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Research paper

Strategies for imaging mitophagy in high-resolution and high-throughput

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ABSTRACT

The selective autophagic removal of mitochondria called mitophagy is an essential physiological signaling for clearing damaged mitochondria and thus maintains the functional integrity of mitochondria and cells. Defective mitophagy is implicated in several diseases, placing mitophagy as a target for drug development. The identification of key regulators of mitophagy as well as chemical modulators of mitophagy requires sensitive and reliable quantitative approaches. Since mitophagy is a rapidly progressing event and sub-microscopic in nature, live cell image-based detection tools with high spatial and temporal resolution is preferred over end-stage assays. We describe two approaches for measuring mitophagy in mammalian cells using stable cells expressing EGFP-LC3 – Mito-DsRed to mark early phase of mitophagy and Mitochondria-EGFP – LAMP1-RFP stable cells for late events of mitophagy. Both the assays showed good spatial and temporal resolution in wide-field, confocal and super-resolution microscopy with high-throughput adaptable capability. A limited compound screening allowed us to identify a few new mitophagy inducers. Compared to the current mitophagy tools, mito-Keima or mito-QC, the assay described here determines the direct delivery of mitochondrial components to the lysosome in real time mode with accurate quantification if monoclonal cells expressing a homogenous level of both probes are established. Since the assay described here employs real-time imaging approach in a high-throughput mode, the platform can be used both for siRNA screening or compound screening to identify key regulators of mitophagy at decisive stages.

1. Introduction

Mitophagy, the selective elimination of mitochondria is an important cellular process required for maintaining a pool of ATP generating functional mitochondria. Since mitophagy is selective and a spatially initiated event, live cell imaging methods have been regularly used to track and visualize these dynamic events. The maintenance of functional mitochondria is a highly regulated process that involves constant fission and fusion events; followed by marking with various adaptors that aid in the selective degradation of damaged mitochondria involving lysosomes or proteasome or by both (Ashrafi and Schwarz, 2013; Yang and Yang, 2011; Youle and Narendra, 2011). This process also plays a critical role in the removal of mitochondria from red blood cells at the time of maturation, and also in T cell differentiation (Gottlieb and Carreira, 2010). Mitophagy received increased attention because of its role in neurodegenerative diseases, hypoxia-mediated cell survival or cell death in cancer and aging-related diseases (Band et al., 2009; Ding and Yin, 2012; Gottlieb and Carreira, 2010). Despite all these, it is difficult to conclude loss or increase in mitophagy contributes to the progression of such diseases. In various types of cancers such as breast, brain, ovarian and prostate, loss of autophagy signaling due to allelic loss of the essential autophagy gene Beclin1 (BECN1) is common (Devenish, 2007; Gong et al., 2013; Zhou et al., 2012b). Studies linking defective autophagy machinery with increased spontaneous

Abbreviations: MAP1LC3, microtubule-associated protein 1 light chain 3; LAMP1, lysosomes associated membrane protein 1; ROS, reactive oxygen species; EGCG, epigallocatechin gallate; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; VDAC1, voltage-dependent anion-selective channel protein 1; PINK1, PTEN-induced putative kinase 1; OPA1, optical atrophy 1; DRP1, dynamin-related protein 1

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tumor formation in mouse models suggest that defects in autophagy promote tumorigenesis (Gozuacik and Kimchi, 2004). Deficiency of autophagy and subsequent loss of mitophagic flux to remove damaged mitochondria with higher ROS may lead to increased ROS levels, (Lemasters, 2005) with an increase in tumorigenicity (Novak, 2012).

The evidence from cancer and aging place mitophagy as an important target for drug intervention (Taylor and Goldman, 2011). Studies using multiple modes of mitophagy induction has helped to identify the sequence of events and the key regulators such as parkin, PINK1, VDAC1, p62 in mediating the process of mitophagy (Klionsky et al., 2016; Lazarou et al., 2015; Vives-Bauza et al., 2010). However, it is increasingly presumed that additional players do exist that can modulate mitophagy in mammalian cells. Owing to its clinical and biological importance, several assays were developed for the detection of mitophagy (Katayama et al., 2011b; McWilliams et al., 2016; Narendra et al., 2010; Sun et al., 2015; Vives-Bauza et al., 2010; Zhang and Ney, 2010). Since mitophagy is a highly dynamic temporally regulated process, live cell approaches with increased throughput are critical in addressing the complex mechanism of mitochondrial removal as well as to identify small molecule modulators of mitophagy.

We describe an image-based approach using the most commonly used probes to visualize autophagosome formation with the inclusion of mitochondria and its fusion with lysosomes as a means of mitophagy detection. Strikingly, we noticed that mitophagy is associated with a decrease in mitochondrial mass with a concomitant increase in lysosomal mass. Hence, a quantitative measure of dual organelle intensity in image-based approach can be utilized as a parameter for quantification purposes; provided, cells with homogenous and stable expression of both fluorescent proteins are ensured. Mitochondrial engulfment by EGFP LC3 has been extensively employed as a tool for mitophagy. However, autophagy-independent aggregation of LC3 and LC3 independent mitophagic response cannot be determined by this approach. The mitochondria-targeted pH-dependent fluorescence of coral-derived protein Keima is the best approach to detect mitophagy in *in vivo* and *in vitro* conditions (Katayama et al., 2011b; Sun et al., 2015). The excitation of mito-Keima shifts from 440 nm to 586 nm while delivered to the acidic lysosomes rendering the mito-Keima a ratiometric imaging probe for mitophagy. Since the spectral properties are highly dependent on lysosomal pH, this approach is not fixation compatible. In addition, partial overlap of excitation spectrum at red and green results in orange color at lysosome making it difficult to interpret the images (Williams et al., 2017). A recently described approach, mito-QC employs tandem mCherry −GFP tag fused to the mitochondrial targeting sequence of FIS. The differential acid sensitivity of GFP and mCherry makes this dual color probe as an innovative tool for mitophagy with red and green color in steady state condition changing to red dominating color upon delivery to lysosomes because of increased acid sensitivity GFP over mCherry (McWilliams et al., 2016). mito-QC is fixation compatible and has been used to determine mitophagy in *in vivo* and *in vitro* models (McWilliams et al., 2016; Williams et al., 2017). Similar to mito-QC, the methods described here are fixation compatible and utilizes the most widely employed probes for targeting lysosome and mitochondria. In addition, it is possible to track the finer details of rapidly progressing mitophagic process in live-cell super-resolution imaging approach with the help of structured illumination microscopy technique.

2. Materials and methods

2.1. Cell lines and maintenance

The ovarian cancer cell line OVCAR-8 was procured from National Cancer Institute, USA. The cells were maintained in RPMI 1640 (Invitrogen, #72400-047) containing 10% Fetal Bovine Serum (FBS) (PAN-Biotech GmbH, #3302) in a humidified CO_2 (5%) chamber at 37 °C.

2.2. Plasmids and generation of stable cells

pEGFP-LC3 vector was described previously (Kabeya et al., 2000). LAMP1-RFP plasmid (Addgene plasmid #1817) was described earlier and procured from Addgene (Sherer et al., 2003). Expression vector for DsRed-Mito (#6975-1) was purchased from Clontech Laboratories. Expression vector for Mito-EGFP (#558718) was purchased from BD Pharmingen™. The cells were transfected with Lipofectamine LTX (Invitrogen, #15338-100) as per the manufacturer's protocol followed by selection in 500 μg/ml of Geneticin® (Invitrogen, #11811-031) for three to four weeks. Single cell clones stably expressing the first gene was further transfected with the second gene followed by sorting on BD FACSAriaII cell sorter (BD Biosciences) to enrich cells expressing both fluorescent proteins. The single cell clones stably expressing both the transgene were further expanded and validated.

2.3. Chemicals and reagents

Drugs used for high-throughput screening are listed in the Supplementary Table 1 with their effective concentration and known mechanism(s) of action. The chemical compounds were obtained from Santa Cruz Biotechnology, Sigma-Aldrich or Calbiochem. Bafilomycin A1 (#B1793) was obtained from Sigma-Aldrich and used at a final concentration of 20 nM. The LysoTracker® Deep Red reagent was procured from Molecular probes™ and used as per standard protocol provided by the manufacturer.

2.4. Fluorescence time-lapse live cell imaging

Cells grown on chambered eight well cover glass, Lab-Tek™ (Nunc, #155411), were exposed to drug-containing medium, RPMI 1640, 5% FBS. For live-imaging, cells were incubated in a live cell chamber (Tokai Hit) with optimum CO_2, temperature, and humidity. Imaging was carried out using 100X Plan ApoVc oil 1.4 NA objectives on an inverted fluorescence microscope (Nikon Eclipse, Ti) (Nikon Instruments Inc.). Images were captured using RETIGA Exi camera (QImaging) at regular intervals for the indicated time periods. To curtail photo-bleaching, the intensity of light was reduced to less than 25% by intensity iris control.

2.5. High-throughput imaging with automated microscopy

An inverted microscope with motorized XY stage and infrared laser based focus compensation system to maintain stable focus for a longer time was employed for high-throughput imaging applications (Nikon Eclipse Ti-PFS). The microscope was supported by 96 well plate live cell incubation chamber to maintain temperature and CO_2 throughout imaging periods (Okolab). The four different XY positions were identified for each well and memorized. The stably expressing cells were seeded on 96 well glass bottom plates (Whatman, #7706-2370) and allowed to grow for 24 h. Then the wells were added with different drugs and placed on stage incubator. Images were acquired with Cool SnapHQ camera (Photometrics) using NIS Element AR 3.0 software (Nikon Instruments Inc.) at 15 min interval for 24 h or 48 h. Filter combinations used for EGFP, DsRed, and Hoechst-33342, were described previously (Joseph et al., 2011). All high-throughput imaging was carried out using 40X plan Apo 0.95 objective. Analysis of a sequence of images was performed using NIS element software or open source tools, Squassh3C and SquasshAnalyst as described previously (Rizk et al., 2015).

2.6. High-throughput imaging by BD Pathway 435™ Bioimager

For imaging in 96 wells plate format, stably expressing cells were seeded in 96 well glass bottom plates (BD Biosciences, #353219) and after 24 h, medium was removed and drug containing phenol-red free