each unique or overlapping dataset. **(F)** Schematic illustration of the metabolites (shown in grey) and proteins involved in the glycogenesis pathway. All proteins were found in the glycogen dataset. Proteins in aquamarine color are known to be in the endoplasmic reticulum (ER).



Supplementary Figure 8. Network analysis of hepatocyte organelle connections. **(A)** Total number of nodes per cell network and annotated to endoplasmic reticulum (ER), glycogen (Gly), mitochondria (Mito), or lipid droplets (LD) organelles. **(B)** Relative fraction of nodes that have a connection within a 500 nanometer (nm) distance radius. **(C)** Relative fraction of network nodes that either lack connections (i.e., isolated), or have between 1-to-3 (1to3), 4-to-10 (4to10), or more than 10 (10+) node connections. **(D)** Representative organelle network maps displaying connected nodes only. **(E)** Percentage of mitochondrial perimeter covered by LD, or glycogen contact sites. **(F)** Hepatocyte cell size in mice after overnight fasting, 1 or 4 hours of glucose infusion, or during a fed state. **(G)** $^{13}\text{C}/^{12}\text{C}$ ratio in isolated mitochondria (Mito) or mitochondria with ER, LD, or glycogen (Gly) contacts. In (E), p values are shown. In (F), *** $p < 0.001$, and different colors represent data from individual animals. One-way ANOVA with Dunns test post-hoc was used in (F) or Dunns or Kruskal-Wallis test was used in (E and G).

Supplementary Table 1. TM Sprayer parameters for application of enzyme

TM Sprayer M3	
Enzyme	Isoamylase
Solvent	H ₂ O
Temp (°C)	45
# Passes (Count)	15
Flow Rate (ml/min)	0.15
Velocity (mm/min)	900
Track Spacing (mm)	3
Pattern	CC
Pressure (psi)	10
Gas Flow Rate (l/min)	2
Drying Time (s)	0
Nozzle Hight (mm)	40

Supplementary Table 2. Additional MALDI IMS instrument parameters

TM Sprayer M5	
Matrix	CHCA
Solvent	1:1 ACN: H ₂ O (0.1% TFA)
Temp (°C)	72.5
# Passes (Count)	24
Concentration (mg/ml)	5
Flow Rate (ml/min)	0.05
Velocity (mm/min)	1300
Track Spacing (mm)	3
Pattern	CC
Pressure (psi)	10
Gas Flow Rate (l/min)	2
Drying Time (s)	2
Nozzle Hight (mm)	40

Supplementary Table 3. Additional MALDI IMS instrument parameters

Transfer	
Deflection 1 Delta	(-)80.0 V
Funnel 1 RF	350.0 Vpp
isCID Energy	10.0 eV Vpp
Funnel 2 RF	500.0 Vpp
Multipole RF	500.0 Vpp
Collision Cell	
Collision Energy	25.0 eV
Collision RF	3000.0 Vpp
Transfer Time	110 µs
Pre Pulse Storage	15.0 µs

Ion Energy	Quadrupole	25.0 eV
Low Mass		m/z 500.00