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Live cell imaging and analysis of lipid droplets biogenesis in hepatatis C virus infected cells

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ABSTRACT

Lipid droplets (LDs) are regulated neutral lipid storage organelles having a central role in numerous cellular processes as well as in various pathologies such as metabolic disorders, immune responses and during pathogen infection. Due to the growing significance of LDs, extensive efforts are made to study the mechanism and the dynamics of their formation and life history and how are these diverted or modified by pathogens. Real-time visualization of lipid droplet biogenesis can assist in clarifying these and other important issues and may have implications towards understanding the pathogenesis of the associated diseases. Typically, LDs are post-experimentally stained using lipophilic dyes and are visualized under a microscope. Alternatively, overexpression of LD-associated proteins or immunofluorescence analyses are used to identify and follow LDs. These experimental approaches only examine a single end point of the experiment and cannot answer questions regarding LD dynamics. Here, we describe a simple and novel experimental setting that allows real-time fluorescence staining and detection of LDs in cultured living as well as infected cells. This method is quick and simple and is not restricted to a specific dye or cell line. Using this system, the biogenesis of LDs and their growth is demonstrated in cells infected with hepatitis C virus (HCV), confirming the strength of this method and the wide range of its applications.

1. Introduction

LDs are conserved neutral lipid storage depots that provide energy during times of nutrient scarcity and supply building materials for membrane synthesis and restoration (for reviews see [1–3]). LDs form in all types of eukaryotic and prokaryotic cells under conditions of fatty acids excess. In addition to their obvious association with metabolic disorders, LDs were found to have a role in the life cycle of several major pathogens including viruses [4–7], intracellular bacteria [8–10] and parasites [11]. LDs provide lipids to sustain the reproduction of these pathogens and can serve as a platform for viral assembly [4]. As opposed to most organelles in which a lipid bilayer surrounds an aqueous lumen, LDs are composed of a hydrophobic lipid core surrounded by a monolayer of phospholipid membrane [12]. Proteins associate with LDs via a variety of hydrophobic association motifs, amphipathic sequences, lipid modifications or via protein-protein interactions [13]. Most of the LD-binding proteins are associated with regulation of lipid metabolism [14–17]. Despite the substantial progress in the understanding of these steps in LD formation, many questions still remain open. What determines the site of lipid accumulation? Is LD formation a protein-mediated process? At least some LDs seem to remain connected to or tightly associated with the ER (LD-ER junctions). These bridges seem to be essential for LD growth, however little is known how these bridges are formed, regulated and for how long are they preserved [18–21]. Here we present a straightforward method to label LDs in living intact cells starting at an early stage of their formation and allowing to follow their life history. HCV, a positive strand RNA virus, is a major viral pathogen. Infection with HCV can cause liver hepatitis that can lead to a chronic disease resulting in liver cirrhosis and hepatocellular

Abbreviations: LDs, lipid droplets; ER, endoplasmic reticulum; HCV, hepatitis C virus; GFP, green fluorescent protein; NS5A, non-structural protein 5A; OA, oleic acid.

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carcinoma [22]. HCV replicates in replication complexes composed of host and viral proteins that reside on ER-derived viral induced membrane alterations. The production of infectious virus particles depends on their export from the cell coupled with lipoprotein complexes [23,24]. This explains the need for a large mass of lipids. Several viral proteins are localized to LDs, one of which is the non-structural protein 5A (NS5A) [25]. Viral replication and assembly are thought to occur in the vicinity of LDs [4,25–27].

Here, we propose a relatively simple yet versatile method for real-time live cell analysis of LD formation. In this approach, a 35 mm tissue culture plate with a glass bottom is mounted to a microscope stage under a constant temperature of 37 °C. The plate is connected via silicon tubing to a 30–50 ml reservoir containing low concentrations of the lipophilic dye BODIPY® 558/568 C_{12}. Using a peristaltic pump, the circulating media containing the lipophilic dye continuously samples the living cells in the chamber and allows continuous labeling of newly forming LDs in real time. Thus, prolonged time-lapse imaging experiments analyzing the formation, growth and movement of LDs can be carried out. This system is versatile as it can be used with any type or combination of any lipophilic dye suitable for staining of living cells as well as LD-relevant fluorescent tagged protein of choice. Here we preferred the use of the lipophilic dye BODIPY® 558/568 C_{12} which is essentially a fatty acyl that is incorporated into triglycerides and phospholipids. Compared to dyes such as Nile red or BODIPY® 493/503 which partition into the neutral lipid cores of fat droplets, the BODIPY® 558/568 C_{12} may be more specific. Although one drawback might be that LD precursors and small droplets may be below the resolution limit of light microscopy [28], with this approach it is possible to visualize LDs from an early stage using conventional equipment found in most laboratories. Co-labeling of LDs with an overexpressed LD-associated protein and a lipophilic dye could be carried out as well. Moreover, existing LDs could be differentiated from newly forming ones using a combination of two lipophilic dyes with different spectra. In this case, the existing LDs are primarily labeled with one dye, while the second dye will be constantly added using the pump as described. Using this technique, the kinetics of LDs formation were quantified with high spatial and temporal resolution and the details of LDs biogenesis were visualized in cells infected with a GFP-tagged HCV genome [29]. This demonstrates the strength and flexibility of this method for studying LD formation and dynamics.

2. Materials and methods

2.1. Reagents and solutions

BODIPY® 558/568 C_{12}, (4,4-Difluoro-5-(2-Thienyl)-4-Bora-3a,4 a-Diaza-*s*-Indacene3-Dodecanoic Acid) and Nile Red (9-diethyla mino-5H-benzo[alpha]phenoxazine-5one) were purchased from Invitrogen (Carlsbad, CA). Oleic acid was administered complexed to fatty acid free bovine serum albumin (BSA) (both purchased form Sigma-Aldrich, St. Louis, MO). Oleic acid-BSA complexes were prepared as described [30]. The plasmid pEGFP-TBC1D20 R105A is described elsewhere [31].

2.2. Cell culture and transfections

Huh7 and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal calf serum (Sigma), 5% (v/v) glutamine and 1% (v/v) of penicillin and streptomycin (Biological Industries). Huh7.5 cells were supplemented with 1% (v/v) non-essential amino acids (Biological Industries). For transfection, sub-confluent COS-7 and Huh-7 or Huh7.5 cells were plated in glass bottom 35 mm plates (Greiner Bio-one, Monroe, NC) and transfected with Lipofectamine 2000 reagent (Invitrogen) or Fugene 6 (Roche, Indianapolis, IN). Confocal laser-scanning microscopy experiments were carried out 24 h after transfection.

2.3. In vitro transcription and electroporation of HCV RNA

In vitro transcripts of the JC1/GFP (kindly provided by R. Bartenschlager, University of Heidelberg, Heidelberg, Germany) were generated by linearizing the plasmid using MluI [29]. The linearized DNA template, was purified using phenol-chloroform extraction and ethanol precipitation and resuspended at a final concentration of 1 μg/μl. The template was then transcribed with T7 RNA polymerase using a MEGAscript T7 kit (Ambion, Austin, TX) according to the manufacturer's instructions. After transcription, synthesized RNA was treated with DNase I. The integrity of the RNA was analyzed by nondenaturing agarose gel electrophoresis, and the yield was determined spectrophotometrically. RNA was stored at −70 °C until electroporation. For the electroporation, Huh7.5 cells were grown to 60–80% confluence, trypsinized, and washed twice in cold RNase-free phosphate-buffered saline (PBS; BioWhittaker Inc., Walkersville, MD). Cells were resuspended in cold PBS at a concentration of 1.5×10^7 cells/ml; 0.4 ml of the cell suspension was mixed with 5 μg of in vitro-transcribed JC1/GFP RNA. The mixture was dispensed into a 2-mm-gapwidth cuvette (BTX, San Diego, CA), and electroporation was performed using a BTX model 830 electroporator (820 V, five 99 μs pulses given at 220 ms intervals). Cells were left to recover for 15 min at room temperature and then mixed with 10 ml of pre-warmed (37 °C) growth medium. Cells were then seeded in 35 mm glass bottom plates and examined at 72 h post-electroporation.

2.4. Live cell microscopy

Cells were imaged in Hybernate-A, a CO_2-independent cell culture media (Thermo-Scientific, Waltham, MA, USA), but supplemented with 10% (v/v) fetal calf serum (Sigma-Aldrich, St. Louis, MO, USA) and 1% (v/v) of penicillin and streptomycin (Biological Industries, Bet-Haemek, Israel). Images were acquired using an either the Zeiss LSM Pascal or the LSM800 confocal laser-scanning microscope (Carl Zeiss MicroImaging, Jena, Germany) hooked to an inverted microscope containing an Aquastop-sleeve (Carl Zeiss MicroImaging, Jena, Germany) to prevent damage from spillage or leakage. Fluorescence emissions resulting from 488 nm excitation for GFP and 543 nm excitation for BODIPY® and Nile-red were detected using the manufacturer supplied filter sets. The confocal and time-lapse images were captured using a Plan-Apochromat X40/NA1.4 objective (Carl Zeiss MicroImaging). The temperature on the microscope stage was held stable during time-lapse sessions using an electronic temperature-controlled airstream incubator. Images and movies were generated and analyzed using the Zeiss LSM Zen software and ImageJ software (W. Rasband, National Institutes of Health, Bethesda, MD). Long time-lapse experiments were carried out using the autofocusing function integrated into *the advanced time series macro set* (Carl Zeiss MicroImaging). For presentation purposes, confocal images were exported in TIFF and their contrast and brightness optimized in Photoshop CS5 (Adobe Systems, Mountain View, CA, USA). Cells were used for real-time data collection for a maximum of 3 h.

2.5. Imaging of lipid droplet biogenesis

A glass-bottom culture dish is placed on the microscope stage which was pre-equilibrated to 37 °C using a temperature-controlled airstream incubator. To accommodate the silicone tubing for the in and out-flow, two holes were drilled into the lid of