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Bioloc 3D (B3D) : Protocol for 3D automated analysis of colocalization in microscopy V.1

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Thibault Dhellemmes[1], Rémi inet[1], Fabrice Cordelières[2], Marc Landry[1]

[1]Université de Bordeaux - Institut des Maladies Neurodégénératives (IMN) / UMR 5293;
[2]Bordeaux Imaging Center

Bioloc3D (B3D)

Thibault Dhellemmes
Institut des Maladies Neurodégénératives (IMN)

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External link: https://github.com/Bioloc3D

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Protocol status: Working
This protocol has been used in different published or on-going different studies. In addition, the results obtained in the methods paper can be considered as an experimental validation.

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Abstract

This protocol introduces Bioloc3D, a fully open-source tool designed to quantify fluorescent labeling across entire stacks and automate statistical analysis. It offers a solution to the lack of tools capable of colocalization quantification between multiple identified elements in 3D. Expected results includes graphical representations of the results through images and estimation plots.

Materials

Raw image stacks with fluorescent signals to analyze in any format adapted to ImageJ.

Linux/Mac/Windows computer with ImageJ, Excel and Bioloc3D-Plt installed. ImageJ installation file is available online at https://imagej.net.

Before start

To operate in the best condition, the minimal requirement for colocalization analysis is a stack of 2 channels with 2 images (i); in 8-bit color depth (ii); preferably in TIFF format (iii)

Installation

3m

1 Download the *B3D-Imaging (B3D-Img)* ImageJ macro at https://github.com/Bioloc3D/B3D-Img

2 Install *B3D-Img* in ImageJ macro library, by drag/drop the .ijm file in the toolsets folder of the application (*Fiji/ImageJ → macros → toolsets*)

3 Install latest version of *3D Objects Counter* plugin available online on GitHub, by drag/drop the .jar file in the plugins folder of the application (*Fiji/ImageJ → plugins*)

4 Launch ImageJ software

Software	
ImageJ (Fiji)	NAME
Windows 10	OS
National Institutes of Health (USA)	DEVELOPER
https://imagej.net/software/fiji/downloads	REPOSITORY
https://imagej.net/software/fiji/downloads	SOURCE LINK

5 Through ImageJ install *MorphoLibJ*, *3D ImageJ Suite* and *Read and Write Excel* additional plugins available in plugin list (*Help → Update... → Manage Update Sites → tick plugins → Apply and Close)*

6 Close ImageJ software

7 Download the *B3D-Plt* application depending of the user operating system at https://github.com/Bioloc3D/B3D-Plt

Software	
Bioloc3D	NAME
Windows / MacOS	OS
Thibault Dhellemmes / Rémi Kinet	DEVELOPER
https://github.com/Bioloc3D	REPOSITORY
https://github.com/Bioloc3D	SOURCE LINK

Image analysis : *B3D-Imaging (B3D-Img)*

8 **Launch B3D toolset in ImageJ software** 30s

8.1 Start ImageJ software

8.2 Click on the "More Tools" menu (≫) and select *B3D-Img*

9 **Defining the correct parameters** 2m 30s

9.1 Click on B3D parameters icon (cf. Supplementary Fig. 1A)

9.2 Select the stack to test the parameters and the folder in which test data will be saved. Parameters must be tested in an iterative matter, select one channel from the stack (cf. Supplementary Fig. 1C, Step 1 of the diagram).

9.3 Click "OK"

9.4 Define the threshold adapted to the signal of the selected channel (cf. Supplementary Fig. 1C, Step 2 of the diagram).

9.5 Click "OK"

9.6 Use the threshold defined in step 11 and define minimal and maximal size threshold to test (Supplementary Fig. 1C, Step 3 of the diagram). If needed, define minimal area for soma detection (cf. Supplementary Fig. 1C, Step 4 of the diagram)

9.7 Click "OK": Test analysis is launched

10 **Analysis of one stack and raw data export**

! CAUTION As discussed in the “advantages and limitations” section, time of analysis depends on several aspects, including computation power of the machine, size, potential number of objects to count per channel and signal-to-noise ratio of your images. To estimate the time of each step of the procedure, we used a stack presenting the following characteristics: size= 1024 × 1024; z= 25; objects counted per channel= 1000. In addition, sample is presenting a moderate signal-to-background (SBR) but a low signal-to-noise ratio (SNR).

10.1 Click on B3D analysis icon (cf. Fig. 2A)

10.2 Select the folder containing stack(s) to analyze and numbered your batch of analysis (Fig2C, section 1 on the diagram). Normalization of the name of exported data files is compulsory for the automatic computation. If more than one file is present in this folder, "headless" option must be defined on "Yes"
! CAUTION Select folder must contains only tiff file(s) before starting the analysis in ImageJ.

10.3 Click "OK"

10.4 Enter parameters defined in step 9, in the GUI (Fig. 2C, section 3 on the diagram) and select options for quantification based on expected information

10.5 **Option A :**
Simple quantification (3D-Simple) of primary channels, without quantification of additional features (control of colocalizations sites and soma)
(i) Select "Yes" for "Simple segmentation of C1 & C2:"

30s

(ii) Select "No" for "Check colocalization with an additional channel"
(iii) Select "No" for "Complete counting with an additional channel"

10.6 **Option B :**
Complete quantification (3D-OC) of primary channels, without quantification of additional features
(i) Select "No" for "Simple segmentation of C1 & C2:"
(ii) Select "No" for "Check colocalization with an additional channel"
(iii) Select "No" for "Complete counting with an additional channel"
3m

10.7 **Option C :**
Simple quantification (3D-Simple) of primary channels, including quantification of additional features
(i) Select "Yes" for "Simple segmentation of C1 & C2:"
(ii) Select "Yes" for "Check colocalization with an additional channel"
(iii) Enter parameters for colocalization control (threshold and size)
(iv) Select "Yes" for "Complete counting with an additional channel"
(v) Enter parameters for soma counting (minimum area and threshold)
40s

10.8 **Option D :**
Complete quantification (3D-OC) of primary channels, including quantification of additional features
(i) Select "No" for "Simple segmentation of C1 & C2:"
(ii) Select "Yes" for "Check colocalization with an additional channel"
(iii) Enter parameters for colocalization control (threshold and size)
(iv) Select "Yes" for "Complete counting with an additional channel"
(v) Enter parameters for soma counting (minimum area and threshold)
3m 30s

10.9 Click "OK"

10.10 (Optional) Click “Help” to be redirected to GitHub repository

11 **(Optional) Proofreading of 3D quantification on one stack**
5m

! CAUTION If only one stack is present in the folder, illustrations of the results can be displayed by selecting “No” for headless mode (Fig2C, section 2 on the diagram).

11.1 For a more precise proofreading apply option B or D (Steps 10.6 or 10.8)

11.2 Overview the different output stacks with the excel of the data aside to check if it counts what you wanted

Computation, plotting and statistical analysis : *B3D-Plotting (B3D-Plt)*

12 Open the B3D-Plt application

13 Click on "Load file" and select the folder containing the Excels to analyze

14 Click on "Save file" and select the folder in which graphs and analysis will be saved

15 Click on the drop-down menu and select the number of conditions you have to analyze

16 In the bars that appear, enter the name of the condition and the color you want to associate with it (seaborn or hexadecimal #FF0000 style, but can stay empty)

Seaborn color link : https://seaborn.pydata.org/tutorial/color_palettes.html

17 Click on "Launch analysis"

15m

! CAUTION Timing indicated for this step has been tested on a file containing 60 excel files

18 The name of the document currently analyzed is print to monitor progress

19 Analysis end is notified through a pop-up window

20 Find the figures and Excel in the folder selected in step 14