A simple method of Analyzing Lipid Droplets combining Oil Red O Staining and Confocal Microscopy

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ABSTRACT

Localizing, identifying and quantifying lipid droplets are important features to be assessed in the research of mechanisms involved in diseases such as steatosis, obesity, diabetes, myopathies and arteriosclerosis. Lipid-droplet staining with Oil Red O is widely used combined to either light field, conventional fluorescence microscopy and phase contrast microscopy. Here, for the first time, we report an easy, fast and precise protocol for the quantitative evaluation of lipid droplets staining with Oil Red O in HepG2 cells in vitro using confocal laser scanning microscopy associated with maximum intensity projection technique and counting point method. Our methodology was compared to a previous described protocol to measure lipid droplets, based on phase contrast microscopy and binarization. Our protocol substantially enhanced the quality of lipid droplets images compared to phase contrast microscopy by different reasons, such as: 1-Use of maximum intensity projection technique; 2- Out-of-focus light absence; 3-Increased contrast; 4-Enhanced lipid droplet definition. Thus, the use of this protocol increases the sensitivity of lipid droplets quantification, showing morphological results that can be underestimated using other approaches.

Keywords: Confocal laser scanning microscopy, lipid droplets, maximum intensity projection technique, oil Red O.

Um método simples de Analisar Gotas Lipídicas combinando Coloração Com Oil Red O e Microscopia Confocal

RESUMO

A localização, identificação e quantificação das gotas lipídicas são características importantes a serem avaliadas na pesquisa de mecanismos envolvidos em doenças como a esteatose, obesidade, diabetes, miopatias e arteriosclerose. A coloração de gotas lipídicas com Oil Red O é amplamente utilizada combinada às microscopias de campo claro, fluorescência convencional e contraste de fase. Neste estudo relatamos, pela primeira vez, um protocolo fácil, rápido e preciso para a avaliação quantitativa de gotas lipídicas coradas com Oil Red O em células HepG2 *in vitro* usando microscopia confocal de varredura a laser associada à técnica de projeção máxima de intensidade e método de contagem de pontos. Nossa metodologia foi comparada a um protocolo descrito anteriormente para quantificar gotas lipídicas, com base em microscopia de contraste de fase e binarização. Nosso protocolo melhorou a qualidade das imagens de gotas lipídicas substancialmente em comparação com a microscopia de contraste de fase por diferentes razões, como: 1-Uso da técnica de projeção máxima de intensidade; 2- Ausência de luz fora de foco; 3-Aumento do contraste; 4-Definição de gotas lipídicas aprimorada. Assim, o uso deste protocolo aumenta a sensibilidade da quantificação de gotas lipídicas, mostrando resultados morfológicos que podem ser subestimados por outras abordagens.

Palavras-chave: Microscopia confocal de varredura a laser, gotículas lipídicas, técnica de projeção máxima intensidade, Oil Red O.

INTRODUCTION

Lipid droplets (LD) are active organelles that play a central role in cellular metabolism through lipids collection, storage and supply according to cellular needs [1]. However, when there is an excess of fatty acids, progressive lipotoxicity occurs, characterized by organelle membrane destruction, stress pathway activation, metabolic dysregulation and apoptosis [2]. Several

diseases, such as steatosis, obesity, diabetes, myopathies and arteriosclerosis, are related to lipids accumulation [1,3]. Thus, localizing, identifying and quantifying LD shape are important features to be assessed in the study of the mechanisms involved in diseases that present, as a characteristic, lipids accumulation, as occurs in steatosis [1,3]. However, a methodology for images analysis capable of LD measuring, using Oil red O (ORO) staining and confocal microscopy, has not yet been fully developed.

The HepG2 cells are widely used as an in vitro model for steatosis research, considering that it accumulates lipids in the cytoplasm similarly to hepatocytes [4-6]. Oleic acid is a monosaturated omega-9 fatty acid that is widely used to induce LD accumulation in HepG2 cells [5-7]. Among the available methods, staining lipids with the classic ORO dye represents a low-cost, easy-to-prepare method of visualizing and quantifying LD. ORO stains neutral lipids (mainly triglycerides) with an orange-red tint [8]. ORO-stained LD are commonly visualized and quantified using either light field, conventional fluorescence microscopy and phase contrast microscopy associated with binarization [3,9].

All these previously cited microscopy techniques present some limitations, including low resolution (0.2 µm), out-of-focus information which often blurs the image, inability to combine its use with other fluorescent labeling techniques, as 40,6-diamidino-2-phenylindole (DAPI) nuclei stain, and difficult in defining a threshold in the binarization process [10,11].

The confocal laser scanning microscope represents an important tool in the field, considering it can be used to produce optical sections of the specimen at relatively shallow depths of field (0.5–1.5 µm), eliminating or reducing the background (which leads to image quality loss) allowing information collection from a well-defined plane, rather than from the entire specimen thickness. Moreover, eliminating out-of-focus light results in increased contrast, clarity, and detection sensitivity. These advantages make it possible to obtain high quality images with optimum resolution [10].

The aim of our study was to analyze LD in HepG2 cells, treated or not with oleic acid and stained with ORO and DAPI, describing a new image analysis protocol, using confocal laser scanning microscopy associated to maximum intensity projection technique and counting point method. Additionally, our protocol was compared with a traditional protocol, also for LD analysis in HepG2 cells, based on phase contrast microscopy associated to binarization.

MATERIALS AND METHODS

Materials

The human hepatocarcinoma cell line (HepG2) was obtained from Rio de Janeiro Cell Bank, UFRJ, Rio de Janeiro, Brazil. Dulbecco's Modified Eagle Medium and fetal bovine serum were obtained from Gibco, Life Technologies. Streptomycin (100 mg/mL) and penicillin (100 units/mL), 40,6-diamidino-2-phenylindole (DAPI) and Clearmount solution were obtained from Invitrogen, Carlsbad, CA. Oil Red O was supplied by Sigma-Aldrich, USA. Oleic acid was supplied by Synth, Brazil and isopropanol were supplied by Nuclear, Brazil. Coverslips were obtained from Assistent, Germany.

Cellular culture and treatment

HepG2 cells were cultivated in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1% streptomycin (100 mg/mL) and penicillin (100 units/mL), 2 g/L HEPES buffer (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) and 3.7 g/L NaHCO3 in a humidified atmosphere with 5% CO2 at 37 °C. Stock solutions of oleic acid were prepared in isopropanol and dilutions were prepared in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. For the ORO and DAPI staining assays, using confocal microscopy, round 18-mm diameter coverslips were first washed with detergent solution Extran® 2%, rinsed with distilled water

three times and subsequently sterilized in an autoclave. Cells were cultured for 24 hours before treatment under sterile coverslips placed in 6-well culture plates (6.0 x 104 cells/well). For the ORO staining assay, using phase contrast microscopy, cells were seeded into 24-well plates (12 x 10^4 cells/well) and cultured for 24 hours before treatment, in all cases, to allow cell adhesion on the plates. Cells were treated with 100 µM oleic acid for 24 hours. Control wells contained Dulbecco's Modified Eagle Medium and 10% fetal bovine serum.

Oil Red O and DAPI staining for confocal microscopy

The protocol described below was adapted [8]. After the treatment period, culture medium was discarded and cells were washed twice with phosphate buffered saline (PBS). Subsequently, cells were fixed with 4% paraformaldehyde for 30 min. Then, fixed cells were washed thrice with PBS and subsequently rinsed with 60% isopropanol.

A fresh 0.3% ORO solution was made as follows: 0.5% ORO stock solution (0.5 g ORO and 100 mL isopropanol) was prepared by dissolving the ORO (Sigma-Aldrich, USA) in isopropanol (solvent) under slight heating followed by filtering (filter 0.22 µm). Then, the 0.5% ORO stock solution was diluted in distilled water to obtain the 0.3% concentration, waited 10 minutes and the solution was filtered again (filter 0.22 µm). Afterwards, cells were stained with this freshly prepared 0.3% ORO solution for 15 min. Thus, cells were rinsed with 60% isopropanol, washed thrice with PBS and subsequently stained with a solution containing 300 nM DAPI for 30 min.

After that, cells were washed once with PBS and Milli Q water. The coverslips were removed from the culture plates and dried at room temperature protected from light. Glass slides 26mm×76 mm were assembled with overlapping coverslips and Clearmount solution mounting medium.

Oil Red O staining for phase contrast microscopy

Cells were cultured and treated as aforementioned. After the treatment period, culture medium was discarded and cells were washed twice with PBS. Afterwards, cells were fixed with 4% paraformaldehyde for 30 min, washed thrice with PBS and subsequently rinsed with propylene glycol. Then, cells were stained with freshly prepared solution of propylene glycol containing 0.5% ORO (previously filtered with a 0.45 µm filter) for 15 min. Thus, cells were rinsed with 60% propylene glycol and washed with distilled water.

Image acquisition for confocal microscopy

Images were captured under a confocal laser scanning microscope (Leica TCS SP8, Leica Microsystems, Germany) with a Leica Application Suite X (LASX) software. Digitized images from 50 single confocal sections with a z-step size of 0.1 µm were acquired with a 63× (numeric aperture 1.40) oil-immersion objective (HC PL APO CS2 63X/1.40 OIL, Leica Germany). In these images, the maximum intensity projection technique was applied, converting this 3D data, obtained from these 50 images, into a single 2D image. This technique takes the brightest pixel in each layer and display that pixel intensity into 2D image.

For each sample, images of four fields were acquired and processed. The ORO fluorescence was measured after being excited by a 552 nm laser beam and emission scan collected at 660 nm. The DAPI fluorescence was measured after a laser exciting at 390 nm and emission collected at 460 nm.

Image acquisition for phase contrast microscopy

Digitized images were acquired, via Bel software, in a phase contrast inverted microscope (INV100, BEL Engineering, Italy) coupled to a Bel Photonics camera. The images were obtained using an Infinity Long Working Distance Plan Achromatic Phase objective (40x/0.6 WD 2.6mm, BEL Engineering, Italy). For each sample, two images from different sample fields were analyzed.

Lipid droplets quantification in confocal laser scanning microscopy and in phase contrast microscopy

The following morphometric parameters were estimated in control HepG2 cells and HepG2 cells treated with oleic acid; stained with ORO and analyzed in confocal laser scanning microscopy or in phase contrast microscopy: (1) Area covered by LD (2) HepG2 cell density (3) Area covered by LD/ HepG2 cell density relation. Our main goal is to compare two techniques and image analysis protocols, thus, to normalize data, in these 3 morphometric parameters, the mean values obtained in controls were considered as 100% and oleic acid groups are presented as a percentage of controls.

The analysis of maximum intensity projection images obtained in confocal laser scanning microscopy were performed using Image Pro Plus 6.0 software (IPP6–Media Cybernetics, Silver Spring, MD). While the analysis of planar images obtained from phase contrast microscopy were performed in Image J software, as an adaptation of a previous described protocol [12].

In confocal laser scanning microscopy images, the area covered by LD was estimated using a stereological tool, the point counting method, in this, a grid mask (a grid of 588 crosses with equidistant intervals) was placed over the images, each cross corresponding to one counting point. When the upper right quadrant of the cross hit LD images they were counted. To a more quantitative evaluation, the area covered by LD can be obtained by the following equation $A = a/p . N$, where A= area; a/p= area point per cross; and N= number of crosses counted [13,14] (fig. 1).

In order to estimate the area covered by LD in phase contrast microscopy, the images were converted to a 8 bit images (0-255), from the green channel image. Analyzing the digitized image and its respective gray level histogram, a threshold value was selected to distinguish LD from other image elements, and then the image was binarized in this threshold value.

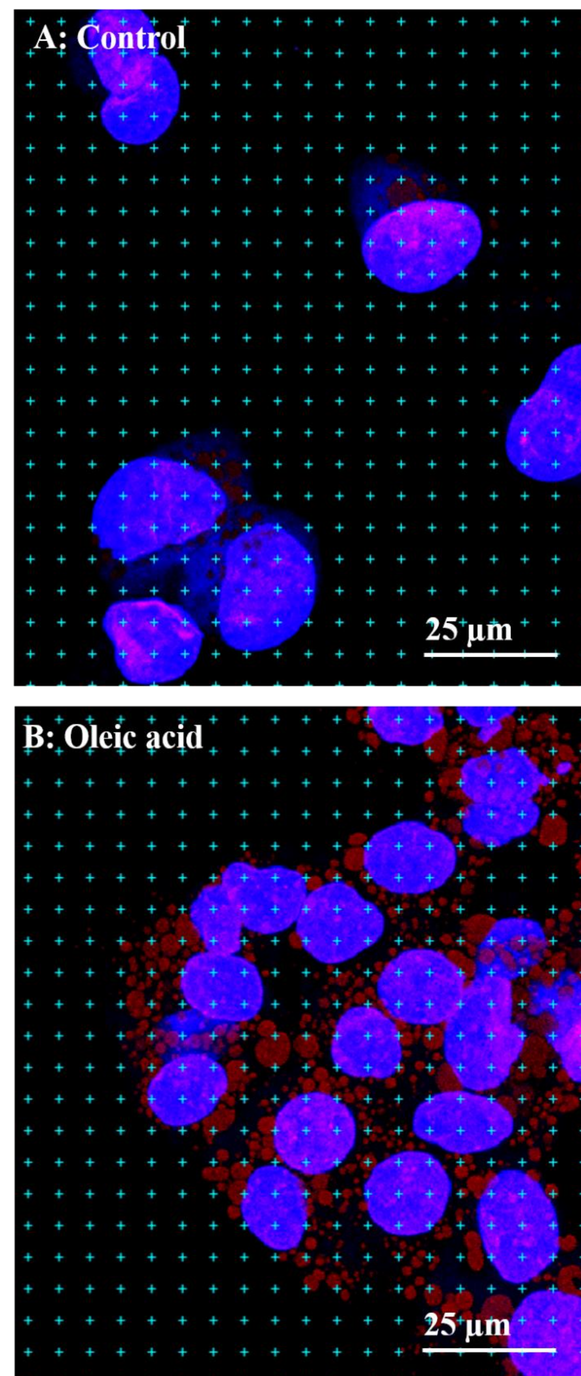

Fig. 1. Representative digitized images of nuclei maximum projections and LD from HepG2 cells through confocal laser scanning microscopy. The grid mask used to estimate the percent of LD is presented. (A) Control cells and (B) Cells treated with 100µM oleic acid (1B). LD were stained with Oil Red O and the nuclei with DAPI. Calibration bar = 25 µm.

Pixels above that threshold value are considered as area covered by LD, while pixels below are considered as area not covered by LD.

To quantify the HepG2 cell density, a quantitative analysis, similar to the Neubauer chamber, was performed in both, confocal scanning microscopy and phase contrast images [15]. Briefly, the overall image area was considered as the Area of interest (AOI), nuclei located inside each image or intersected by the upper and/or left edges of the AOIs were counted, nuclei intersected by the lower and/or the right edges of AOIs were not counted. The cell density can be obtained using the following equation: Cell density = number of counted cells/ AOI area.

To estimate the area covered by LD/ HepG2 cell density relation in confocal scanning microscopy, the number of crosses that hit LD in the AOI is divided by the number of cells counted in the same AOI; while in phase contrast microscopy, the number of pixels above that threshold value found in AOI is divided by the number of cells counted in the same AOI.

Figure 2 summarizes the procedures applied from cell culture to image analysis in these two protocols.

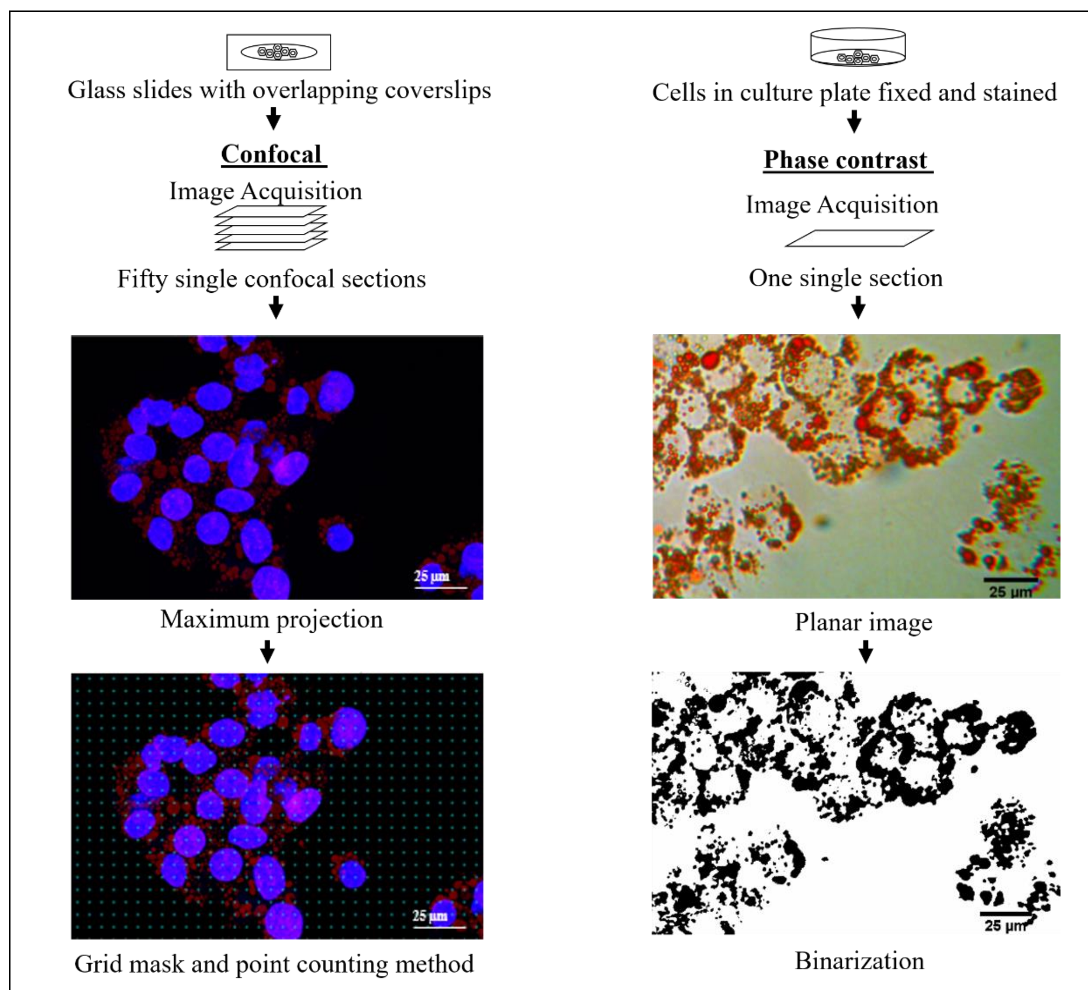

Fig. 2. Procedures applied from cell culture to image analysis using different methods (confocal or phase contrast microscopy). Calibration bar = 25 µm.

Statistical analysis

The three estimated parameters: 1-Area covered by LD; 2-HepG2 cell density; 3-Area covered by LD/ HepG2 cell density relation were compared into four groups: 1-Confocal/ Control/; 2- Confocal/Oleic Acid/; 3- Phase contrast/Control; 4- Phase contrast/Oleic Acid using One-

Way ANOVA, followed by Tukey Test. All results are presented as mean ± SD ($p<0.05$). Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Firstly, in a qualitative analysis, comparing the images obtained using confocal and phase contrast microscopes, is possible to note that in confocal microscopy, the brightness of OR staining is less intense, however, LD boundaries are well defined when compared to phase contrast. Another point to be considered is that maximum intensity projection technique provide high resolution images compared to single section image obtained in phase contrast microscopy, this difference facilitated LD quantification by image analysis, planar morphometry or stereological approaches. In phase microscopy, the cell limits were not so distinguishable in some images, making HepG2 cell density estimation a not easy task, while in confocal scanning microscope, using DAPI staining, this information can be easily obtained (figures 1 and 2).

In a semi-quantitative evaluation, observing data obtained from confocal scanning microscopy associated to maximum intensity projection technique and counting point method, oleic acid promote a strong increase in the three morphometric parameters analyzed: area covered by LD ($p<0.001$), HepG2 cell density ($p<0.05$) and area covered by LD/ HepG2 cell density relation ($p<0.001$) when compared to confocal-control group. These increases could not be observed in phase contrast microscopy, associated to image analysis by binarization. Additionally, when the confocal-oleic acid and phase contrast-oleic acid groups are compared, the first presents higher values in these three parameters: area covered by LD ($p<0.001$), HepG2 cell density ($p<0.01$) and area covered by LD/ HepG2 cell density relation ($p<0.01$) (fig. 3).

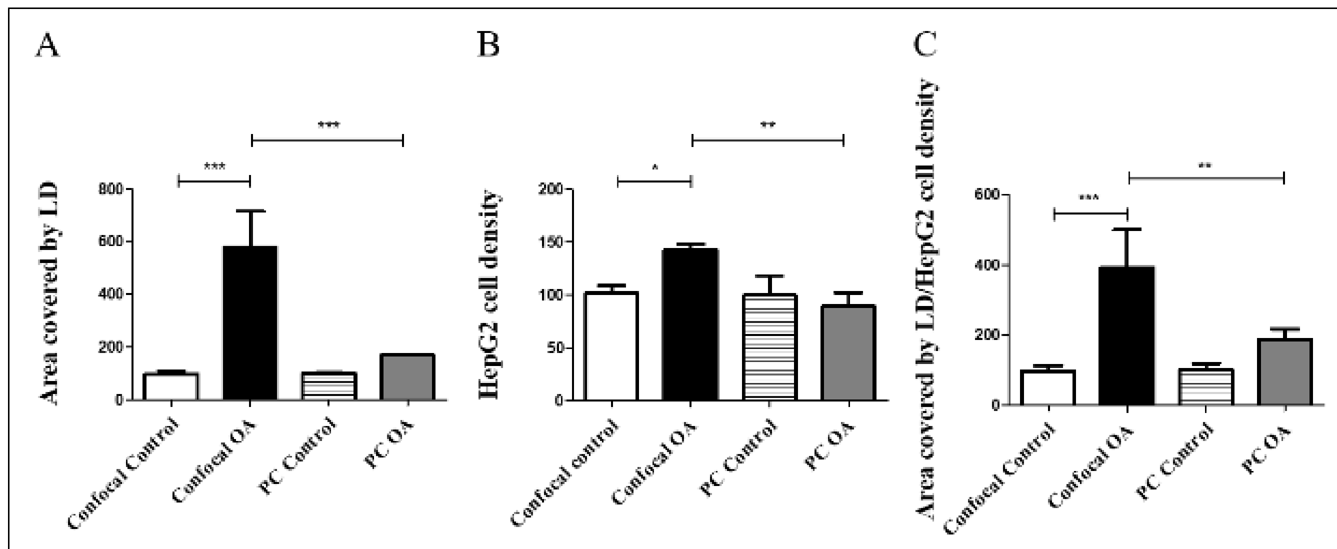

Fig. 3. Comparison between results obtained using confocal microscopy/maximum projection technique/counting point method and phase contrast microscopy/binarization. The following morphometric parameters were analyzed: (A) Area covered by LD; (B) HepG2 cell density; (C) Area covered by LD/ HepG2 cell density relation. In these three parameters, controls were considered as 100%. Legend: Confocal control: control group analyzed by confocal microscopy, Confocal OA: Group treated with oleic acid analyzed by confocal microscopy, PC Control: Control group analyzed by phase contrast microscopy, PC OA: Group treated with oleic acid analyzed by phase contrast microscopy. In the oleic acid treatment, cells were treated with 100μM oleic acid for 24 hours. In confocal microscopy, oleic acid treatment promotes a strong increase in the three morphometric parameters analyzed: area covered by LD ($p<0.001$), HepG2 cell density ($p<0.05$) and area covered by LD/ HepG2 cell density relation ($p<0.001$) when compared to confocal-control group. The same differences could not be observed in phase contrast microscopy. Data are presented as mean + SD from three independent experiments. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

The first obvious conclusion of our study is that confocal laser scanning microscopy associated to maximum intensity projection technique and counting point method presents different qualitative and semi-quantitative results when compared to phase contrast microscopy evaluated by binarization, at least in the *in vitro* model used in our study. These differences will be discussed below.

Regarding the differences in qualitative results, the high resolution images obtained in confocal microscopy associated with maximum intensity projection technique facilitated LD and nuclei delineation. In fact, in some monolayer cell cultures, 2D image acquisition, as used in phase contrast microscopy, is often partially in focus, while a 3D stack contains more information and the information needed to reconstruct a focused 2D image [16].

Considering the semi-quantitative results, the most relevant questions regarding our findings are: Is the first approach promoting an overestimation of LD and HepG2 density? Or the second protocol is responsible for an underestimation in the same parameters?

In fact, both protocols tested here are semi-quantitative evaluations inspired on surface area estimation [17,18]. The main goal of these protocols is not to be so precise and unbiased as other stereological approaches as the optical dissector or Cavalieri Method [19,20,21]; but show relevant morphological differences between cells, tissues and treatments. In this way, confocal microscopy associated to the maximum intensity projection technique is able to show differences in 3D structures that could be slight in 2D images obtained from a single section and cannot be detected by planar morphometry.

This search for a better protocol for LD quantification is not recent, a previous study describing a protocol for conventional fluorescence microscopy using ORO staining show better results in comparison to bright-field microscopy, although with limitations in image quality inherent to the conventional fluorescence microscope [9]. The advantages of this protocol were the elimination of phospholipid interference in the automated quantification of LD and the possibility of performing multi-immunofluorescence staining on a single section [9]. Another study using a Nile Red lipophilic dye and conventional fluorescence microscopy also obtained images with low contrast and poor LD definition [22], showing the need of a more suitable method to improve LD image quality and quantification.

Most studies that use OR staining do not involve quantitative assessment of the images [5,7,23]. Some use the staining protocol only to show representative images of OR-stained LD stained [23] or dissolve the LD with subsequent optical density reading in a microplate reader [5,7]. Our study proposed the application of the point counting method to directly quantify the area covered by LD, eliminating possible limitations regarding dissolution and lipid stability or low sensitivity for samples with small LD amounts. Additionally, the counting point method is highly accurate and allows the exclusion of possible artifacts in the image, which is often impossible using automated protocols in image analysis software [19].

Interestingly, there was an increase in cell percentage after treatment with oleic acid through the analysis of images obtained by confocal microscopy. Our results are in agreement with another study showing that nonalcoholic fatty liver disease in an animal model correlates with an increase in proliferating hepatocytes number in the liver [24]. A study that used HepG2 cells treated with oleic acid (0.1-2mM) for 24h showed an excellent correlation between lipid accumulation and optical density measured using the OR-based colorimetric quantitative assay [5]. However, no image analysis was performed in that study. Image-based LD quantification is important to corroborate the results obtained by colorimetric assays.

CONCLUSIONS

The present study developed a simple, fast and precise protocol to LD semi-quantification by ORO staining using

confocal laser scanning microscopy combined with maximum projection technique and counting point method. This method provided great gain in LD analysis when compared to phase contrast microscopy associated to binarization. We hope that this protocol, and any adaptations of it, may help researchers in future studies to obtain more detailed and reliable results regarding LD evaluation and other histological challenges.

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