Cryo-Correlative Light and Electron Microscopy Pipeline for Nanoscale Mapping of Endoplasmic Reticulum–Mitochondria Contact Sites in Hypoxia-Stressed Human Hepatocytes

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Abstract. Endoplasmic reticulum–mitochondria contact sites (ERMCS) are ultrastructural platforms governing calcium flux, lipid transfer, and mitochondrial dynamics under physiological and pathological conditions. Despite their recognized role in hepatocellular stress responses, precise nanoscale quantification of ERMCS remodeling during hypoxia has remained technically intractable. Here, we present an optimized cryo-correlative light and electron microscopy (cryo-CLEM) workflow integrating fluorescence-guided cryo-focused ion beam milling, dual-axis cryo-electron tomography, and automated segmentation via a retrained nnU-Net convolutional architecture. Applied to HepG2 and primary human hepatocytes exposed to 1% O_2 for 6–48 hours, this pipeline resolved ERMCS geometry with sub-5 nm precision, revealing hypoxia-induced tethering distance compression and a 2.3-fold expansion of contact-site area. Quantitative proteomics of immunopurified ERMCS fractions confirmed concurrent enrichment of VDAC2, MFN2, and IP_3R1. This methodology constitutes a reproducible, broadly transferable framework for organelle-contact-site research in disease-relevant cellular models.

Keywords: endoplasmic reticulum–mitochondria contact sites, cryo-correlative light and electron microscopy, cryo-focused ion beam milling, cryo-electron tomography, organelle ultrastructure, hepatocellular hypoxia, mitochondria-associated membranes, nnU-Net segmentation, calcium signaling microdomains

Introduction

Intracellular organelle communication is now recognized as a central axis of eukaryotic cell physiology, with membrane contact sites (MCS) serving as discrete, proteinaceous nanojunctions that coordinate metabolite exchange, signaling cascades, and organelle

biogenesis without vesicular fusion. Among the repertoire of characterized MCS subtypes, endoplasmic reticulum–mitochondria contact sites (ERMCS) have emerged as particularly critical determinants of cellular homeostasis. Spanning tethering distances of 10–30 nm, ERMCS facilitate the rapid, spatially restricted transfer of Ca^{2+} from IP_3-sensitive ER stores into the mitochondrial matrix via voltage-dependent anion channels (VDAC) and the mitochondrial calcium uniporter (MCU) complex, thereby coupling ER stress responses directly to mitochondrial bioenergetics and apoptotic commitment. Simultaneously, ERMCS constitute the principal loci for the non-vesicular transfer of phosphatidylserine and ceramide, positioning these junctions at the nexus of both lipid anabolism and lipotoxic cell death pathways. The hepatocyte represents an especially instructive cellular model for ERMCS biology. The liver is chronically subjected to oscillating oxygen tensions along the periportal-perivenous sinusoidal gradient, and pathological hypoxia—as encountered in ischemia-reperfusion injury, metabolic-dysfunction-associated steatohepatitis (MASH), and hepatocellular carcinoma progression—is increasingly understood to precipitate profound ultrastructural reorganization of the ER–mitochondrial interface. Hypoxia-inducible factor 1α (HIF-1α) stabilization drives transcriptional reprogramming that affects core ERMCS tethering proteins including Mitofusin-2 (MFN2), PTPIP51, and sigma-1 receptor (SIG-1R), yet the spatiotemporal consequences of this reprogramming at genuine nanoscale resolution have not been systematically resolved. This gap persists largely because conventional fluorescence microscopy lacks the spatial resolution to delineate individual contact-site geometry, while classical room-temperature electron microscopy introduces fixation-induced artifactual membrane collapse that confounds quantitative morphometry. Cryo-electron tomography (cryo-ET), when preceded by targeted cryo-CLEM-guided lamella preparation using cryo-focused ion beam (cryo-FIB) milling, now offers an uncompromising route to visualizing hydrated, near-native cellular ultrastructure at sub-nanometer resolution. Recent methodological advances in automated lamella milling, direct electron detector technology, and deep-learning-assisted organelle segmentation have together elevated cryo-ET from a technically prohibitive specialty to a tractable platform applicable to disease-relevant human cell models. However, no integrated, end-to-end protocol has yet been benchmarked specifically for the quantitative analysis of ERMCS remodeling under metabolic stress conditions in human hepatocyte systems. The present study addresses this methodological lacuna by establishing and rigorously validating a cryo-CLEM pipeline—from live-cell fluorescence targeting through cryo-FIB lamella fabrication, dual-axis cryo-ET data acquisition, and convolutional-network-assisted three-dimensional segmentation—optimized for the quantitative morphometric and proteomic characterization of ERMCS in hypoxia-stressed hepatocytes. The article is structured as follows: Section 2 details all materials, cell culture conditions, and instrumentation parameters; Section 3 describes the stepwise cryo-CLEM workflow and computational segmentation strategy; Section 4 presents quantitative morphometric and proteomic findings; and Section 5 contextualizes these results within current models of hypoxia-

driven hepatocellular pathophysiology and evaluates pipeline transferability to additional MCS subtypes.

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