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Extended-resolution imaging of the interaction of lipid droplets and mitochondria

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ABSTRACT

Physical contacts between organelles play a pivotal role in intracellular trafficking of metabolites. Monitoring organelle interactions in living cells using fluorescence microscopy is a powerful approach to functionally assess these cellular processes. However, detailed target acquisition is typically limited due to light diffraction. Furthermore, subcellular compartments such as lipid droplets and mitochondria are highly dynamic and show significant subcellular movement. Thus, high-speed acquisition of these organelles with extended-resolution is appreciated. Here, we present an imaging informatics pipeline enabling spatial and time-resolved analysis of the dynamics and interactions of fluorescently labeled lipid droplets and mitochondria in a fibroblast cell line. The imaging concept is based on multispectral confocal laser scanning microscopy and includes high-speed resonant scanning for fast spatial acquisition of organelles. Extended-resolution is achieved by the recording of images at minimized pinhole size and by post-processing of generated data using a computational image restoration method. Computation of inter-organelle contacts is performed on basis of segmented spatial image data. We show limitations of the image restoration and segmentation part of the imaging informatics pipeline. Since both image processing methods are implemented in other related methodologies, our findings will help to identify artifacts and the false-interpretation of obtained morphometric data. As a proof-of-principle, we studied how lipid load and overexpression of PLIN5, considered to be involved in the tethering of LDs and mitochondria, affects organelle association.

1. Introduction

Compartmentalization of specialized biochemical reactions and metabolic pathways in membrane-confined organelles is a key feature of all living cells and the intracellular transport of metabolites is essential to maintain cellular homeostasis and vital functions. Beside vesicular transport and diffusion through the cytosol, molecules can be transported from organelle to organelle via physical interaction of membrane structures. These specialized membrane contact sites (MCSs) are characterized by the accumulation of specific proteins and lipids to promote the exchange of molecules and to stabilize the association of contact sites, such as those between the endoplasmic reticulum and other organelles [1–4]. Lipid droplets (LDs) are the main lipid storage depots for neutral lipids ('fat'), mainly triacylglycerols (TAGs) and cholesteryl ester, in all eukaryotic cells. Synthesis, storage, and mobilization of neutral lipids are critical cellular processes to maintain cellular lipid and energy homeostasis and their breakdown is mediated by LD-associated lipases (lipolysis) or through lipophagy [5]. Physical contact between LDs and mitochondria appear to have an important role for adenosine triphosphate (ATP) production through mitochondrial β-oxidation. This is reflected by a particular intimate physical association of these organelles in tissues with high energy demand such as skeletal and cardiac muscle [6,7]. Furthermore, the direct flux of fatty acids from LDs to mitochondria has been observed under starvation conditions [8] as well as increased fatty acid flux from LDs induces mitochondrial biogenesis [9]. These findings suggest that physical interactions between LDs and mitochondria play an important role in channeling fatty acids to oxidation and presumably to prevent high concentrations of putative toxic fatty acids. Thus, deeper insights into the function and maintenance of LD-mitochondria contacts may help to uncover mechanisms controlling energy homeostasis.

Fluorescence microscopy as well as organelle markers such as organic reference dyes and green-fluorescent-protein (GFP) fusion proteins and variants thereof are key tools to identify and study organelle

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contacts in living cells. However, the resolution of optical microscopy is limited to ~200 nm laterally and ~500 nm axially due to the diffraction of light [10,11]. This physical barrier frequently impairs detailed target acquisition. Furthermore, organelles such as LDs and mitochondria show oscillating and directional movements as well as can undergo significant morphological alternations. In addition, the space between compartments can be limited due to the complexity of organelle networks as generated by mitochondria and the endoplasmic reticulum. Thus, random contacts between LDs and mitochondria cannot be excluded. Consequently, time-resolved high-speed imaging with extended-resolution is appreciated to gain more detailed insights into the dynamics and interactions of these subcellular structures.

Recent benchmark technologies such as structured illumination microscopy (SIM) and light-sheet microscopy have provided new possibilities to address such challenging issues. Sophisticated methods have been developed allowing the time-resolved and spatial analysis of organelle contacts in multi-labeled samples. In this respect, super-resolution SIM has allowed the spatial tracking of physical lysosome-mitochondria interactions in human epithelial cells over extended periods of time [12]. Furthermore, light-sheet microscopy has been applied to study the spatio-temporal organization of the entire organelle interactome in a fibroblast cell line. The latter approach includes critical image processing steps such as deconvolution to improve the quality of acquired image data as well as image segmentation as the basis for morphometric analysis of organelles including quantification of organelle contacts [13].

Confocal laser scanning microscopy (CLSM) has been a key tool in life sciences for decades. This imaging technique has been traditionally considered to be slow due to the limited speed of standard scan systems. However, the development of high-speed resonant scanners has allowed confocal imaging at video rates [14]. Interestingly, confocal microscopy has also been recognized as an instrument for “superresolution” microscopy in live samples. For example, extended-resolution beyond the diffraction limit in the ~140–170 nm range (x/y) has been achieved by different adaptions of the confocal setup [15,16]. Remarkably, it has been demonstrated that the CLSM has the potential to resolve structures even below the 100 nm range laterally, however, in a cell-free system only. In this early study, improvement of the resolution was achieved in two sequential steps: acquisition of 3D image data using a small pinhole size and second, post-processing of acquired spatial image data using iterative maximum-likelihood estimation (IMLE) deconvolution [17]. In CLSM, the spatial resolution is a product of the point-spread-function (PSF) of the microscope system and the size of the pinhole. The PSF describes the three-dimensional diffraction pattern of light emitted from a point source in the specimen. Due to practical reasons, the pinhole size is typically set to the size of the central feature of the diffraction pattern (Airy disk; Airy unit (AU) 1). In theory, upon closing the pinhole to diameters of AU 0.2 the resolution of a CLSM is increased to ~ 170 nm laterally and ~350 nm axially [18]. However, minimizing the pinhole size has the drawback that the number of detected photons is significantly decreased. This effect is enhanced when scanning is performed at increased scan speed due to shorter integration times per pixel. Consequently, both a minimized pinhole size and resonant scanning will decrease the signal-to-noise-ratio (SNR) of acquired images which may interfere with the resolution and detection of subcellular structures in biological samples. In this respect, newer detector systems such as hybrid photon detectors (HyDs) combining photomultiplier and semi-conduction diode technology, show low detector noise and relatively high quantum efficiency. Thus, such photon detectors are potentially useful for imaging at sub-Airy pinhole conditions. Confocal imaging at small pinhole sizes (> AU 0.5) and/or image deconvolution have been successfully applied to improve the resolution of spatial confocal images [19]. However, this approach has not been applied using a minimized pinhole size potentially providing maximum resolution, and has been considered to be incompatible with high-speed and time-resolved imaging so far.

Image restoration using deconvolution is a technique to improve the contrast as well as the resolution of spatial images generated by different microscopic techniques. Popular mathematical concepts such as IMLE are applied for this purpose. In physical terms, a microscope convolutes the object by the intrinsic PSF of the system to generate the image. IMLE partially reverses the convolution made by the optical system with a theoretical or measured PSF, thereby increasing contrast and resolution. This process is typically accompanied by a significant reduction of image noise. IMLE deconvolution improves the lateral and particularly the axial resolution of 3D image objects. However, this deconvolution method requires several critical input parameters such as estimation of the SNR value of the acquired spatial images as well as the number of iterations [20–22]. The quantification of the deconvolved data can be performed at the pixel level or on bases of segmented image objects. In the latter case, the structures of interest are extracted from the spatial images. For this purpose, a large number of different segmentation algorithms is available. Global thresholding techniques such as the Otsu method are frequently applied to separate image objects from the background. This method assumes that the background and the object class follow a bi-modal histogram, whereas segmentation is performed by maximizing the between-class variance [23]. The application of IMLE deconvolution typically results in high-contrast images, which is advantageous for the segmentation of image objects. However, the effects of important deconvolution parameters such as the SNR estimate and the number of iterations on morphometric analyses including the quantification of organelle contacts have been barely studied.

In this work, we present an expandable image informatics pipeline enabling the generation of time-resolved high-resolution live cell data of LDs and mitochondria in COS-7 cells. We show that deconvolution parameters such as the SNR estimate and the number of iterations significantly affect absolute values obtained from quantitative analyses, which may lead to a false-interpretation of generated morphometric data. In this context, factors leading to an overestimation of putative organelle contacts are discussed. Considering the outlined drawbacks of these widely applied image processing routines, the presented methodology provides an excellent descriptive and semi-quantitative tool for the analysis of the spatiotemporal organization and interaction of organelles in multi-labeled samples. As a proof-of-principle, we applied the approach to study LD-mitochondria association in fatty acid treated cells as well as in cells overexpressing PLIN5, considered to be involved in the tethering of LDs and mitochondria.

2. Materials and methods

2.1. Cell culture

African green monkey kidney fibroblast (COS-7) cells were obtained from the American Type Culture Collection. The cells were seeded on 20 × 20 mm coverslips (Menzel #1; 0.17 mm thickness), placed in Petri dishes (Mattek, Inc.) and cultivated for 24 h at 37 °C, 6% CO_2 and 95% humidity in 2 ml of standard growth medium consisting of Dulbecco's Eagle modified medium supplemented with 2 mM L-Glutamine and 10% v/v fetal bovine serum (Sigma, Inc.). Coverslips were mounted on standard microscope slides prior to imaging. Acquisition of fluorescently labeled cells was performed within ~ 15 min including manipulation time.

2.2. Overexpression of PLIN5

The expression vector pEYFP-C1 (Clontech, Inc.) containing the coding sequence of murine PLIN5 was used for overexpression of EYFP-PLIN5 encoding murine Plin5 cDNA in COS-7 cells. For this purpose, the cells were seeded in specialized dishes with cover slip bottom for high-resolution imaging (Ibidi, Inc.) transfected with Metafectene® (Biontex GmbH, Munich, Germany) according to the manufacturer's instructions