BIOCELL
2017 41(2-3): 55-58

ISSN 1667-5746
ELECTRONIC

Freely Available Tool (FAT) for automated quantification of lipid droplets in stained cells

Diego Masone[1,2], Aldana D. Gojanovich[1], Yesica R. Frontini-Lopez[1], Samanta DEL Veliz[1], Marina Uhart[1], Diego M. Bustos[1,3*]

[1] Laboratorio de Integración de Señales Celulares. IHEM-CONICET, Universidad Nacional de Cuyo Mendoza, Argentina.
[2] Facultad de Ingeniería, Universidad Nacional de Cuyo, Mendoza, Argentina.
[3] Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Cuyo, Mendoza, Argentina.

Key words: Adipogenesis, 3T3-L1 cells, human stem cells, automatic image processing.

Abstract: In this study, we propose an automatic procedure for digital image processing. We describe a method that can efficiently quantify and characterize lipid droplets distributions in different cell types in culture. Prospectively, the lipid droplets detection method described in this work could be applied to static or time-lapse data, collected with a simple visible light or fluorescence microscopy equipment. Fully automated algorithms were implemented in Octave, a freely available scientific package.

Introduction

The number and size of lipid droplets are important quantities to classify in *in vitro* studies, like stem cell differentiation, effects of altered nutritive culture conditions and insulin resistance (Olofsson *et al*., 2008). Also, these parameters may serve as biomarkers for metabolic responses to diet, pharmacological intervention, or exercise.

The analysis of microscopy images is a powerful research tool, especially if it is simple, quantitative and automatic. The simplest method to analyze the amount of lipid droplet-stored triglycerides and cholesterol oleates in cells is Oil Red O staining (Mehlem *et al*., 2013). A useful protocol to quantify this methodology is to dissolve the lipid droplet retained dye in isopropanol and read the absorbance at 510 nm. However, the intrinsic information of lipid droplets number and sizes is lost. To obtain a more detailed quantitative analysis of absolute lipid droplet frequency distribution, additional equipment with proprietary software is required to segment and evaluate lipid droplets or to implement Raman spectroscopy-based approaches (Nan *et al*., 2003), which are expensive and require high expertise. Several methods were published to analyze lipids droplets in stained or unstained images (Deutsch *et al*., 2014; Sims *et al*., 2015; Varinli *et al*., 2015). However, to the best of our knowledge none of them are neither fully automatic nor were tested on different cells types. This later issue is important given that cell characteristics could increase false positive rates. Fig. 1 shows human stem cells and 3T3-L1 mouse cells after 10 or 7 days of differentiation, respectively, stained with Oil Red O. Three main type of circular o near-circular objects could be observed: (a) lipid droplets, (b) lipid droplets in the process of fusion and (c) non-lipidic vesicles. The number of each cellular compartment is a characteristic of the cell type and state (*i.e.*, stem, progenitor, differentiated).

Here, we introduce a fully automatic algorithm capable of analyzing hundreds of images and of quantifying cells and lipid droplets of different origins, using the freely available Octave package (Eaton *et al*., 2015). Our method can be used on both standard Oil Red O light microscopy or fluorescent images, to extract a bulk of biologically relevant information. Also, it is perfectly applicable to screenings to elucidate the functions at systems level by investigating the phenotypes of a large number of cells in culture.

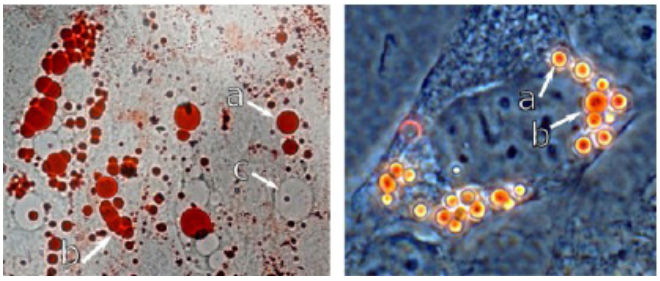
Methods

To implement an automatic algorithm capable of quantifying cells or nuclei, we used an edge detection method to identify the points of the image that determined the perimeters of the nuclei. Edge detection allows for identification of points in a digital image where abrupt discontinuities take place (Marr and Hildreth 1980). Also, changes in intensity characterize boundaries of objects of interest. There are many approaches for edge detection with different capabilities to deal with diverse image issues (Maini and Aggarwal 2009). For example, effects such as refraction or focus problems

* Address correspondence to: Diego M. Bustos, dbustos@mendoza-conicet.gob.ar
This paper belongs to the 60th Anniversary Collection of the Instituto de Histología y Embriología de Mendoza (IHEM)

may result in poorly defined boundaries and therefore more difficult edge detection (Maini and Aggarwal 2009). Recently, an algorithm for digital image edge detection based on the dispersive phase stretch transform was developed by Asghari and Jalali (2015), which allows for efficient edge detection of microscopy cell images.

Our protocol was implemented in Octave, a freely available scientific package which provides easy to use mathematical functions to manipulate images, to adjust colors and to count pixels. Using functions implemented in Octave, we were able to automatically and equitably quantify the amount of cells in each image together with the amount of pixels of an specific color. The main parts of the codes used for image processing are shown under Supplementary Material. Fully functional scripts for Octave are provided upon request.



(a) (b)

FIGURE 1. (a) 40x magnification of human stem cells stained with Oil Red O after 10 days of differentiation. (b) 100x magnification of 3T3-L1 mouse cells stained with Oil Red O after 7 days of differentiation.

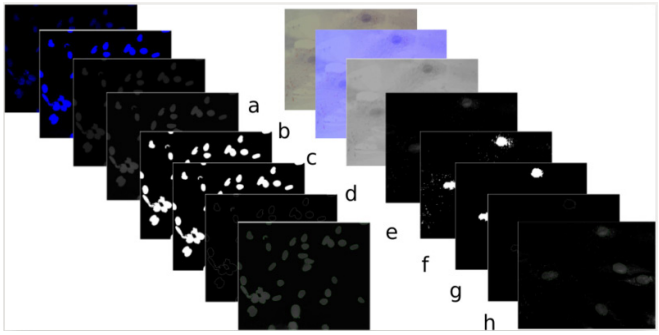


FIGURE 2. Sequential image processing: (a) original image (left: DAPI, right: hematoxilin/eosin) (b) color adjustment, (c) gray-scale, (d) equalization, (e) black and white, (f) filled holes and small dot selection, (g) perimeters determination, (h) final superposition.

Fig. 2 shows the implementation of our automatic protocol for cell nuclei edge detection and counting. It is observed how cells are recognized and their perimeters highlighted in green. At these point, little programming is needed to implement further modifications to quantify any geometrical characteristics of the objects recognized. The intermediate results as produced by the algorithm are also shown in Fig. 2. First, image colors are adjusted to intensify the ones of scientific interest. Second, image is converted to gray-scale. Third, image contrast is enhanced using histogram equalization with Octave function *imadjust*. Fourth, image is converted to black and white. Fifth, all holes are filled and small dots are deleted using the function *bwareaopen* (this step avoids false object counting). Sixth, perimeters in the black and white image are detected using the function *bwperim*. Seventh, a new image is created merging the perimeters found in the black and white mask and the gray-scale version of the original image, so that edges are highlighted in color. Full steps are summarized as a block diagram in Fig. 3.

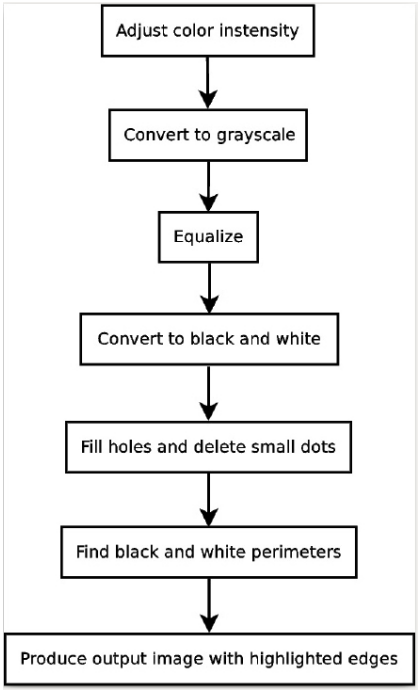


FIGURE 3. Automatic edge detection complete protocol steps (for more details, see Supplementary Material).

Results and Discussion

Different cell types, including voluntarily donated adipose derived human mesenchymal stem cells and murine 3T3-L1 preadipocytes were differentiated into mature adipocytes during 10 and 7 days, respectively. Intracellular lipid droplets were stained with Oil Red O (Gojanovich *et al.*, 2016). Images of adipocytes were captured with a Nikon 80i microscope equipped with a DS-F1 charge-coupled device (CCD) camera and 40X RC and 100X RC objectives. Highly dense packed clusters could influence results in microscopy-based image analyses. A proposed method to avoid this problem is the manual separation of overlapping droplets by using the ImageJ pencil tool (Deutsch *et al.*, 2014). Biologically, overlapping droplets (fused) are one cellular compartment and must be treated as one (big) lipid droplet. Also, based on the fact that the number of lipid droplets is high enough to be treated statistically, we developed an entire new approach to obtain the frequency of Oil Red O-stained lipid droplets. After the conversion of 8-bit RGB (Red Green Blue) images to binary black and white, we sequentially and automatically calculated the area of a window to spawn lipid droplets sizes from the largest to the smallest. The software takes this information and plots it. In order to validate this approach we compared results from Deutsch *et al.*, (2014) and the distribution obtained on the same image by our method. Fig. 4 depicts this comparison.

To test the usability of our method, we analyzed a time-course adipogenesis experiment of human mesenchymal stem cells. Cells were grown on DMEM supplemented with

fetal bovine serum and adipogenesis inductors following the protocol from Gojanovich et al., for details see supplementary information. At days 7, 10 and 14, the same days of insulin addition and one day before drug supplementation, respectively, cells images were taken. Cells showed lipid accumulation after staining with Oil Red O (see Fig. 5 and Tab. 1).

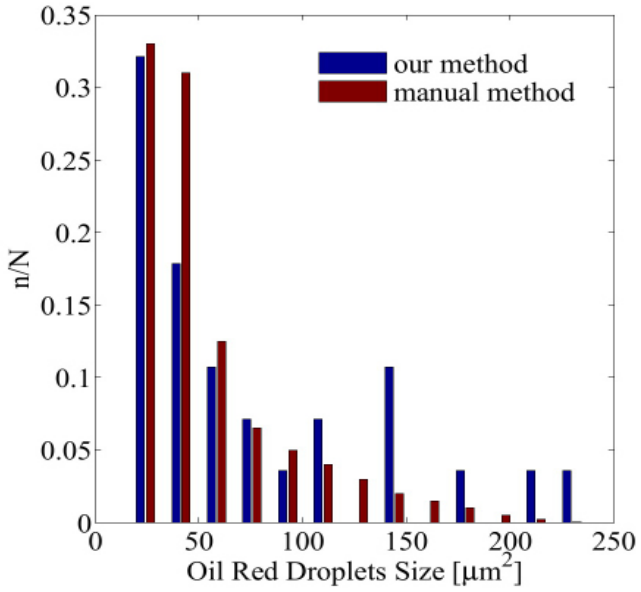


FIGURE 4. Comparison of two different methods to analyze lipid droplet size and distribution. n = number of specific events, N = total number of events.

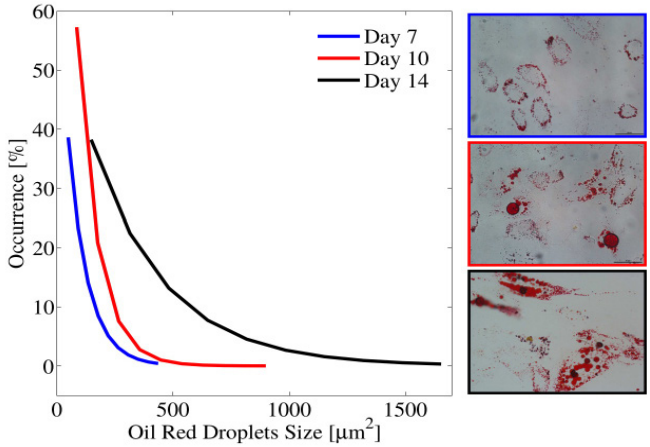


FIGURE 5. Automated analysis of lipid droplets during human stem cell differentiation. Cells were cultured on DMEM + FBS and differentiated using the previously optimized drug cocktail (Gojanovich *et al.*, 2016,).

Finally, we compared the frequency distributions of lipid droplet sizes on bright-field and fluorescent images of the same cells. Because these techniques have different sensitivities, automatic methods could generate disproportionated distribution curves, where smaller or larger droplets could be over represented. However, our results show that the algorithm is independent of the method used to get the images (see Fig. 6).

As expected, lipid droplets distributions (curves) are shifted to the right as the days passed, showing an increase of droplet sizes as differentiation progressed. The fact that at the end of the differentiation protocol, the size of the smaller droplets is larger than the size measured at early stages could mean that lipid accumulation, or the fusion of existing droplets, is favored over the formation of new ones. The information acquired from the images reflects the expected changes in the size of lipid droplets during adipogenesis. These data demonstrate that a simple computational image analysis of Oil Red O-stained cells, is capable of yielding precise quantitative information on size and frequency distribution of lipid droplets.

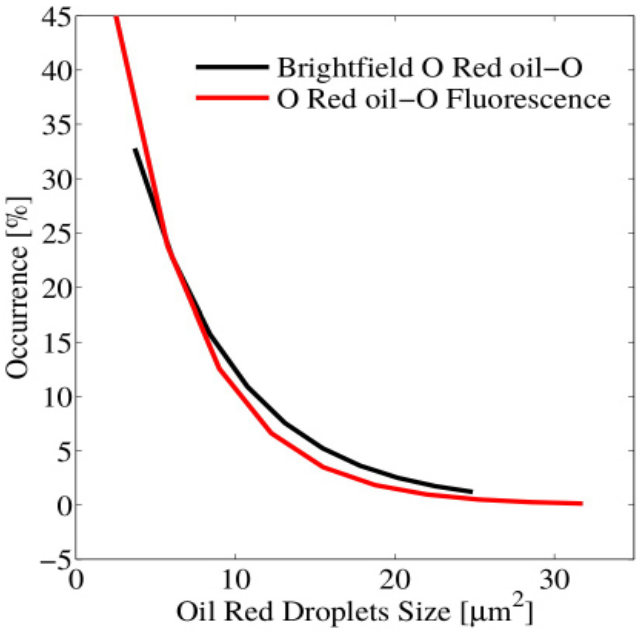


FIGURE 6. Comparison of our method performance on bright field and fluorescence images of the same cells.

Besides the distribution of lipid droplets sizes, our method also allows to quantify the number of red pixels on each image, for both, bright field and fluorescent microscopy techniques. This number results to be directly proportional to the triglycerides and cholesterol esters contained in the totality of the lipid droplets per image (an example is given in Tab. 1). This quantification gives similar information as the one obtained by discoloration of the lipid droplets-bound dye with isopropanol and absorbance measurement at 510 nm, with the following advantages: i) time saving: it does not require additional experimental steps of discoloration and absorbance measurement; ii) budget saving: the same samples that are mounted and imaged can be quantified. In the discoloration method, extra samples must be prepared exclusively for quantification. iii) Automation and uniformity: hundreds or even thousands of images are quantified after setting the parameters for one of them. In contrast, the discoloration process is not always achieved to the same level in different samples. If the number of cells/image is not equal, the number of red pixels should be normalized by the number of cells per image using our cells (nuclei) counting application. Either fluorescence (DAPI or Hoechst) or any bright field dye (*i.e.* Hematoxylin) may be used for nuclei staining.

Frequently, in a screening method it is necessary to consider many images and to plot the information in the most comparable way. Fig. 7 shows the results of a drug screening performed on 3T3-L1 mouse preadipocyte cells

undergoing adipogenic differentiation. Three images were taken on biological quadruplicates of six different conditions (drug cocktails), resulting in 72 images. These were analyzed by our software and automatically plotted in a bar chart, see Fig. 7 (presented as).

TABLE 1

Red pixels quantification and cell counting

	Total number of red pixels [10^5]	Cell count	Norm [10^4]
Day 7	0.55	11	0.5
Day 10	1.52	11	1.4
Day 14	1.44	8	1.8

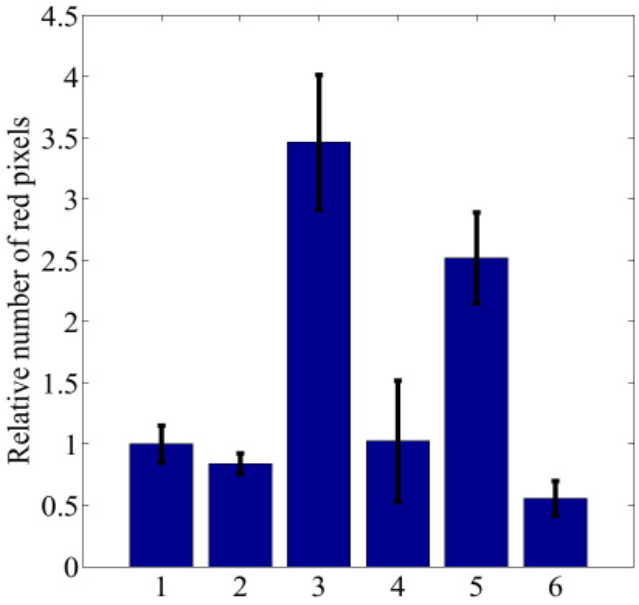


FIGURE 7. Automated analysis of lipid droplets during adipogenic differentiation of 3T3-L1 mouse preadipocyte cells. 1: non-treated cells, 2: non-optimized drug cocktail, 3: optimized drug cocktail (Gojanovich et al., 2016), 4: GLP-1 x insulin, 5: GLP-1 x IBMX 6: GLP-1. See supplementary material for more details.

Conclusions

Our results suggest that the image processing method described in this article can be used to accurately quantify and to analyze size distribution of cellular lipid droplets. As the processing method is capable of detecting lipid droplets in both bright-field and fluorescent images, it should be straightforward to extend the analysis to time-lapse microscopy and/or screening.

Lipid droplets are present in a wide range of cell types both in health and disease. Their distribution and growth is related to lipid metabolism, which is unbalanced in insulin resistance, obesity and other lipid related human diseases. Our freely available tool will undoubtedly contribute to advance research in these fields.

Acknowledgments

This work was supported by grants from ANPCyT (PICT-11 1450, PICT-12 2498 and PICT-14 1659), MinCyT (Iniciativa de Proyectos Acelerados de C\'alculo) IPAC 2016 (SACT 017-00721036) and IPAC 2017 (2017-75-APN-SECACT\#MCT), CONICET (PIP-13CO01), SeCTyP-UNCuyo (J051) and the Roemmers Foundation. Supercomputing time granted by the Sistema Nacional de Computación de Alto Desempeño (SNCAD-MinCyT) in clusters Mendieta (CCAD-UNC), Piluso (UNR), Coyote (CIMEC) and Pirayú (CIMEC) is gratefully acknowledged as well as GPU hardware granted by the NVIDIA Corporation. DM, MU and DMB are members of CONICET. ADG, YRFL and SDV are fellows of the same institution. The authors would like to thank the staff of Javier Cattaneo Clinic and Lagomaggiore Hospital for adipose tissue donations.

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