Methods to Analyze Lipid Bodies by Microscopy

Abstract

Lipid Droplets (LDs) have recently been recognized for their relevance in numerous biomedical processes. While formerly LDs were regarded as mere lipid stores, over the recent two decades it has become clear that LDs are actually a very heterogeneous group of organelles with highly variable dynamics, size, number, protein content and lipid composition playing important roles in metabolism, immunology and growth control. We have recently revisited the existing methods and provide here some refined protocols for microscopy work with LDs, allowing automatized quantification of number and area of LDs per cell, thus enabling the use of LDs as quantitative markers for research and drug screening. Detailed protocols for some of the most popular and efficient contrasting methods are provided.

Introduction

Lipid Droplets (LDs), also known as lipid bodies, oil bodies or adiposomes- are dynamic, multifunctional organelles implicated in cellular processes relevant to diverse areas of biomedical research. LDs dynamics, size, number, protein content and lipid composition can vary widely between cell types, or within the same cell in response to different stimuli or metabolic states viral, microbial and protozoan pathogens -and some of their components- as well as immunogenic and metabolic messengers can induce the accumulation of LDs in cells [1,2,3,4,5,6, 16]. Here we review a number of contrasting strategies to enhance LD-specific contrast for subsequent semi-automatically quantification of cellular LD responses.

Contrasting Strategies

LDs are mainly composed of triacylglycerides and cholesteryl esters surrounded by a phospholipid monolayer and certain proteins specifically associated with it [3]. Physical, chemical and immunological properties have been exploited to distinguish LDs from their surroundings. The high refractive index difference of neutral lipids compared to cytoplasm, RI~1.45 to RI 1.35, respectively [7], can be visualized using phase contrast optics (compare fig 2). Light scattering at LD-cytoplasm boundaries can be visualized using darkfield optics. Specific vibrational states of neutral lipids can be detected by Raman microscopy [8].
Osmium tertraoxide binds phospholipid head regions and has been used for lipid droplets contrasting in both electron and light microscopy [17]. Osmium precipitates generate a broad absorption contrast in brightfield images. Because of the high atomic number of osmium, it also is a suitable contrasting agent for nano-computed tomography (nanoCT) [9] and electron microscopy [10], allowing for correlative microscopy analyses. Oil red O, (1-(2,5-dimethyl-4-(2,5-dimethylphenyl) phenyldiazenyl) azonaphthalen-2-ol) is a fat-soluble diazo dye of the group of Sudan dyes used for staining of neutral triglycerides and lipids, it is used as a contrasting agent for both, brightfield and fluorescence microscopy [3]. A number of proteins are specifically associated with the coat of LDs, the perilipin protein family is best characterized immunohistochemically. Perilipin 1 (PLIN1) is the major perilipin in adipocytes but in the majority of other cells studied, the main parilipin present is perilipin 2 (PLIN2/ADRP) [18]. A commercially available antibody against perilipin 2 (PLIN2) is often used target fluorescent reporters to LDs [11].

Protocols

General Remarks
Because neutral lipids are soluble in organic solvents like Methanol, Ethanol or Isopronol and are sensitive to detergents, all used reagents and surfaces getting in contact with either sample or reagents have to be kept free of organic solvents and detergents. At 37 degree Celsius, lipid bodies are in a so called smectic liquid crystalline state, i.e. they are partially liquid and crystalline. LDs have melting points between 20-53 degrees C [12], reflecting the heterogeneity of LD constituents. Necessarily, LDs will transition to a more crystalline state when processed at room temperature. Because the transition are assumed to be fully reversible, it may not be critical to store or process samples at lower temperatures, however samples have to be warmed up to room temperature before imaging. Because organic solvents have to be critically avoided, aldehyde based fixation
protocols are to be used which prevent extraction of neutral lipids from LDs. Aldehydes fix proteins by crosslinking, thereby desactivating proteases and preserving the overall structure of cells and tissues; they do not fix lipids though. Because of the refractive index of LDs, mounting the samples in aqueous solutions will provide a good phase or darkfield contrast, while mounting samples in glycerol based media will mostly abolish phase or scattering contrast.

**Cell Sample Preparation**
Sample preparation is the first step for all following staining protocols.
1. Prepare no.1.5 (170+/−20=µm) coverglasses with about 105 cells per cm²; either let immortalized cells grow to 2/3 confluency or use a cytocentrifuge and spin an equivalent amount of cells onto the coverglass. Spin non-adherent cells onto cover glasses or glass slides at 18–23 g for 5 min.
2. Blot off excess fluid using a suitable filter paper. Do not let the sample dry out completely.
3. Submerse the sample in freshly prepared 3.7% formaldehyde solution for 10 min at room temperature.
4. Rinse sample carrier in deionized water. Blot off excess fluid but do not let the sample dry out completely.
5. Immediately proceed with the staining protocol.

**Required Materials and Stock Solutions**
1. 3.7% (para-)formaldehyde OH(CH₂O)nH(n=8-100) solution. Read the material data safety sheet before handling (para-)formaldehyde. Dilute 3.7% w/v paraformaldehyde in modified Hanks-buffered salt solution without calcium chloride and magnesium chloride (Ca²⁺/Mg²⁺-free HBSS) Check buffer pH 7.4 before use. Use commercially available liquid aliquots of (para)formaldehyde if available, else handle paraformaldehyde powder with extreme care under a suitable fume hood. Prepare fresh (para-)formaldehyde solution immediately before use. (Para)formaldehyde is unstable. Check fixation results using a brightfield microscope at 1000x magnification. Adapt fixation times.
2. Glass slides and #1.5 (0.17+/−0.02 mm thickness) coverglasses for microscopy.

**Osmium Staining**
Osmium tetraoxide is volatile and toxic. It has to be handled with the appropriate care, e.g under a fume hood. Critically avoid skin contact and inhaling of osmium vapors. Please consult the material data safety sheet before use.
1. Prepare fixed cell samples as described above in ‘Cell sample preparation’.
2. Place 0.1 ml of each 50 mM cacodylate buffer and of 1.5% osmium tetroxide solution into a single drop onto a kinkfilm. Mix by slowly aspirating the drop with a micropipette. For cells on glass slides, carefully place the drop over the
sample area. A hydrophobic marker pen can be used to facilitate this.
3. Place the coverglass, sample facing the osmium solution, onto the drop and incubate for 30 min. Carefully handle the osmium solution. Samples on glass slides: carefully place the drop over the sample area. A hydrophobic marker pen can be used to facilitate this.
4. Rinse sample carrier in deionized water. Blot off excess fluid but do not let the sample dry out completely.
5. Place 0.2 ml of thiocarbohydrazide solution in a separate drop on a kinkfilm and place the coverglass sample facing the thiocarbohydrazide solution for 5 min at room temperature. Samples on glass slides: carefully place the drop over the sample area. A hydrophobic marker pen can be used to facilitate this.
6. Rinse twice with deionized water.
7. Re-incubate with osmium tetraoxide as above for 3 min.
8. Rinse twice with deionized water.
9. Let the slides dry. Blot off excess fluid. Do not let the sample dry out completely.
10. Mount with aqueous or glycerol based mounting medium. Use an appropriate spacer to avoid damaging the sample.
11. Dispose off the remaining solutions and used materials into suitable waste containers.

**Required Materials and Stock Solutions**

1. 0.2 M cacodylate stock buffer (4x). Dissolve 4.28 g of sodium cacodylate in 90 ml of distilled water. Add 5.4 ml 0.2 M HCl in order to obtain pH 7.4 after dilution to 1x; make up to 100 ml with distilled water. Dilute to 50 mM (1x) cacodylate buffer on the day of use. Check for pH 7.4.

2. 1.5% osmium tetraoxide (OsO₄) solution. Read the material data safety sheet before handling osmium tetraoxide. Preferentially purchase osmium tetraoxide solution ready made. Keep osmium teraoxide solution on ice to reduce evaporation. To prepare stock solution from powder, carefully dissolve 1.5 g of osmium tetraoxide in 100 ml of 50 mM sodium cacodylate buffer pH 7.4. Store 2ml aliquots in air tight screw cap glass tubes at 4–8°C for up to 3 month. Protect from light. Open a new aliquot on the day of use.

3. 1% Thiocarbohydrazide ((NH₂NH)₂CS) solution. Read the material data safety sheet before handling thiocarbohydrazide. Carefully dissolve 50 mg of thiocarbohydrazide in 5 mL of distilled water. Heat if required to speed up the dissolving process. Cool to room temperature before use. Prepare freshly on the day of use.

4. Aqueous or glycerol based mounting medium.

5. For aliquots use 10 ml screw cap glass vessels.
**ADRP Staining**
Perilipin2/ADRP is a protein ubiquitously and specifically associated with cytoplasmic lipid bodies in various types of cells.
1. Prepare fixed cell samples as described above in ‘Cell sample preparation’.
2. Permeabilize the cells by placing the sample carrier into 0.1% Triton X-100 in Ca$^{2+}$/Mg$^{2+}$-free HBSS for 10 min at room temp. Note that cells have to be permeabilized for antibodies to enter. In our experience, neutral lipid staining is not affected by this permeabilization protocol, however the detergent used may also partially dissolve the lipids in the lipid droplets; the ADRP is expected to stay put because it is crosslinked to neighbouring proteins.
3. For mouse, rat, human or bovine cells incubate with guinea pig anti-human ADRP polyclonal antibody diluted to 1 μg/ml final concentration in 50 mM cacodylate buffer. Prepare 0.25 ml antibody solution per cm$^2$ sample.
4. Working with samples on coverglasses, place 0.2 ml antibody solution into a single drop onto a kinkfilm.
5. Place the coverglass, sample facing the osmium solution, onto the drop. For cells on glass slides, carefully place the drop of antibody solution over the sample area. Circumscribing the sample region using a hydrophobic marker pen can help to keep the drop in place.
6. Incubate for 30 min.
7. Wash three times in Ca$^{2+}$/Mg$^{2+}$-free HBSS.
8. Incubate with fluorescent-labeled secondary antibody for 1 h room temperature.
9. Wash three times in Ca$^{2+}$/Mg$^{2+}$-free HBSS. Optionally include 10-100 nM DAPI or alternative nuclear stains.
10. Mount in glycerol based mounting medium for fluorescence microscopy. Use appropriate spacers to avoid sample damage during the mounting process. Commercially available mounting media typically contain addition to prevent rapid fading of fluorescence. Make sure the mounting medium is compatible with the fluorophores used.

**Oil Red O Staining**
Oil red O belongs to the polyazo group of dyes which also includes the ‘Sudan’ series of dyes. It accumulates in lipid-rich compartments. Oil red O staining can be readily visualized in both, bright field and fluorescent microscopy.
1. Prepare fixed cell samples as described above in ‘Cell sample preparation’.
2. Wash twice in deionized water.
3. Place slides in absolute propylene glycol for 5 min.
4. Stain in 0.5% oil red O solution for 10 min at room temperature.
5. Rinse cells in 85% propylene glycol solution for 3 min.
6. Wash twice in deionized water.
7. Optionally counterstain with hematoxylin solution for brightfield microscopy (like shown in fig.1) or with DAPI for fluorescence microscopy: Add 10-100 nM DAPI into the first washing step. For hematein staining, incubate the sample for about 30 sec in haematoxilin solution. Stop staining by washing out the dye once intense staining is achieved.

8. Wash hematein counterstained samples thoroughly in tap water. Because most alum hematoxylin formulae are fairly acidic, the nuclei will initially be stained with a purplish color. Tap water 6.0 - 6.8 is considerably more alkaline than the pH of most hemalums (pH 2.6 - 2.9), which results in bluing of the hematein stain and generates better contrast against the Oil Red O. Overstaining with hematein will generate a background that may be difficult to deal with for automated image processing.

Required Materials and Stock Solutions
1. 0.5% Oil Red O: 1-(4-(Xylylazo)xylyl]azo)-2-naphthol, MW 408.49. Add 5 ml of propylene glycol (100%) to 0.5 g of oil red O with stirring and gradually complete the volume with propylene glycol to 100 mL. Heat the solution until 95°C, but do not boil. Filter through paper filter. The solution can be stored at room temperature.
2. Hematoxylin solution. Use Mayer's or Gill's Hemalum formulations.
3. Aqueous mounting medium.
5. Mount with aqueous mounting medium.
6. 11 mM DAPI (C16H15N5 · 2C3H6O3) stock solution. DAPI is a mutagen, handle with care. Avoid skin contact. Add 10 mg of DAPI dilactate to 2 ml of deionized water. For long-term storage the stock solution can be aliquoted and stored at -20°C. For short-term storage the solution can be kept at 2–6°C, protected from light. When handled properly, DAPI solutions are stable for at least six months. Use DAPI at final concentrations of 10-100 nM, depending on the intensity of the other stains in the sample.

Alternative Protocols
A number of alternative protocols for lipid droplets exist, which are detailed elsewhere. These include Lipid Tox red, BODIPY, Nile Red and P96.

Computational Image Analysis
We have used the macro language of the open source image analysis platform Fiji [13] to automatically extract and quantify image information from microscopy images. For the separation of different color information from Oil Red O/hemain we used the Color Deconvolution plugin [14] and for the generation of the extended focus projection of the brightfield image in figure 2, a plugin from [14] was used. The complete macro code to analyze LDs is available from the authors upon
request. We are currently implementing a fully automated image acquisition and analysis pipeline.

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