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Quantification of fluorescence intensity of antisense constructs within Bodo saltans and its intracellular symbiont

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We use this protocol and it's working

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Abstract

The purpose of this Protocol is to quantify the intensity of fluorescence resulting from antisense molecules within a microeukaryote *Bodo saltans* and its intracellular symbiont *Candidatus* Bodocaedibacter vickermanii[1] following incubation. This protocol is specific to two-channel fluorescent images of objects contained within larger objects. It can be used directly to analyse images of objects of similar size.

The images were recorded using a confocal laser scanning microscope as single focal plane images, containing three channels: 1) fluorescent antisense molecule, 2) DAPI, 3) transmitted light.

The antisense molecule intensity in channel 1 is to be measured. The DAPI channel allows the segmentation of both, the *B. saltans* nucleus and the symbionts. The transmitted light channel is used for general reference.

This Protocol.io contains two sample files (.czi), which open in Fiji and can be re-analyzed using the steps described here. A separate word file with all the steps listed can also be found attached to this Protocol.io.

Attachments

FILE
sample_image.czi
642KB

FILE
sample_image2.czi
652KB

DOCX
CHROSTEK-Protocols_i...
28KB

Materials

B. saltans cultures
100 and 8 µm filters
cerophyll medium
Low melting temperature agarose
PBS
96-well plates
microscope slides and coverslips
Fluorescent antisense molecules
Hoechest 33342 (or other DNA dye)
Vectashield (or other mounting medium)

Standard laboratory equipment: pipettes, hemacytometer, tabletop centrifuges, rotary shaker
Confocal microscope
Computer with FIJI software

Incubation of *B. saltans* with antisense molecules

1 Filter B. saltans culture through 100 and 8 µm filter.

2 Harvest the cells by centrifugation at 1200 × g for 12 mins at 19 °C.

3 Wash the cells with 10-15 ml sterile filtered (SF) 1×PBS and centrifuge as above.

4 Re-suspend the cells in 5 ml SF 1×PBS, count the cells using hemacytometer and take the volume of cells which contains 5×106 cells for every 10 samples (5×105 cells per sample is required). Preparing at least duplicates for each treatment and including the sense, scrambled and no-treatment control as well as 20% excess in case of pipetting errors means that at least 10 samples have to be prepared for each experiment.

5 Centrifuge at 1200 × g for 12 mins at 19°C.

6 Remove the PBS and resuspend the cells in SF cerophyll medium so that the volume of the medium and the molecules tested is 100 µl per sample. E.g. if you add 10 µl of molecules under investigation per sample you have to use 90 µl of medium per sample. For ten samples, resuspend the cells in 900 µl of medium.

7 Move 90 µl of the cell suspension to a sterile 2ml tube.

8 Add the tested molecules to the B. saltans cells in 2 ml tube. To have final concentration of 50 µM of the molecule we use 10 µl from the500 µM stock. Mix well the tested molecule and cells by pipetting and then place the tubes in the incubator.

9 After 24 h prepare samples for imaging. Centrifuge the 2 ml tube containing the cells (1200 × g for 12 mins at 19°C), remove the supernatant and leave 30 µl of liquid in the tube. Resuspend the cells and divide the volume in two wells of a 96 well plate, 15 µl in each well. Add 15 µl of low melting temperature agarose (e.g. Thermo Fisher Scientific) to each well and mix. Let it set for a few seconds. The following steps will be done similarly for both wells in order to make two slides for each treatment.

10 Add Hoechest 33342 (Thermo Fisher, 1:2000 in PBS) to the well for 10 minutes.

11 Rinse and wash 1x with PBS.

12 Remove the agarose from the well with clean forceps and place it on a microscope slide.

13 Add a drop of a mounting medium (eg. Vectashield, Vector Laboratories), and flatten the agarose as much possible using the coverslip.

Imaging *B. saltans* using an LSM 880 Laser Scanning Confocal (Zeiss)

14 When using a Confocal microscope, always follow local facility rules. Microscopes are expensive, sensitive, and choosing wrong settings can lead to artifacts and non-processable results.

15 Turn the microscope and all the systems on according to local rules. The order of turning individual components on matters.

16 View your samples using the eyepiece and light source, to make sure you can find your samples and
focus. Due to its small size, *B. saltans* is best visualized using a high numerical aperture (e.g. 63×, 1.40 NA) objective.

17 Switch to scanning mode, acquire images of all of your samples and negative controls using the same settings.

Image preprocessing

18 Open raw image in Fiji[2]

19 Duplicate DAPI channel [Shift + D].

20 Subtract the background (out-of-focus light and/or autofluorescence) via rolling ball Background subtraction with a radius of 25 pixels.

Image segmentation

21 Duplicate background subtracted DAPI channel.

22 Use global thresholding algorithm [Fiji > Image > Adjust > Threshold > IsoData][3] to segment the areas
positive for DNA staining, which are the symbionts and the *B. saltans* nucleus, from the background and convert image to a binary mask.

23 Run [Fiji > Process > Binary > Watershed] operation to separate touching objects.

24 Run connected component analysis [Analyze Particles] with a size thresholding of 0-300 pixels, the [Add to manager] and [Exclude on Edges] options enabled. The size exclusion should ensure that the identified objects are only the symbiont and not the *B. saltans* nucleus. his generates a set of regions of interest (ROIs).

25 Save the bacteria ROIs.

26 Select raw image and duplicate the fluorescent antisense molecule channel [Shift + D].

27 Use global thresholding algorithm [Fiji > Image > Adjust > Threshold > Mean] [4] to segment the areas
positive for antisense staining from background and convert image to a binary mask.

28 Run connected component analysis [Analyze Particles] with a size thresholding of 1000-Infinity pixels and the [Add to manager] and [Exclude on Edges] options enabled. The size exclusion should ensure that the identified objects are only the symbiont and not the *B. saltans* nucleus. This generates a set of regions of interest (ROIs), usually only one.

29 Rename the ROI, e.g. to “cell”.

30 The *B. saltans* ROI and the individual symbiont ROIs are combined using the XOR operator to generate a single ROI containing the *B. saltans* cytoplasm only, excluding the symbiont. With two inputs, XOR is true if and only if the inputs differ (i.e. one is true, one is false).

31 Rename the ROI to “cell-minus-bacteria”.

Measurements

32 Activate the following parameters to be measured via [Fiji > Analyze > Set Measurements…]:
1. Area
2. Mean gray value
3. Modal gray value
4. Integrated density
5. Median
6. Activate Display Label option

33 Select raw image.

34 Set channel to antisense channel.

35 Deselect ROIs in the ROI Manager (or select all).

36 Measure set parameters by [ROI Manager > More > Multi Measure].

36.1 Inactivate [Measure all 3 slices] and [One row per slice].

37 Save results table.

Automation

38 The whole process can be automated using a Fiji macro script: https://github.com/Marien-kaefer/General_Fiji_macros/tree/main/BodoSaltans

References

39
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