SOFTWARE TOOL ARTICLE

ContactJ: Lipid droplets-mitochondria contacts characterization through fluorescence microscopy and image analysis [version 1; peer review: 2 approved with reservations]

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Abstract

Lipid droplets (LDs) are the major lipid storage organelles of eukaryotic cells and together with mitochondria key regulators of cell bioenergetics. LDs communicate with mitochondria and other organelles forming “metabolic synapse” contacts to ensure that lipid supply occurs where and when necessary. Although transmission electron microscopy analysis allows an accurate and precise analysis of contacts, the characterization of a large number of cells and conditions can become a long-term process. In order to extend contact analysis to hundreds of cells and multiple conditions, we have combined confocal fluorescence microscopy with advanced image analysis methods. In this work, we have developed the ImageJ macro script ContactJ, a novel and straight image analysis method to identify and quantify contacts between LD and mitochondria in fluorescence microscopy images allowing the automatic analysis. This image analysis workflow combines colocalization and skeletonization methods, enabling the quantification of LD-mitochondria contacts together with a complete characterization of organelles and cellular parameters. The correlation and normalization of these parameters contribute to the complex description of cell behavior under different experimental energetic states. ContactJ is available here: https://github.com/UB-BioMedMicroscopy/ContactJ/tree/1.0

Keywords

Contact sites, Lipid Droplets, Mitochondria, Image Processing and Analysis, ImageJ, Fluorescence Microscopy, Bioimaging, Interactome
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Introduction

Lipid droplets (LDs) are the major lipid storage organelles of eukaryotic cells and together with mitochondria key regulators of cell’s bioenergetics. They supply essential lipids to produce signalling molecules, membrane building blocks, and the metabolic energy needed to survive during nutrient poor periods.  

In order to achieve their functions, LDs communicate with mitochondria and other organelles (endoplasmic reticulum, endosomes, peroxisomes and vacuoles) forming membrane contact sites, “metabolic synapses”, to ensure that lipid provision occurs where and when necessary. Contact sites between these organelles have been described and characterized by transmission electron microscopy (TEM) as it resolves at the membrane scale where these contacts take place. Whereas TEM allows accurate and precise characterization of contacts, their analysis on a large number of cells and conditions can become a long-term process. On the other hand, confocal fluorescence microscopy combined with advanced image analysis methods enable to extend contact analysis to hundreds of cells and multiple conditions.

Methods

Sample preparation and imaging

Sample preparation and imaging have been previously described in detail. Briefly, HEK293 cells were grown in fibronectin coated glass coverslips. Cells were fixed for 60 min in 4% paraformaldehyde, permeabilized in 0.15% Triton X-100 for 10 min, followed by blocking with 1% BSA (A7906, Sigma-Aldrich), 0.1% Tween in PBS for 15 min. Labeling was achieved by incubating cells for 1 hour at room temperature with rabbit polyclonal anti-TOM20 (1:500; ab186734, Abcam) diluted in blocking solution. Primary antibody was detected with donkey anti-rabbit IgG AlexaFluor 555 (A321094) from ThermoFisher Scientific, diluted 1:250 in blocking solution. Finally, cells were labeled with DAPI (1: 4000; ThermoFisher) and LDs were stained with BODIPY 493/503 (1:1000; Molecular Probes) for 10min at room temperature, washed twice with PBS and coverslips were mounted with Mowiol (475904; Calbiochem, Merck).

Imaging of LDs, mitochondria and nuclei was performed using a LSM880 laser scanning spectral confocal microscope equipped with an AxioObserver Z1 inverted microscope. DAPI, BODIPY 493/503, and Alexa Fluo 555 images were acquired sequentially using 405, 488 and 561 nm lasers, dichroic beam splitters, emission detection ranges of 415-480 nm, 500-550 nm and 571-625 nm, respectively, and the confocal pinhole was set at 1 Airy Unit (AU). Spectral detection was performed using 2 photomultipliers and 1 central GaAsP. Images were acquired in a 1024 × 1024 format, pixel size at 93 × 93 nm, and integration time of 0.51 microseconds. Sample preparation and image acquisition of TEM image from Figure 3a has been previously described in detail.

Implementation

We have developed ContactJ, a macro script for the open-source image analysis software ImageJ. This macro automatically and rapidly quantifies confocal images that are saved in a folder and returns the database of the resulting measurements, images and Regions of Interests (ROIs) in a “Results” folder. Thus, inexperienced users with no prior image analysis experience will find it easy to use. As can be seen in Figure 1, the flowchart illustrates how the macro automatically detects and measures LD-mitochondria linear contacts by combining standard and machine learning segmentation processes and the novel use of colocalization together with skeletonization methods from a large number of fluorescence images.

First, ContactJ macro performs the segmentation of the cell, LD and mitochondria separately. For the segmentation of the cell, channels from mitochondria and LD were intensity compensated and added to a binary mask from nuclei. The resulting image was used to find local maxima (with prominence of 100) and to obtain subsequently the segmented particles binary image. Segmented particles limits were encoded as 0 value on the binarized image from the three merged channels. Limits between cells allowed to accurately segment, individualize and store cells as ROIs (see Figure 2a).
LD segmentation was achieved through a Trainable Weka Segmentation classifier on LD channel image (see Figure 2b) and mitochondria were segmented by intensity thresholding (autothreshold method “Otsu”) (see Figure 2c).

On one hand, contact regions between mitochondria and LD were first obtained using the Colocalization plugin. This plugin highlights the colocalized “contact” points between mitochondria and LD 8-bits images (or stacks). The plugin generates an 8 bit binary image with only the colocalized points (Display value = 255). Two points are considered as colocalized if their respective intensities are strictly higher than the threshold of their channels (autothreshold methods “Yen” for LD channel and “Otsu” for mitochondria channel), and if their ratio (of intensity) is strictly higher than the ratio setting value (set to 50%: ratio (0-100%)). On the other hand, the regions of individualized LD are obtained using the Find Maxima tool with Segmented particles result from the LD mask. Finally, individualized contact regions were converted to a contour line section by performing the skeletonization of the minimum image calculation from the colocalization mask and the segmented particles result from LD. Contact perimeter and contact counts (a contact is defined as a continuous contact line) were quantified, obtaining the linear LD-mitochondria contact of each cell (see Figure 2d).

Finally, along the execution of the macro, all the data is stored in arrays (cell, LD and mitochondria areas and perimeters, contact perimeter, number of contacts, etc). Moreover, this data is stored in a .txt database file allowing the traceability of the results for each cell and each image.

Operation
ImageJ/Fiji with the Colocalization and WEKA plugins should be installed and ContactJ run from ImageJ macro editor. The software can be tested with the sample data provided (in Underlying data). First, the user should prepare a set of images and organize them into a folder. In this images folder the user should create a subfolder named “Model” with the data and model files obtained specifically for the segmentation of LD channel using the machine learning WEKA...
Once ContactJ runs, macro asks to the user the folder to analyse. Automatically, ContactJ opens the images one by one analysing them, cell by cell, and saving ROIs and all the measurements data obtained (areas, intensity, contact, perimeter …) in a .txt file as a data base.

**Use cases**

ContactJ has been developed and tested for the contact analysis of hundreds of HEK293 cells treated or untreated with lipopolysaccharide (LPS) and expressing or not a protein of interest PLIN5.4

Taking advantage of fluorescence multilabelling, the cells have been segmented and all parameters can be expressed per cell individually. The macro segments the nucleus, LDs and mitochondria from each cell and it obtains the following parameters that are stored in a data base table: Cell Area, number of LDs and Mitochondria, LDs and Mitochondria Total Area, Mean LD Area per cell, Standard deviation of the LD mean Area, Mean LD Perimeter and Total Mitochondria and LD Perimeter.

**Figure 2. Results of ContactJ macro.** Hek293 cells were labelled with anti TOM20 antibody (mitochondria) in red, Bodipy493/505 (LDs) in green and DAPI (nuclei) in blue. The different regions of interest resulting from segmentation are highlighted in white (a) Cell Segmentation, (b) LD segmentation, (c) mitochondria segmentation and (d) LD-mitochondria contacts. Insert in d) shows a detail of how contact regions found by ContactJ are accurate and individualized per LD.
The main novelty and distinctive feature from ContactJ is that it creates a contact line corresponding to each contact site between mitochondrion and LD. In order to obtain the contact site, ContactJ first generates a colocalization region corresponding to the overlapping fluorescence from both organelles, using the Colocalization plugin. Then, this shape is skeletonized generating a line of equidistant points to its boundaries representing the contact site. The macro stores in the data base file also the total length of the contact sites, the mean length of each contact and the number of contacts detected per cell. In the mentioned work, the results were expressed as Total Contact Length/Cell and compared between cell populations and treatments.

Discussion

One of the innovative and distinctive features of ContactJ is that it creates a linear contact region on the mid plane of the LD in close proximity to Mitochondrion, representing the most probable contact site between both organelles. Although light microscopy resolution limit prevents assertion of true interaction, the analysis of inter organelle contacts by fluorescence microscopy is accepted as an indicator of possible communication between these two organelles, bringing many advantages when performing contact analysis at a high scale of samples and conditions. TEM is used to measure contacts between organelles as it resolves at the membrane scale where these contacts take place, as can be seen in Figure 3(a). ContactJ measurements of contact perimeter between LD and mitochondria are 2-3 times bigger compared to TEM measurements (Figure 3). The main reasons for this difference are, first, that 2D confocal microscopy image represents a projection of approximately 500nm sample thickness, compared to the 70nm of the ultra-thin TEM lamella and consequently, it is collecting a higher proportion of membrane and contacts. Secondly, the intrinsic difference in resolution would affect more directly the measurements of small contacts by light microscopy over-estimating them. Therefore, the contacts obtained with ContactJ can be considered reliable compared to those observed by TEM.

Obtaining contact regions, together with multiple morphological parameters of organelles, allows the calculation of descriptive statistics that would help describing cellular response. In front a metabolic or pathogenic event, cells need to regulate the transfer and communication between organelles. Among many possible cell reactions, they may change the contact surface between organelles together with the organelle size, number and distribution. For example, in this case, the quantification with Contact J of the contact length and the LD perimeter would allow the calculation of the LD-Mitochondria “transfer or communication” efficiency for each cell (Contact length/LD Perimeter Length) helping in the comparison between cells response.

In conclusion, the described image analysis workflow unveils a wide range of possibilities in the automatic quantification of LD and mitochondria contacts and it also has been tested, and it is applicable, to the study of other organelles in 2D and 3D images. Obtaining contact regions together with multiple cell and organelles parameters allow building descriptive statistics of the cells response. Moreover, its application in a large number of images enables the use of High Content Screening and Analysis, highly increasing the quality and statistical confidence of the results.
Data availability
Underlying data
This project contains the trained model and data for Weka plugin and example images.

Software availability
Source code available from: https://github.com/UB-BioMedMicroscopy/ContactJ/tree/1.0
Archived source code as at time of publication: http://doi.org/10.5281/zenodo.4569935
License: CC0

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Data presented in this work is part of the published article.4

This paper is dedicated to the living memory of Anna Bosch.

References
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The authors present ContactJ, an ImageJ macro, to identify and quantify contacts between lipid droplets (LD) and mitochondria.

This type of analysis is usually performed using electron microscopy. The authors have found a way to carry out a similar study (taking into account the limitations of resolution) using confocal microscopy images.

The use of this type of image along with the combination of different analytical techniques and machine learning make this macro an interesting and novel work.

The authors clearly describe the workflow of the macro. I would like to highlight the effort that has been put into establishing a clear distinction between each part of it, which helps to monitor and check the results at all times. The figures shown are very clear and leave no room for doubt. Another strength of this work is that this macro has already been used to analyse the work done in a publication.

However, I want to comment on some minor aspects that could be subject to improvement:

Note that the link in reference 11 is broken. Please replace with: https://imagej.nih.gov/ij/plugins/colocalization.html

In the Implementation section, the macro configuration is clearly defined and the parameters that have been used in each step are well described. However, the type of machine learning algorithm used for the LD segmentation is missing.

In the Discussion section, it is mentioned that the study can be carried out for 3D images. Adding an example of this type in the test images file is recommended.

In this same section, the authors mention that this macro can be used to analyse a large number
of images (High Content Screening and Analysis). For this type of analysis, it would be helpful that the option to check if the cells are well-segmented could be activated or deactivated. This way, it could be used to check some images and then run the macro over the entire experiment unsupervised.

The macro is fast, and its code is well commented, which makes it easy to read and understand. Considering the different domains that a single type of image may have (such as changes in the intensity of the sample or noise), I suggest that the configuration parameters of the different methods become variables that are described at the beginning of the macro. Therefore, the macro gives the user the possibility, if needed, to quickly and easily change the parameters, avoiding changes within the code.

Similarly, in addition to converting the channels used each time into a variable, it is recommended to comment on the code and refer to the channels not only by their colour but also by what they contain, as sometimes the experts use different criteria for the colour that each channel represents.

As the macro progresses, the user can watch how several images are opened, which represent the intermediate steps. This intermediate image display slows down the macro, and it is suggested to use the “setBatchMode (true)” option whenever possible to avoid this effect.

In addition, it is observed that the intermediate images that have been generated in the different steps remain open until the final result is obtained. To improve the performance of the macro, it is recommended that they are automatically closed when they are no longer needed.

Regarding the use of GitHub to upload the macro, I recommend that the authors complete the README section, providing information on the installation of the macro, the requirements that are needed for the correct functioning of the macro, and a brief description of its use.

The macro gives a robust set of results, which offer the possibility of obtaining interesting relationships, as the authors have commented. However, I recommend adding the calculation of the standard deviation for both the mean of the perimeter of the LD and the mean of the length of the Contact. Since both means are already calculated, it is convenient to calculate the standard deviations for each one.

All this would improve and facilitate the use of this macro by others in a general, simple and clear way.

Is the rationale for developing the new software tool clearly explained?
Yes

Is the description of the software tool technically sound?
Yes

Are sufficient details of the code, methods and analysis (if applicable) provided to allow replication of the software development and its use by others?
Partly
A novel ImageJ macro script called ContactJ that allows for the automatic determination of contacts formed between lipid droplets (LD) and mitochondria in cells is presented. An interaction that is usually assessed by electron microscopy, involving a reduced number of biological events analyzed, is assessed in this work by the combination of fluorescence microscopy with automatic image analysis. This approach boosts the number of biological events analyzed. It turns the analysis more time-efficient by using colocalization methods combined with the segmentation of the LD, which extracts the contact region on the midplane of the LD that is in close proximity of mitochondria.

The limitation of using light microscopy to look at very small contacts is clearly stated. Nevertheless, the use of this imaging technique as an indicator of possible contact between different organelles is well accepted in the community. Light microscopy was already shown to provide more information about the contacts between LD and mitochondria due to the sample thickness that is bigger than the sample prepared for transmission electron microscopy.

ContactJ is presented as a well-established workflow. The authors combine tools previously
developed in an exquisite way, such as the trainable Weka segmentation classifier and the colocalization plugin, with the conventional object segmentation. Figure 1 is very self-explanatory of the workflow followed by the macro script. Another positive aspect is the detailed output of the macro script that allows for a complex and quantitative analysis of the contacts established between the LD and mitochondria. The Operation section description is well written, and no problems were found while running the macro script; it is a very simple and straightforward process.

Despite the positive aspects mentioned above, there are fewer positive points that need to be checked:

Regarding the strategy applied for the segmentation of the cells (page 3), most probably extra cell labeling could be used to facilitate this step. Usually, the staining of a cytoskeleton protein or the cell membrane staining is used to define the cells' boundary facilitating the cell segmentation step. The authors may consider such an approach in the sample preparation and imaging.

Not so thorough is the description of the code behind the workflow. The authors are sparse in describing the different steps that make up the workflow. Some of those steps are not even mentioned in the Implementation section of the manuscript, being only perceptible by reading or running the code line by line, which jeopardizes reproducibility. Likewise, the comments along the macro script are also sparse. A more detailed and extensive workflow description is needed, both in the manuscript and in the macro script. ImageJ good code practices suggest that you mention the ImageJ version and cite it in the associated publication.

The citation "channels from mitochondria and LD were intensity compensated" should be explained to be more explicit. The authors commented in the script (line 143) "calculate addition 0.2LD+0.2mitochondria", meaning that the intensity compensation was 20% of the original values. Is there a reason for assigning this value? Should it be considered as a variable dependent on the dataset?

In the Implementation section (page 4), it is stated that “the colocalized “contact” points between the mitochondria and LD 8-bit images (or stacks)”. It is not clear if an 8-bit image is mandatory for determining the contact points or the number of channels that may compose the image. These details should be clearly stated. Additionally, as the reference to use a stack of images is made, the dataset available to test the macro script should contain this type of image.

The available dataset for testing the code is unfortunately diminished. Only two very similar images are available for testing the script. The dataset for testing this tool should be bigger and the images must represent a real, heterogeneous population of cells to test the proposed script's execution and performance.

Following the same lines, the script should be tested for quality and performance and presented in the manuscript. A previous publication shows the analysis of hundreds of HEK293 cells by the application of ContactJ. However, a comparison between the ContactJ results and another analysis method (such as manual quantification or using proprietary software) is not presented. Moreover, the usage of ContactJ to look at LD-mitochondria contacts in different cells (primary cells such as neurons, or other cell lines such as PC12 or CAD cells) could be included. Such validation is of extreme importance to show robustness and accuracy on the data obtained by the application of
ContactJ.

Throughout the manuscript, the term *database* refers to the script output .txt file created upon the analysis (page 5). In our understanding, this terminology is incorrectly used since a database is a set of related tables. The authors can use the term *table* since the output is indeed a table.

Looking closer to the presented macro in ImageJ, some adjustments should be made. Along with the code, several hard-coded values are used, such as thresholding values and filter parameters. It is unclear how these values may be applied in different images or conditions. Maybe the authors should consider building a graphical user interface for prompting these parameters. Plus, there is no code error handling, not testing, for example, if the input is an 8-bit image with 3 channels. The algorithm premises that images are 8-bit depth, although this is not mentioned nor guaranteed along with the code.

There is likely an error in the option of removing cell ROIs (lines 180-190). When deleting the cells, the results table with cell measurements is not updated and the deleted ROIs are the last ones on the table and not the ones corresponding to the correct position in the image. This error affects the results obtained, so it should be corrected. Furthermore, the *Resulting txt file* needs to be truncated or deleted from the results folder. Otherwise, it will append the results of further analysis.

Running the ContactJ macro script takes some time (this should also be measured). The authors could improve the code performance by reducing the number of *for*-cycles and by closing the images while the code is running (not only at the end). Also, the analysis could be set in batch mode (true), showing only the images for quality control.

While ContactJ is being presented as a tool to look for contacts between LD and mitochondria, the translational application of this script is not obvious. It might strengthen the work if more broad applicability of the ContactJ is mentioned or, at least, speculated. We see the potential for the application of ContactJ on image analysis where interaction between different cell types (oligodendrocytes-neuron interaction, microglia-neuron interaction, yeast-macrophage interaction.), or between different cellular organelles is done. May the authors comment on these possible applications and add them to the Discussion section?

**Is the rationale for developing the new software tool clearly explained?**
Yes

**Is the description of the software tool technically sound?**
Yes

**Are sufficient details of the code, methods and analysis (if applicable) provided to allow replication of the software development and its use by others?**
No

**Is sufficient information provided to allow interpretation of the expected output datasets and any results generated using the tool?**
Partly
Are the conclusions about the tool and its performance adequately supported by the findings presented in the article?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Light microscopy image analysis specialist. Neuroscience.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

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