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BRIEF REPORT



Live visualization of dynamic autophagy processes in LC3 reporter mice using enhanced two-photon microscopy

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ABSTRACT

We developed a computationally enhanced intravital imaging framework for high-resolution analysis of macroautophagy/autophagy dynamics in vivo. By integrating two-photon microscopy with an optimized tissue stabilization method, motion artifacts were minimized, enabling real-time imaging of LC3-positive structures in the liver and skeletal muscle under physiological stress conditions, including starvation, ischemia, and ethanol exposure. Image quality was further improved using a trained denoising model and enhanced Super-Resolution Radial Fluctuations (eSRRF), enabling nanoscale visualization of autophagy-related compartments. To differentiate increased autophagosome formation from impaired degradation, autophagic flux was assessed via chloroquine-mediated lysosomal inhibition, enabling functional interpretation beyond static structural measurements. Together, this pipeline overcomes major technical limitations of intravital imaging and provides a robust framework for integrated structural and functional investigation of autophagy and related intracellular processes in vivo.

Abbreviations: CQ: chloroquine; eSRRF: enhanced super-resolution radial fluctuations; EtOH: ethanol; GFP: green fluorescent protein; I/R: ischemia-reperfusion; MAP1LC3/LC3: microtubule associated protein 1 light chain 3; MTR: MitoTracker Red; rFRC: rolling Fourier ring correlation.

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Introduction

Autophagy [1,2] is an evolutionarily conserved intracellular degradation pathway responsible for the turnover of damaged organelles, protein aggregates, and other cytoplasmic components [3] through the formation of double-membraned vesicles known as autophagosomes [4,5]. As a fundamental mechanism for maintaining cellular homeostasis, autophagy plays essential roles in adaptation to environmental and metabolic stressors, including nutrient deprivation, hypoxia, oxidative stress, and toxin exposure [6–10]. Dysregulation of autophagy has been implicated in numerous pathological conditions, including cancer [11], neurodegeneration disorders [12], cardiovascular diseases, and metabolic syndromes [13]. Consequently, understanding the spatial and temporal regulation of autophagy within living tissues is critical for elucidating its roles in both physiological adaptation and disease progression [14].

A hallmark of the autophagic pathway is the formation of MAP1LC3/LC3-positive phagophores that encapsulate cytoplasmic material, maturing into autophagic vesicles (~150 nm in diameter) that deliver the cargo for lysosomal degradation [15–17]. These structures, including phagophores, autophagosomes and early autolysosomes, are highly dynamic and transient, with characteristic dimensions approaching or falling below the diffraction limit of conventional light microscopy. Their small size, rapid turnover, and heterogeneous distribution within tissues

present significant challenges for direct visualization in vivo [18]. Although electron microscopy provides ultrastructural information at nanometer-scale resolution, it is inherently limited to fixed specimens and cannot capture dynamic cellular processes in living animals. Conventional fluorescence microscopy techniques, including wide-field and confocal microscopy, are further constrained by limited imaging depth, photobleaching, and optical diffraction limit (~250 nm) [19,20], restricting their ability to resolve autophagy-related structures within intact tissues.

Two-photon microscopy [21] has been introduced as a powerful modality for intravital imaging owing to its use of near-infrared excitation, reduced phototoxicity, improved optical sectioning, and increased penetration depth in biological tissues [22]. These characteristics have enabled visualization of cellular and subcellular processes in living animals with minimal tissue disruption. Despite these advantages, the spatial resolution of conventional two-photon microscopy is still diffraction-limited, while weak fluorescence signals and substantial background noise often obscure small intracellular structure. Furthermore, physiological motion arising from respiration, cardiac pulsation, vascular activity, and tissue deformation introduces image instability and motion artifacts that complicate quantitative analysis and long-term observation of dynamic organelles such as autophagosomes [23,24].

To address this, we employed a mechanical stabilization strategy using a custom-designed imaging window that gently anchors the target tissue, minimizes displacement, and reduces motion artifacts while preserving physiological function [25]. By improving image stability, this technique enhances the reliability of image analysis which is essential for resolving subcellular dynamics *in vivo*.

Recent advances in computational image restoration and super-resolution microscopy have provided new opportunities for overcoming these limitations. Deep-learning and self-supervised denoising methods can substantially improve image quality by suppressing noise while preserving biologically relevant structural information [26,27]. In parallel, fluctuation-based super-resolution approaches such as enhanced Super-Resolution Radial Fluctuations (eSRRF) [28] enable resolution enhancement from conventional fluorescence image sequences without requiring specialized optical hardware. These methods have demonstrated considerable success in cultured cells and fixed specimens [29,30]; however, their application to intravital imaging remains relatively limited due to the additional challenges of motion artifacts, signal instability, and tissue heterogeneity encountered in living animals [31,32].

Beyond imaging limitations, interpretation of autophagy *in vivo* presents additional biological challenges. Accumulation of LC3-positive structures alone does not necessarily indicate increased autophagic activity, as similar observations may result from either enhanced autophagosome formation or impaired lysosomal degradation. Consequently, assessment of autophagic flux remains important for biological interpretation of LC3-based imaging data [33,34]. Complementary approaches, including pharmacological modulation using lysosomal inhibitors such as chloroquine and molecular analyses of autophagy-associated markers including LC3-II and SQSTM1/p62, can provide additional insight into the functional status of the autophagic pathway [35].

To address these challenges, we developed an integrated intravital imaging framework for high-resolution visualization of LC3-positive compartments in living tissues. The platform combines GFP-LC3 transgenic mice [36], tissue stabilization, two-photon microscopy, self-supervised image denoising, and eSRRF-based computational resolution enhancement to improve the visualization and quantification of autophagy-related structures *in vivo* [37]. Using this approach, we investigated autophagic responses under physiological and stress conditions, including starvation, ischemic injury, and pharmacological modulation of lysosomal function. In addition, complementary molecular and histological analyses were incorporated to support interpretation of the imaging findings. Together, this framework provides a practical and accessible strategy for studying autophagy and mitophagy dynamics in living tissues with improved spatial resolution, image quality, and quantitative capability.

Results

To enable high-resolution visualization of subcellular structures in living tissues, we developed an integrated intravital imaging platform combining tissue stabilization, two-photon

microscopy, self-supervised denoising, and computational super-resolution reconstruction, Figure 1A. The image-processing workflow consisted of a U-Net-based self-supervised denoising algorithm applied directly to raw image stacks [38], followed by eSRRF reconstruction to improve structural resolvability and image quality [28].

Physiological motion arising from respiration and cardiac activity remains a major limitation for high-magnification intravital imaging. As shown in Figure 1B, unstabilized tissues exhibited substantial periodic displacement and image distortion, whereas stabilization markedly reduced pixel motion and improved signal consistency over time. Analysis of representative pixels further demonstrated markedly reduced signal fluctuations following stabilization, indicating improved preservation of image fidelity over time Figure 1C. Motion artifacts are apparent in the unstabilized condition and become particularly severe at higher magnification.

The performance of the computational pipeline was evaluated using quadriceps muscle tissue Figure 1D. While the original two-photon images contained weak and poorly resolved signals, the processed images revealed myofiber organization and fine structural features that were difficult to distinguish in the raw data. Pixel intensity profiles of two selected regions Figure 1E also highlight clearer peak separations and structural discrimination in the reconstructed images. Quantitative analysis confirmed a significant increase in signal:noise ratio (SNR) following processing Figure 1F, reflecting improved image quality through noise reduction while preserving signal intensity. Furthermore, to assess the effect of image processing on spatial resolution, rolling Fourier ring correlation (rFRC) analysis [39] was performed Figure 1G. The processed datasets exhibited a substantial improvement in estimated effective resolution, from approximately 640 nm in the original two-photon images to 116 nm after denoising and eSRRF reconstruction. These values represent computationally derived estimates of effective structural resolvability rather than direct optical resolution. Collectively, these results demonstrate that the combination of tissue stabilization and computational enhancement significantly improves image quality and enables reliable visualization of subcellular structures *in vivo*.

To evaluate the ability of the imaging platform to detect autophagy-related responses *in vivo*, GFP-LC3 mice were subjected to nutrient deprivation and imaged longitudinally in skeletal muscle, as a classical physiological trigger for systemic autophagy induction [40,41]. Representative images are shown in Figure 2A. Compared with control animals, starvation increased the abundance of LC3-positive puncta after 1 day of fasting, whereas fewer puncta were observed after 2 days of starvation. This reduction may reflect physiological adaptation to prolonged nutrient deprivation, resulting in a smaller autophagy pool size, potentially with enhanced autophagy flux [7]. Quantitative analysis confirmed these changes in puncta abundance across the starvation time course Figure 2B. CQ treatment further increased LC3-positive compartment accumulation at both starvation time points, consistent with impaired lysosomal degradation and accumulation of autophagic compartments.

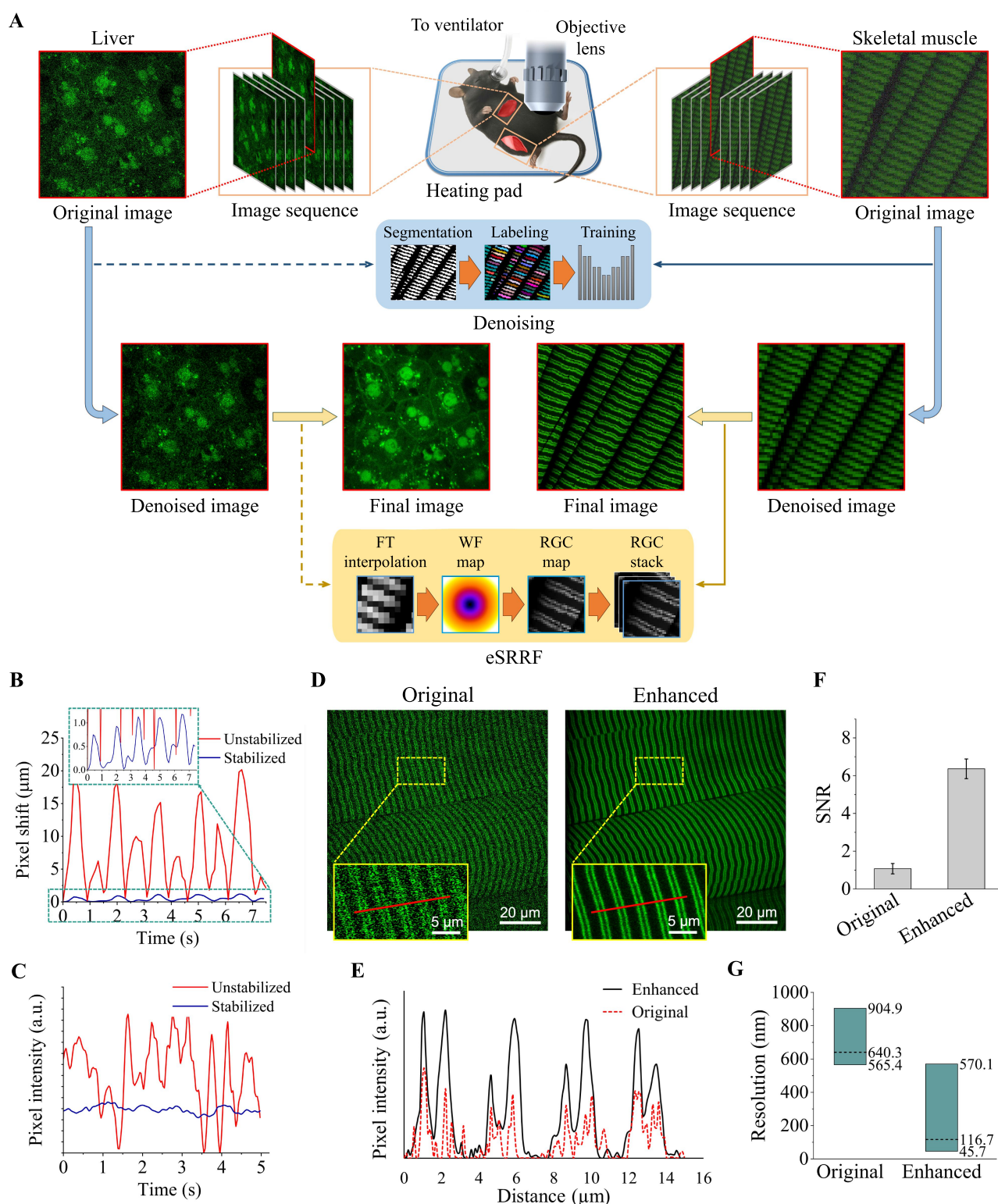


Figure 1. Intravital imaging workflow, tissue stabilization, and computational image enhancement. (A) Schematic illustration of the imaging and processing workflow used for GFP-LC3 intravital imaging. Raw image sequences were subjected to denoising, motion correction, and eSRRF-based resolution enhancement to generate high-resolution images for downstream analysis. (B) Representative pixel-shift traces demonstrating tissue motion in unstabilized and stabilized imaging conditions. Mechanical stabilization markedly reduced motion amplitude and improved image stability during acquisition. (C) Pixel intensity measurements over time from representative regions of interest, showing improved signal stability in stabilized tissue compared with unstabilized tissue. (D) Representative raw and enhanced images of quadriceps muscle from a GFP-LC3 mouse, demonstrating improved visualization of myofiber structures following image processing. (E) Pixel intensity profiles extracted along the red line shown in (D), illustrating reduced background fluctuations and improved separation of structural features after processing. (F) Quantitative comparison of signal-to-noise ratio (SNR) between original and enhanced images. (G) Comparison of estimated effective spatial resolution obtained by rFRC analysis, showing minimum, mean, and maximum resolution values before and after image enhancement. Lower values indicate improved structural resolvability. Scale bar: 20 μm (5 μm , inset). (FT: Fourier transform; WF: weighting factor; RGC: radial gradient convergence).

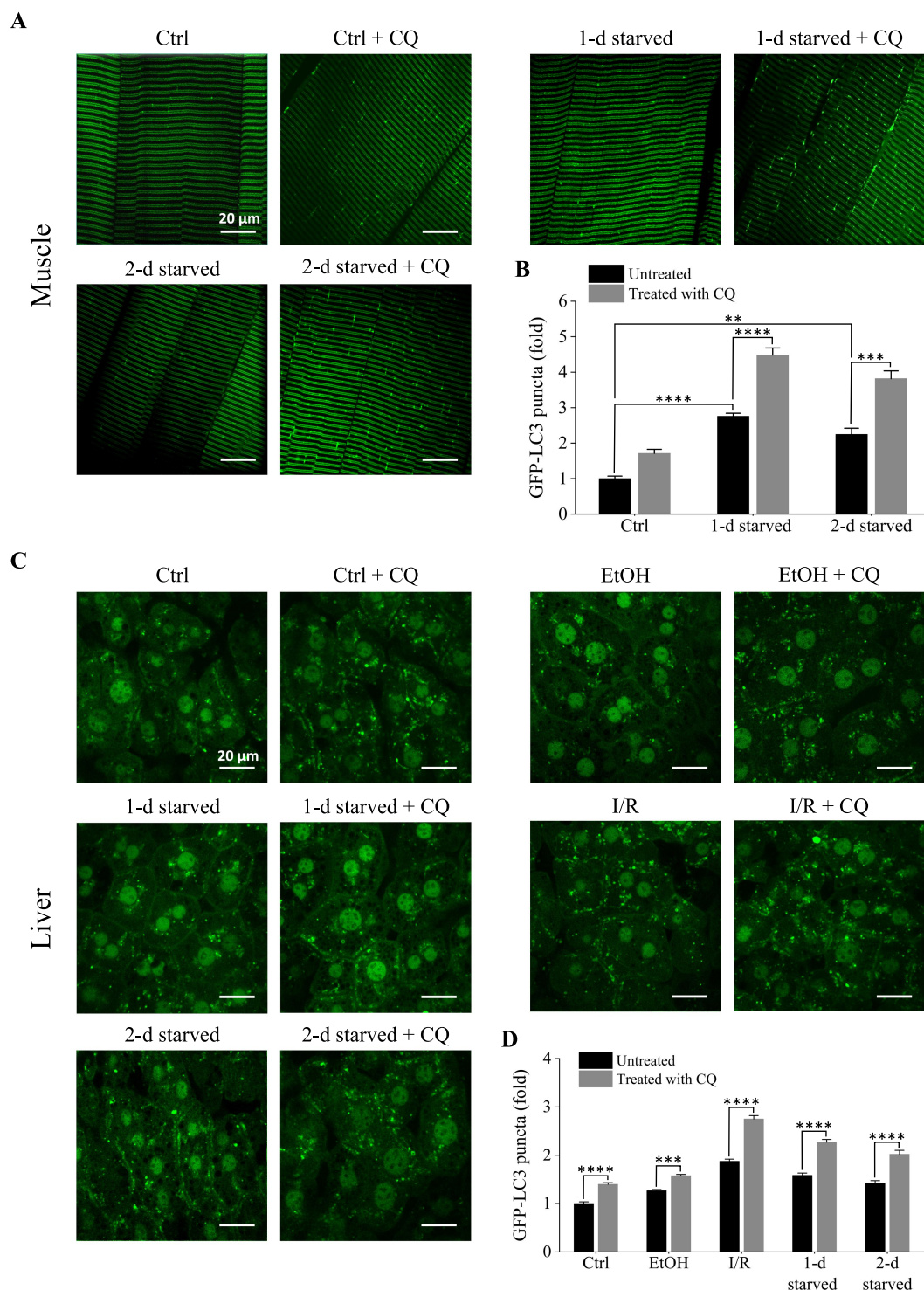


Figure 2. In vivo visualization and quantification of LC3-positive compartments in skeletal muscle and liver of GFP-LC3 mice with and without CQ treatment. (A) Representative skeletal muscle images showing LC3-positive compartments under control (ctrl) conditions and following 1- and 2-day starvation, with or without CQ treatment. (B) Quantitative analysis of LC3-positive compartment abundance in skeletal muscle across control and starvation groups. (C) Representative liver images showing LC3-positive compartments under control conditions and following starvation, ischemia-reperfusion (I/R) injury, or ethanol (EtOH) exposure, with or without CQ treatment. (D) Quantitative comparison of LC3-positive compartment abundance across the different hepatic stress models, normalized to the control group. Data are presented as mean $\pm$ SEM. Scale bar: 20 μ m (5 μ m, inset). Statistical significance was determined using two-way ANOVA followed by Tukey's post hoc test. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

We next investigated whether similar responses could be visualized in the liver and compared autophagy-related changes across multiple stress conditions, including starvation, ethanol (EtOH) exposure, and ischemia-reperfusion (I/

R) injury. Representative intravital images are shown in Figure 2C. All stress models produced increased accumulation of LC3-positive compartments relative to control animals, although distinct spatial patterns and magnitudes were

observed. Starvation increased puncta abundance after 1 day of fasting; however, puncta numbers were reduced on day 2 while remaining elevated relative to control levels, suggesting adaptation to prolonged nutrient deprivation. Ethanol exposure generated a moderate but reproducible increase in LC3-positive structures [42,43], whereas I/R injury [44,45] elicited the most pronounced response, characterized by dense and spatially localized puncta accumulation within affected hepatic regions.

Quantitative analysis of liver datasets confirmed these observations **Figure 2D**. Among all conditions, I/R injury produced the greatest increase in LC3-positive structure abundance, followed by starvation and ethanol exposure. Across all stress models, CQ treatment resulted in additional puncta accumulation, supporting continued autophagosome formation and flux through the autophagic pathway.

Mitophagy in hepatocytes

Mitophagy, the selective autophagic clearance of damaged mitochondria, was next assessed in hepatocytes from control and 1-day starved mice. Mitochondria were labeled with MitoTracker Red (MTR), enabling simultaneous visualization of mitochondrial networks and GFP-LC3 signals by two-photon microscopy **Figure 3A**. Overlap between mitochondrial and LC3 signals was observed under both control and starvation conditions, with a noticeable increase following nutrient deprivation. Quantitative analysis **Figure 3B** demonstrated an increase in the number of mitochondria-associated LC3-positive compartments per cell in starved animals, indicating enhanced spatial association between mitochondria and autophagic compartments during nutrient deprivation. This effect was further accentuated in CQ-treated groups, where accumulation of mitochondria-associated LC3-positive compartments was consistent with inhibition of lysosomal degradation. To distinguish the mitophagy-associated compartment from the overall autophagy compartment, the total number of LC3-positive compartments per cell was also quantified and compared with the mitochondria-associated population. While starvation increased both total LC3-positive compartments and mitochondria-associated LC3-positive compartments, CQ treatment further amplified their accumulation.

Immunostaining and western blot validation

To provide independent validation of the intravital imaging results, liver tissues from the different experimental groups were subjected to SQSTM1 immunostaining and western blot analysis. Representative staining results are shown in **Figure 4**. Immunofluorescence revealed a clear increase in signal intensity under all stress conditions **Figure 4A**. Compared with control tissues, all stress conditions exhibited increased SQSTM1 immunoreactivity, with the most pronounced changes observed following ischemia-reperfusion injury. Starvation produced a progressive increase in staining intensity, whereas ethanol exposure resulted in a more moderate but consistent response. Across all experimental conditions,

CQ treatment further enhanced SQSTM1 accumulation, consistent with inhibition of lysosomal degradation **Figure 4B**.

Western blot analysis revealed similar trends. Relative to control samples, SQSTM1 levels increased under starvation, ethanol exposure, and ischemia-reperfusion injury, with the largest changes again observed in the I/R group **Figure 4C**. CQ administration resulted in additional accumulation of SQSTM1 across all conditions, reflecting reduced degradation of autophagy-associated cargo following lysosomal inhibition. Quantification of band intensities confirmed these observations **Figure 4D**.

Overall, the histological and biochemical findings were consistent with the intravital imaging data. The observed accumulation of LC3-positive compartments, together with increased SQSTM1 levels following CQ treatment, supports altered autophagic compartment dynamics and reduced lysosomal clearance under the investigated stress conditions.

Together, these results demonstrate that the proposed intravital imaging framework enables high-resolution visualization and quantitative analysis of LC3-positive compartments in living tissues, providing a powerful tool for investigating autophagy-related processes *in vivo*.

Discussion

This study establishes an intravital imaging framework that integrates two-photon microscopy, mechanical stabilization, and computational post-processing to resolve subcellular structures in living tissues. Physiological motion is a major limitation in intravital imaging; here, stabilization minimized drift and motion blur, preserving spatial and temporal fidelity during longitudinal acquisition and enabling reliable visualization of LC3-positive compartments over extended imaging periods. Following acquisition, images were processed using a sequential pipeline of denoising, drift correction, and eSRRF-based super-resolution reconstruction. Quantitative rFRC analysis demonstrated a substantial improvement in estimated effective spatial resolution, from approximately 640 nm to 116 nm, enabling visualization of fine structural features that are difficult to resolve in conventional two-photon images.

Compared with established super-resolution techniques, this approach is well suited for intravital applications. While super-resolution structured illumination microscopy (SR-SIM) achieves ~100 nm resolution and single-molecule localization methods such as dSTORM reach higher precision (~30 nm), both are largely restricted to fixed samples due to demanding labeling requirements and limited suitability for deep-tissue imaging. In contrast, the present framework provides improved effective spatial resolution while maintaining the imaging depth and intravital compatibility of two-photon microscopy, with greater tolerance to tissue motion and lower phototoxicity.

Despite advances in autophagy research, direct intravital imaging remains limited. Most GFP-LC3-based studies rely on fixed or *ex vivo* tissues, and real-time, high-resolution imaging in intact soft organs such as the liver remains technically challenging because of motion and optical constraints [7,46,47]. In this context, our approach enables

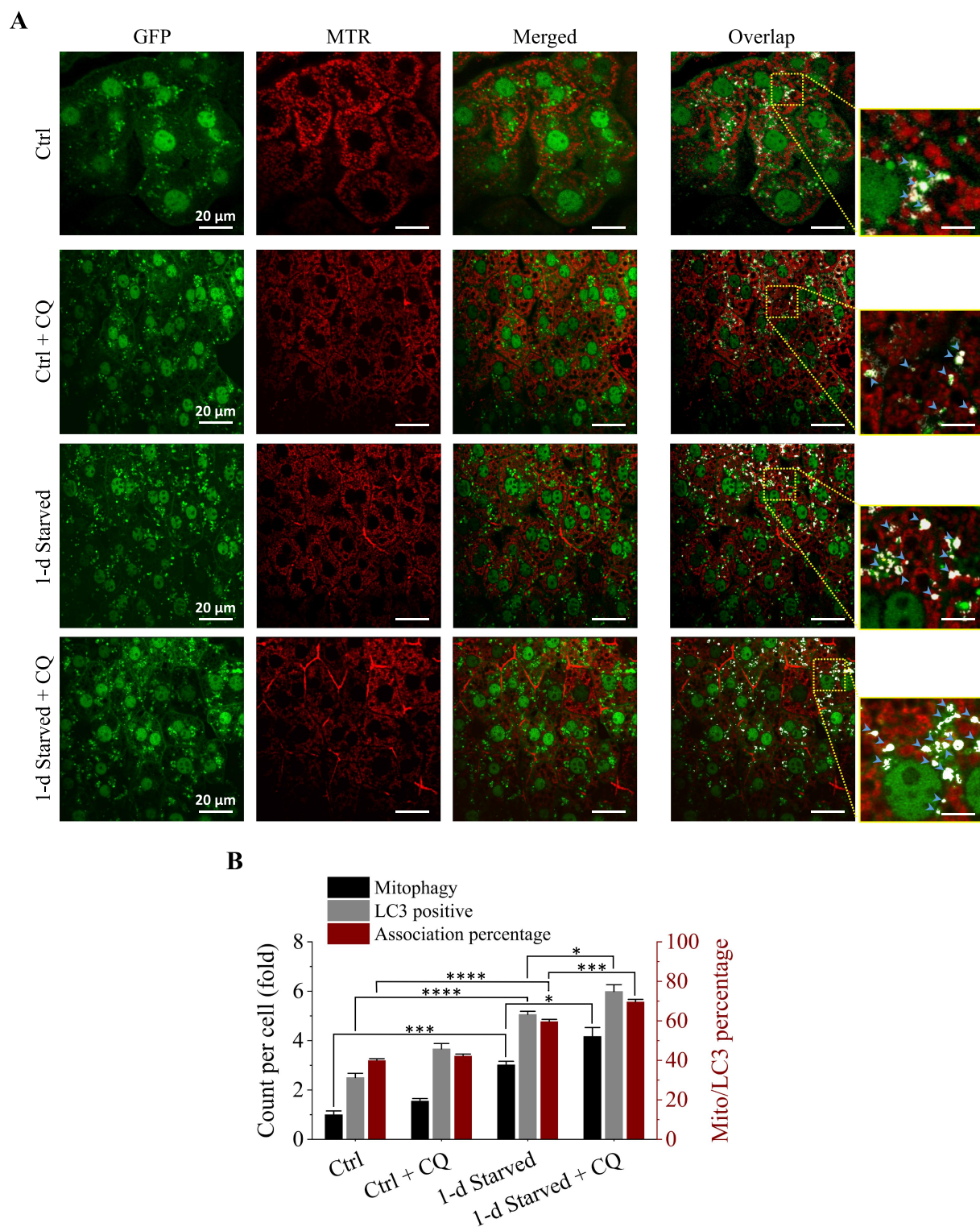


Figure 3. Spatial association between mitochondria and LC3-positive compartments in hepatocytes from control and 1-day starved GFP-LC3 mice with or without CQ treatment. (A) Representative two-photon images showing LC3-positive compartments (green, GFP-LC3), mitochondria (red, MitoTracker red), and the corresponding merged images. Arrowheads indicate regions of spatial overlap between mitochondrial and LC3 signals, consistent with mitochondria-associated autophagic compartments. (B) Quantitative analysis of mitochondria-associated LC3-positive compartments and total LC3-positive compartments per cell, normalized to the mitophagy-associated control group, enabling direct comparison of the mitophagy-associated compartment with total autophagy compartments. Data are presented as mean $\pm$ SEM. Scale bar: 20 μ m (5 μ m, inset). Statistical significance was determined using two-way ANOVA followed by Tukey's post hoc test. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

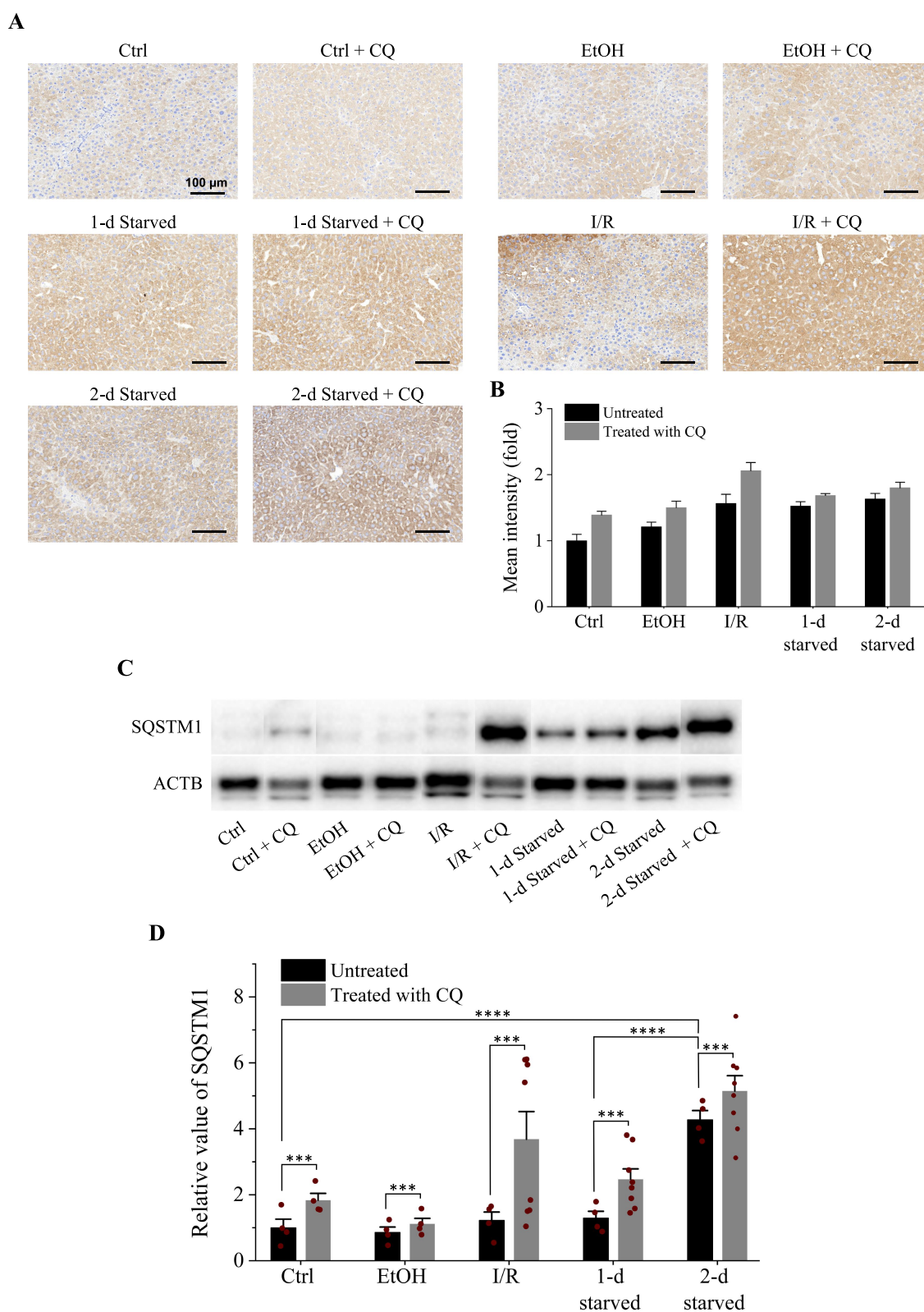


Figure 4. Molecular validation of imaging findings by immunostaining and western blot analysis. (A) Representative immunofluorescence images of liver sections stained for SQSTM1/p62 under control and stress conditions (starvation, EtOH, and I/R), with or without CQ treatment. (B) quantification of SQSTM1 immunofluorescence intensity, normalized to the control group. Increased SQSTM1 signal was observed in stressed groups, with further accumulation following CQ treatment. (C) Representative western blot analysis of SQSTM1/p62 expression across experimental groups. (D) densitometric quantification of SQSTM1 protein levels normalized to ACTB/ β -actin and expressed as Fold change relative to control. Stress conditions resulted in increased SQSTM1 accumulation, which was further enhanced in CQ-treated animals. Scale bar: 100 μ m. Data are presented as mean $\pm$ SEM. Statistical significance was determined using two-way ANOVA followed by Tukey's post hoc test. * $p < 0.001$, ** $p < 0.0001$.

stabilized intravital imaging with quantitative subcellular analysis, extending current capabilities for investigating autophagy-related processes *in vivo*.

The primary aim of this study was to evaluate stress-induced modulation of autophagy-related compartments; however, interpretation of LC3-based imaging requires careful consideration. Static quantification of LC3-positive puncta alone does not distinguish between increased autophagosome formation and impaired degradation. Although GFP-LC3 models enable direct visualization of autophagy-associated structures *in vivo* [7], interpretation requires assessment of autophagic flux rather than steady-state puncta abundance [34,48]. In this study, lysosomal inhibition with CQ was used as a complementary functional readout. The accumulation of LC3-positive compartments and SQSTM1 across stress conditions is consistent with ongoing autophagic turnover and increased autophagic flux, although additional approaches such as tandem fluorescent LC3 reporters or genetically encoded probes [33,49] would further improve flux assessment. Notably, some LC3-positive structures may represent autolysosomes rather than newly formed autophagosomes, highlighting the need for cautious structural interpretation [50]. Furthermore, spatial heterogeneity within the tissue microenvironment may influence both the distribution and apparent dynamics of autophagic compartments.

Using this framework, distinct autophagy-related responses were resolved across multiple physiological stress models. Ischemia-reperfusion injury induced the most pronounced and spatially localized accumulation of LC3-positive compartments, consistent with acute hypoxic and oxidative stress. Starvation produced a systemic increase that partially attenuated over time, suggesting metabolic adaptation, whereas ethanol exposure elicited a more moderate but sustained response. Across all conditions, CQ treatment resulted in additional accumulation of LC3-positive compartments, consistent with continued autophagic turnover and reduced lysosomal degradation.

Mitophagy analysis revealed increased spatial association between mitochondrial and LC3 signals under starvation, which was further enhanced following CQ treatment, indicating accumulation of mitochondria-associated autophagic compartments. Quantitative analysis of mitochondria-associated LC3-positive structures supported this trend; however, spatial overlap alone is insufficient to establish mitophagic flux. Furthermore, LC3 labeling detects both autophagosomes and early autolysosomes, limiting stage-specific interpretation. Integration of lysosomal probes (e.g., LysoTracker) or tandem fluorescent LC3 reporters would enable discrimination of autophagic intermediates and identification of mitolysosomes, providing a more direct assessment of mitophagic flux. In addition, long-term time-lapse imaging or stage-sensitive reporters could further improve characterization of autophagy dynamics *in vivo*.

A key strength of this approach is its accessibility and robustness. The framework requires no specialized hardware modifications and performs reliably under low signal-to-noise conditions, where conventional enhancement approaches such as deconvolution [51] often provide limited recovery of

structural information. Although axial resolution remains diffraction-limited, the combination of mechanical stabilization and computational enhancement provides a practical and broadly accessible strategy for nanoscale intravital imaging. Correlative validation with fixed-tissue sections would be valuable; however, precise spatial registration between intravital and fixed-tissue datasets remains challenging because of tissue deformability, physiological motion, and fixation-induced distortions.

In summary, this platform enables high-resolution *in vivo* visualization and quantitative assessment of autophagy- and mitophagy-associated compartments at the subcellular level, helping bridge the gap between conventional intravital imaging and nanoscale biological analysis. The framework provides a versatile and scalable tool for investigating organelle behavior across physiological and pathological conditions, with potential applications in disease modeling and therapeutic evaluation.

Materials and methods

Animal experiments

All animal procedures were approved by the Institutional Animal Care and Use Committee at Asan Medical Center (permit number: 2025–10-290). GFP-LC3 transgenic mice (male and female, 6–8 weeks, 20–30 g; Jackson Laboratory) were used for all experiments. Twenty-four mice were randomly assigned to starvation, acute ethanol exposure, or hepatic ischemia-reperfusion (I/R) injury groups. Within each experimental condition, animals received either DPBS (Gibco, 14,190,144) or chloroquine (CQ; Sigma-Aldrich, C6628-25 G) treatment. For anesthesia, a mixture of Zoletil 50 (Virbac, 8800553000104), Rompun (Elanco, 90204280), and DPBS (volume ratio 6:4:90) was administered at a dose of 10 mL/kg. During imaging, mice were intubated and mechanically ventilated using a MiniVent 845 animal ventilator (Harvard Apparatus, USA) to ensure respiratory stability. To maintain physiological stability, the body temperature was monitored and regulated at 37°C using the heating microscope stage.

Imaging procedure

Mice were anesthetized, and the surgical area was shaved and sterilized to provide unobstructed optical access and minimize infection risk. A small abdominal incision was performed, and animals were positioned supine on the microscope stage. For hepatic imaging, the liver was gently exteriorized while avoiding mechanical stress and vascular disruption. For skeletal muscle imaging, the thigh skin was retracted to expose the quadriceps muscle. After imaging, incisions were sutured and sterilized, and mice were placed on a temperature-controlled recovery pad before being returned to their cages for longitudinal experiments.

Intravital imaging was performed using a commercial two-photon microscope (IVM-MS, IVIM Technology, Republic of Korea) equipped with a femtosecond Ti:

Sapphire laser tuned to 920 nm (Coherent Inc., USA). Images were acquired at 1024×1024 pixels (pixel size ~110 nm) at 15 frames/s in a single focal plane. Fluorescence signals were detected using four high-quantum efficiency PMTs with broad spectral sensitivity (185–760 nm), with bandpass filter sets optimized for GFP and MitoTracker emission. A 100× oil-immersion objective (NA 1.45, Olympus, Japan) was used for all high-resolution imaging experiments.

Physiological conditions were maintained throughout imaging by continuous application of sterile PBS to prevent tissue dehydration and by using a temperature-controlled stage. Tissue stabilization was achieved using a custom 3D-printed imaging window holder that applied gentle negative pressure to minimize motion artifacts while preserving physiological function, thereby enabling stable imaging with a high-NA oil-immersion objective.

Image analysis

All image processing was performed using Fiji (v1.54) [52]. To improve signal-to-noise ratio while preserving structural features, image stacks were denoised using the self-supervised Noise2Void (N2V) plugin [53]. Residual motion artifacts were corrected through frame-to-frame registration based on calculated pixel shifts. Resolution enhancement was subsequently performed using the eSRRF algorithm applied to time-series image stacks, with processing parameters optimized through systematic parameter sweeping. Resolution improvement was quantitatively evaluated using rolling Fourier ring correlation (rFRC) analysis (1/7 threshold, block size = 64 pixels) implemented via the PANELJ plugin [39]. rFRC values were used as estimates of effective spatial resolvability following image enhancement.

LC3-positive compartments were quantified using a standardized segmentation workflow. Denoised images were subjected to automated global thresholding using the Triangle method, with thresholds determined from control datasets and subsequently fixed across all experimental conditions. Segmented objects were filtered according to predefined size criteria, while border-touching objects and nonspecific structures were excluded. All processing parameters were applied identically across datasets to ensure unbiased comparison and prevent algorithm-driven inflation of compartment counts.

Autophagy induction and analyses

To complement LC3 imaging with pharmacological perturbation of lysosomal degradation, CQ was administered intraperitoneally (50 mg/kg) in GFP-LC3 mice. Three stress paradigms were investigated: starvation, ethanol exposure, and hepatic ischemia-reperfusion injury, each performed with and without CQ treatment.

Intravital imaging was performed in both the liver and quadriceps muscle. For hepatic imaging, the exposed surface of the left lateral lobe was selected because of its accessibility and stable positioning. Imaging fields were chosen within the hepatic parenchyma while avoiding large vascular structures,

including portal vessels and central veins, thereby enabling consistent sampling of mid-lobular hepatocyte regions and minimized bias toward periportal or pericentral areas across experimental conditions.

Control animals received PBS or CQ 4 h before imaging. For starvation experiments, PBS or CQ was administered 30 min before food withdrawal. Animals were then fasted for either 1 or 2 days with free access to water. A second CQ injection was administered 4 h before imaging.

For ethanol exposures, PBS or CQ was administered 2 h before oral gavage of ethanol (70%, 4 g/kg; Daejung Reagent Chemicals, 64–17-5). Imaging was performed 2 h after ethanol administration to assess ethanol-induced hepatic alteration. For ischemia-reperfusion injury, PBS or CQ was administered 30 min before induction of ischemia by temporary hepatic artery occlusion for 45 min. Following reperfusion for 2 h, intravital imaging was performed to assess stress-induced changes in LC3-positive compartments.

At the end of imaging sessions, liver tissues were collected for immunostaining and western blotting analysis of SQSTM1 to provide independent molecular validation of the imaging findings.

Mitophagy analyses

Following a 1-day starvation in the absence or presence of CQ treatment, mitochondria were labeled via intravenous injection of 0.3 μM MitoTracker Red (MTR; Thermo Fisher Scientific, M7512) 30 min before imaging, enabling simultaneous visualization of GFP-LC3-positive compartments and mitochondrial structures. For association analysis, images were background-subtracted and subjected to automated thresholding to generate binary GFP-LC3 and mitochondrial masks. GFP-LC3 puncta were segmented by particle analysis, and overlap maps were generated using binary masks. The number and percentage of mitochondria-associated GFP-LC3 puncta were quantified and compared across experimental groups. All analysis parameters were applied consistently across datasets in Fiji.

Western blot and immunostaining

Western blotting and immunostaining were performed by an external service provider (OBEN Co., Ltd., Republic of Korea). A rabbit monoclonal anti-SQSTM1/p62 antibody (Abcam, ab109012) was used as the primary antibody for both immunostaining and western blotting analyses. Quantitative analysis was performed according to the provider's standardized protocols.

Ethics statement

All animal experiments were conducted according to the guidelines of the Korean Ministry of Food and Drug Safety under the Laboratory Animal Act of the Republic of Korea. The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Asan Institute for Life Sciences (permit number: 2025–10-290).

Statistical analysis

Statistical analyses were performed using two-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test, as appropriate. Data are presented as mean $\pm$ SEM, and $p < 0.05$ indicated significance.

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Author contributions

CRedit: **Saeed Bohlooli Darian:** Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft; **Jeongmin Oh:** Investigation, Resources, Validation, Visualization, Writing – original draft; **Chan-Gi Pack:** Conceptualization, Investigation, Resources, Validation; **Jun Ki Kim:** Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon a request.

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