Method Article

An improved method for murine laser-induced choroidal neovascularization lesion quantification from optical coherence tomography images ☆

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A B S T R A C T

Laser-induced choroidal neovascularization (L-CNV) in murine models is a standard method for assessing therapies, genetics, and mechanisms relevant to the blinding eye disease neovascular or "wet" age-related macular degeneration. The *ex vivo* evaluation of these lesions involves confocal microscopy analysis. *In vivo* evaluation via optical coherence tomography (OCT) has previously been established and allows longitudinal assessment of lesion development. However, to produce robust data, evaluation of many lesions may be required, which can be a slow, arduous process. A prior, manual method for quantifying these lesions as ellipsoids from orthogonal OCT images was effective but time consuming. We therefore developed an OCT lesion quantification that is simplified, streamlined, and less time-consuming.

Specifications table

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Subject area:	Medicine and Dentistry
More specific subject area:	Animal models of eye disease
Method name:	Murine optical coherence tomography lesion quantification
Name and reference of original method:	R. S. Sulaiman, J. Quigley, X. Qi, M. N. O'Hare, M. B. Grant, M. E. Boulton, T. W. Corson. A simple optical coherence tomography quantification method for choroidal neovascularization. J. Ocul. Pharmacol. Ther. 31 (2015) 447454. https://doi.org/10.1089/jop.2015.0049
Resource availability:	Excel Template
	Video Explanation
	Macro
	ImageJ Software

Method details

Background

Choroidal neovascularization is a key pathobiological aspect of wet AMD. Features of this pathology are recapitulated in the widely used laser-induced choroidal neovascularization (L-CNV) mouse model. The advent of small animal OCT enabled longitudinal assessment of neovascular lesion formation. A previous method established simple OCT quantification of lesions [1]. Assuming an ellipsoid shape of the lesions, it allowed for the generation of lesion volume. This is done by pinpointing X and Y coordinates of 8 borders of the lesion to generate 4 distances, which are then used to calculate the ellipsoid volume of the lesion according to the formula volume $= 4/3 \times \pi \times a \times b \times c$, where *a, b*, and *c* are the 3 radii of the ellipsoid. To generate the 8 points, the former method required the evaluator to rest their cursor over each of the 8 locations and note the corresponding X and Y value which were copied down manually. This was repeated for the remaining points requiring in total 16 X and Y values to generate 1 single lesion volume. The manual notation of each individual border's X and Y coordinates was a bottleneck and prevented the evaluator to evaluate many lesions in a time efficient manner. We sought to make this process faster by introducing a way to click the 8 points and export the 16 coordinates so that they can quickly and easily be pasted into a custom Excel template that then immediately calculates the volume of each lesion.

Required and recommended materials

- Experimenter's OCT images of L-CNV lesions in both horizontal and vertical imaging planes (*i.e.,* perpendicular scans).
- Excel Template (required).
- ImageJ software (required).
- Macro (recommended).
- Video explanation (recommended).

Lesion imaging and image selection

L-CNV is induced as specified previously [2,3]. *In vivo* analysis consists of imaging the lesions from OCT in both horizontal and vertical planes of the fundus image. These planes allow for dimensions of the ellipsoid shape of the lesion to be estimated. The largest lesion diameter in each plane is used to image the lesion on perpendicular planes to allow for visualization of all dimensions of the lesion.
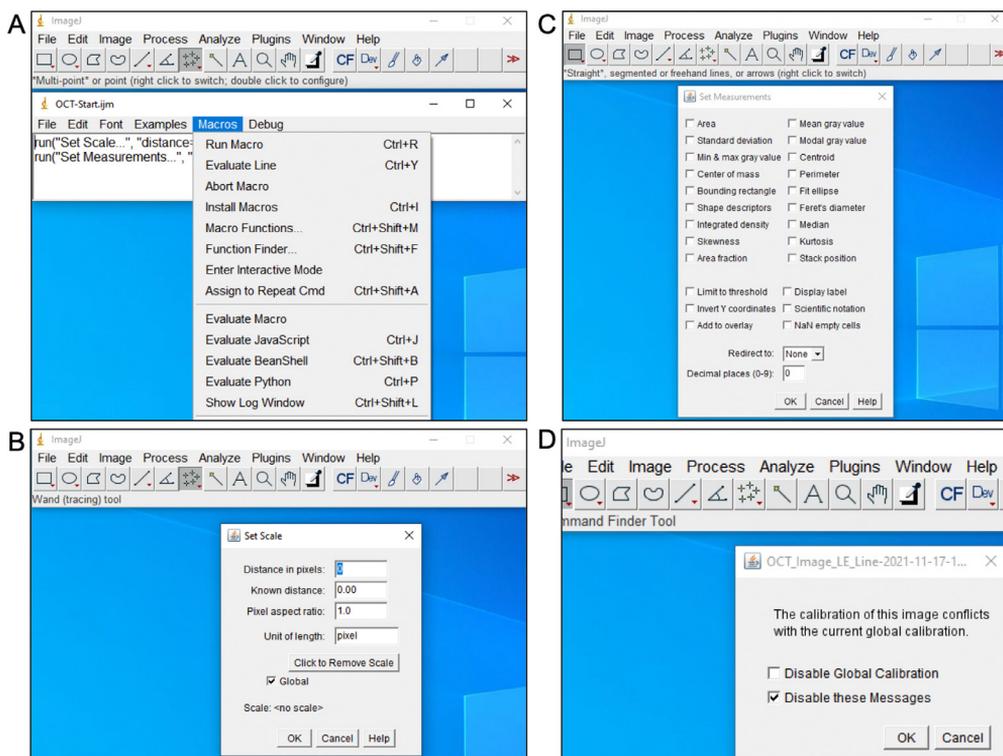
Fig. 1. Setup for image analysis in ImageJ. (A) Demonstration of how to use the macro. After dragging the macro to ImageJ, and pressing "Macros" on the new macro window, a page like this will appear. Press "Run Macro" to initialize macro. (B) Demonstration of setting scale. Ensure that global is checked. Note that if you place 0 for each option here, as is shown in the video, it will revert back to what is shown here, with "1.0″ in pixel aspect ratio, and "pixel" in Unit of length. (C) Demonstration of measurement set up showing that no measurements are checked, and that the decimal places is set to 0. (D) Notice that user will receive after opening their second image after turning on global. Ensure that global calibration is not disabled.

Two images in perpendicular planes will be used to generate the lesion volume. These OCT images of each choroidal neovascular lesion can be captured at various times; we usually assess lesions at day 7 and day 14 after laser treatment [1].

ImageJ set up

The steps within this section take place only the first time after opening ImageJ. They must be repeated only after closing the ImageJ software. With the first lesion image of interest opened within ImageJ, make the necessary settings changes within the software to begin the quantification. Use the macro "OCT-Start" which will set the scale and exporting results setup to work appropriately with the Excel template. To use this macro, simply drag the .ijm file into ImageJ and select "macros > Run Macro" (PC) or "run" (MacOS) (Fig. 1A). The macro will perform the following tasks automatically for you; they can also be done manually following these steps. Click "Analyze" > "Set Scale…" A table will appear; enter 0 for "Distance in pixels:", 0 for "Known distance:", and 1.0 for "Pixel aspect ratio:" Alternatively, click "Click to Remove Scale", then leave the "Unit of length:" as pixel. Turn on the "global" option by ticking the box. Press "OK" (Fig. 1B). Next go to "Analyze" > "Set Measurements…" A table will appear. Have all the possible boxes unclicked. The default in ImageJ will have "Area", "Mean gray value", and "Min & max gray value" turned on. Turn off these measurements and any others you may have turned on. Leave "Redirect" as "None" and set the desired number of "Decimal
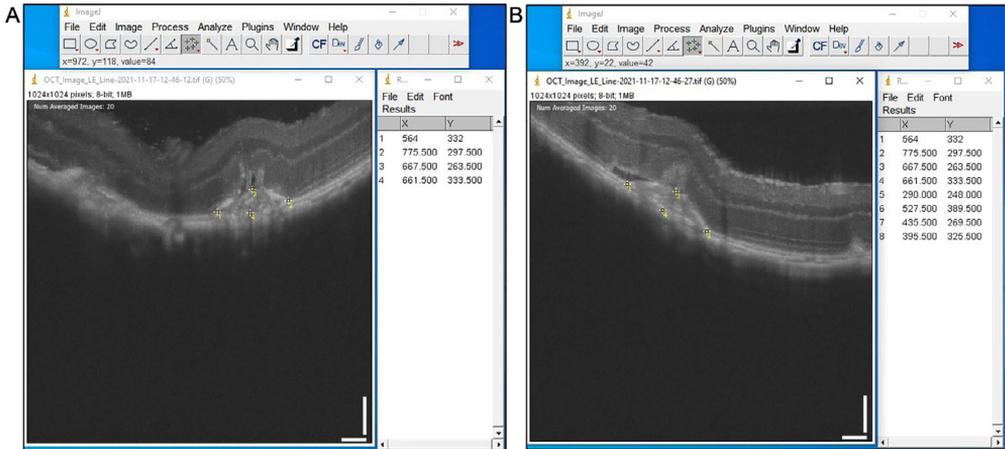
Fig. 2. Demarcation of lesion boundaries. (A) Demonstration of measurement export after 1 of 2 images. (B) Demonstration of measurement export after 2 of 2 images.

places" to 0. Click "OK" (Fig. 1C). These adjustments will allow for the coordinates to be exported as pixels which is the necessary export format for accurate volume calculation within the Excel template. While it may appear that having a larger number of decimal places would increase the accuracy, having this value set to any number other than zero will cause minor discrepancies and be less accurate. After completing the step below of the first image's quantification, you may receive the following notification from ImageJ that reads "The calibration of this image conflicts with the current global calibration." When prompted with this message, turn off "Disable Global Calibration" and turn on "Disable these Messages" This prompt will now no longer appear, and quantification can continue (Fig. 1D). Finally, select the Multi-point tool as this is the tool used for the next steps.

Quantifying the L-CNV lesions from OCT images

Now with the OCT image open in ImageJ and settings as specified in the previous instructions, begin the quantification. To model the lesion as an ellipsoid, we must obtain the pixel coordinates of the left, right, top, and bottom borders of the lesion as shown in Fig. 2A and B. Lesion borders can be defined by the borders of choroidal thickening of the hyperreflective lesion. This is done by clicking on the left-most position of the lesion, followed by the right-most position, followed by the top-most position and finally the bottom-most position. While there is no exact reasoning for this order, this order is imperative for use of the Excel template. Reveal these coordinates by clicking "Analyze" > "Measure" or simply by pressing the letter "m" on the keyboard, which will bring up the Results tab (Fig. 2A).

These measurements provide only half of the necessary information to quantify one lesion. Now, open the corresponding perpendicular OCT image and repeat the steps above (Fig. 2B). Click on any of the rows to select a single row. Select all the results table by pressing "control + a" (PC) or "command + a" (MacOS). With all results selected, cut them from the page with "control + x" (PC) or "command + x" (MacOS). They are now ready to paste into the Excel template.

Using the Excel template

The Excel template has 3 sheet types; the volume summary (VS) sheet, the standard lesion sheets (numbered 1–30 for up to 30 animals), and the Template sheet (T). The VS sheet has a column indicating the sheet name (column F). This column depends upon the actual sheet's name in the Excel document. These labels may be changed to allow for different organization, but it is important to note

that the sheet name (column F) and the actual sheet name must correspond for the VS to draw data from that respective sheet. Once the values are on the clipboard as instructed above, paste them into the Excel document. This is done by placing the cursor over the "1 of the point column (column E) of the appropriate lesion location within the Excel file and pasting from the clipboard with "control + v" (PC) or "command + v" (MacOS). Once the coordinates are pasted, the Excel document will convert pixel coordinates to μm coordinates. The Phoenix Micron IV system we use generates OCT images that have different scaling for X and Y. Specifically, 100 μm is scaled to 74 pixels in the x-axis and scaled to 113 pixels in the y-axis. This Excel document takes this scaling into account and scales the X and Y coordinates appropriately. For other OCT systems, these scaling factors in columns H and I will need to be manually changed. The coordinates then are used to calculate distances in μm and a volume in μm^3. While there are 4 distances measured for a lesion, two of those distances are the same dimensions imaged from different angles. The vertical distance of the horizontal and vertical images represents the same dimension (depth) of the lesion. Only one of these distances is used and, to maintain consistency, only the larger of the two distances is arbitrarily chosen to calculate the volume. The final volume of the lesion will be represented both in the lesion sheet and automatically in the VS sheet. Each lesion sheet allows for quantification of 10 lesions. We typically induce four lesions per eye [2,3] with possibility of up to two eyes per animal, however a location for a 5th lesion evaluation is provided if necessary.

Summary of steps from beginning to end

(1) Open the first OCT image within ImageJ.
(2) Perform the ImageJ setup manually or using OCT-Start macro (only required once per session).
(3) Click the following points on the first lesion in the following order.

   Left-most.
    Right-most.
    Top-most.
    Bottom-most.

(4) Press "m".
(5) Close the image in ImageJ.
(6) Open the corresponding perpendicular image in ImageJ.
(7) Click the following points on the first lesion in the following order.

   Left-most.
    Right-most.
    Top-most.
    Bottom-most.

(8) Press "m".
(9) Select all rows.
(10) Cut all results.
(12) Click the "1 of point column in corresponding Excel sheet.
(12) Paste results.
(13) Lesion volumes are automatically calculated and summarized in the VS sheet.

Method validation

This new method relies upon the formulas and validation of ellipsoidal OCT lesion quantification previously developed [1]. However, the previous method generated lesion quantification only after a few time-consuming steps. Specifically, it included resting the cursor over the lesion borders, manual notation of the lesion coordinates, and manual evaluation of which values to include or exclude. It also did not take into account the scaling variability caused by lesion images that are not horizontal.
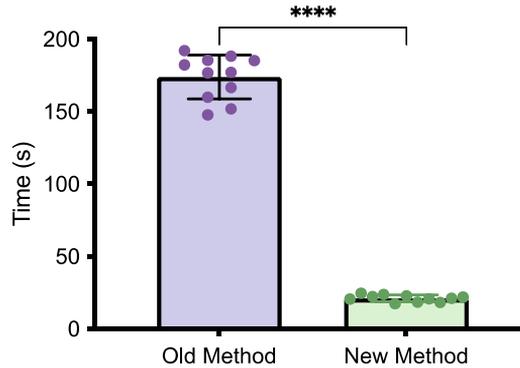
Fig. 3. Time difference to quantify individual L-CNV lesions from OCT images, using published, old method and new method. Mean±SEM, n = 11; ****$p < 0.0001$, t-test.
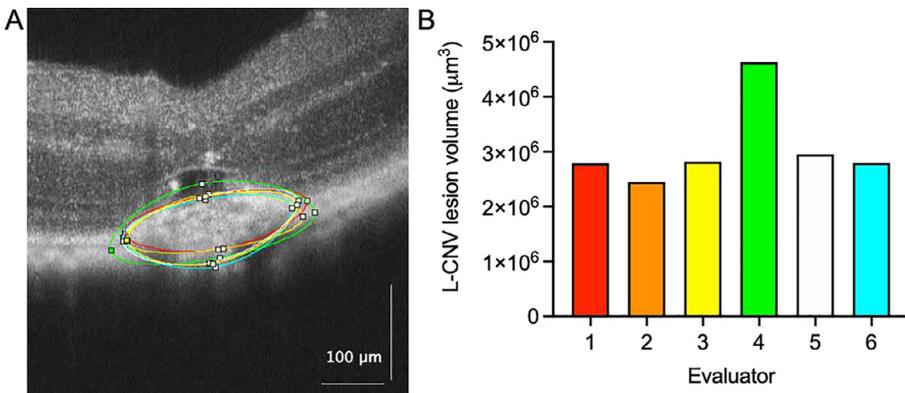
Fig. 4. Different evaluators' border coordinate locations and corresponding volumes. (A) OCT image of a lesion showing six evaluators' coordinate locations. (B) Calculated volumes from these coordinates, with colors matching those in (A).

To validate this new time-saving method, we evaluated several lesions using both the old and new methods. This allowed us to identify that both methods generated identical lesion size. To assess the speeds of the two methods, 11 lesions of 3 eyes were evaluated using both the old and new methods (Fig. 3). The new process demonstrated improved time efficiency and precise quantification of OCT lesion volume. The time to calculate the volume of a lesion previously was 2 min, 54 ± 15 s (mean±SD). The new method averaged 21 ± 2.3 s, or ~8.3 times faster (paired t-test, $p < 0.0001$, $n = 11$). Subjectively, evaluators noted that the new method allowed for greater reliability in lesion border identification.

Limitations of this new method include that this method was developed using the Micron IV OCT system and would potentially need to be adapted to match different OCT imaging software. Additionally, while we anticipate that this method would work with different species used for L-CNV, such as rats, our method thus far has only been tested on the mouse model. The lesion border definition is subjective and is a potential limitation on the consistency of lesion volume if multiple evaluators perform the quantification. We recognized this limitation and to address it, 12 lesions were sent to various trained evaluators. Their lesion borders were all placed on the lesion with their corresponding volumes displayed (Fig. 4). Expert consensus indicated two important factors to maintain consistency between evaluators. First to not include any edema, fluid, or bleb to be part of the lesion. This usually occurs at the superior most border, and it is important to note the

superior border as the beginning of hyperreflective thickening of the choroid. Second, it is important to remember the ellipsoid shape of the lesion as evaluators note each border location. Following these steps to implement the new method will allow users to quantify lesion volume on OCT in a precise, time-efficient manner.

Ethics statement

Animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Female mice were used for the analyses shown, but the methods are equally applicable to male mice.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2022.101809.

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