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FISHji: New ImageJ macros for the quantification of fluorescence in epifluorescence images

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ABSTRACT

Fluorescence in situ hybridization (FISH) is based on the use of fluorescent staining dyes, however, the signal intensity of the images obtained by microscopy is seldom quantified with accuracy by the researcher. The development of innovative digital image processing programs and tools has been trying to overcome this problem, however, the determination of fluorescent intensity in microscopy images still has issues due to the lack of precision in the results and the complexity of existing software.

This work presents FISHji, a set of new ImageJ methods for automated quantification of fluorescence in images obtained by epifluorescence microscopy. To validate the methods, results obtained by FISHji were compared with results obtained by flow cytometry. The mean correlation between FISHji and flow cytometry was high and significant, showing that the imaging methods are able to accurately assess the signal intensity of fluorescence images. FISHji are available for non-commercial use at http://paginas.fe.up.pt/~nazevedo/.

1. Introduction

In science, digital images are a crucial piece of information. The ability to process and analyse the large volume of images produced by the plethora of microscopy techniques available raises the need for specialized software tools [1]. Images should be processed in a systematic and standardized way, such that results are comprehensible and reproducible. There are a number of commercial and open source image processing tools available, and the range of implemented functionalities is significant [2–10]. However, the general purpose of most of these tools hampers domain or application-specific use by laymen, i.e. it is not straightforward for a researcher to use such general software to analyse biological images, without understanding the specifics of the image processing and analysis algorithms. As such, the development of end-user biological image analysis tools is considered useful, essentially for analyzing fluorescence results obtained by diagnostic methodologies for microorganisms. These methodologies or techniques use staining with fluorescent binding dyes which are then visualized using fluorescence microscopy. In order to have a quantitative analysis of the results, the fluorescence intensity needs to be accurately measured.

Fluorescence in situ hybridization (FISH) is one of the methodologies in which a quantitative validation of results is required. FISH has been used as a molecular tool for the analysis and detection of microorganisms [11]. Since the first application in 1989 by Delong et al. [12], this method has undergone several improvements in

Abbreviations: FISH, fluorescence in situ hybridization; LNA, locked nucleic acid; LoG, Laplacian of Gaussian; MFI, mean fluorescence intensity; PI, propidium iodide; ROIs, regions of interest; TSA, trypticase soy agar; 2′OMe, 2′-*O*-methly RNA.

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order to overcome limitations, such as low fluorescence intensity [13,14]. This evolution of the method has led to the use of new types of nucleic acids such as locked nucleic acid (LNA) and 2′-*O*-methly RNA (2′*O*Me) [14,15]. Other chemical modifications within nucleobases, ribose or deoxyribose, and within phosphate moieties have been also introduced to improve the resistance to nucleases present in the cells or body fluids [16–18]. In FISH experiments, the evaluation of the results is based on microscopy, which the quantitative assessment is performed by the researcher, which is dependent of the observer interpretation [19], or on flow cytometry, that provides an automated quantitative assessment but requires the use of expensive equipment and is only applicable for cells in suspension. Therefore, computerized image processing tools offer the most promising and versatile approach to minimize costs and overcome the variability of human microscope analysis. Daime et al. developed a digital image analysis program (DAIME) especially used for microbial ecology [20]. Although, DAIME has different features such as analyzing 2D and 3D microscopy datasets of microorganisms stained by FISH, this program is complex, is generally used for environmental bacteria [21–23] and no validation studies against flow cytometry have been made so for. ImageJ is another well-known and publicly available image processing tool (http://rsbweb.nih.gov/ij/). It has released many plugins and macros useful to biomedical image processing [24], however, also in this case there are no comparative studies against flow cytometry to confirm the use of this software in fluorescence quantification of microscopic images.

This article describes five different methods for the quantification of fluorescent intensity in microscopic images (named FISHji). As a case study, LNA probes were applied in different hybridization conditions, and evaluated by microscopy and cytometry approaches. The biological goal was the detection of 16S rRNA in the clinically relevant bacterium *Helicobacter pylori*. The developed methods are freely available and integrated in the ImageJ software package.

2. Experimental procedures

2.1. Workflow of FISHji methods and validation

In this work, three semi-automatic and two automatic FISHji methods were created (Fig. 1). To validate these methods, the FISH procedure was performed both in bacterial suspensions and in attached bacteria. Eighteen LNA probes specifically designed for *H. pylori* were used at different temperatures, in order to obtain a large range of fluorescence intensities. All images obtained by microscopy (attached bacteria) were analysed by the five FISHji methods and these results were compared with cytometry data (bacterial suspensions) hybridized under the same conditions (temperature and buffer). Afterwards, all results were statistically analysed and the correlation between each FISHji method and flow cytometry was performed. Finally, an extra validation was then performed using another fluorescence staining procedure (propidium iodide) for the FISHji methods.

2.2. Fluorescence analysis and FISHji architecture

In terms of operational mode, there are three semi-automatic (FISHji1, FISHji2 and FISHji3) and two automatic approaches (FISHji4, and FISHji5) (Fig. 2). For all methods, the channels of the original RGB (red, green, and blue light) images were separated in order to analyse the channel where fluorescence is emitted.

The methods consist of three sequential steps: optimization, segmentation and analysis, and measurement steps. In the optimization module, pixel-based treatments are performed in order to highlight the regions of interest (ROIs) and allow the removal of artefacts. In the segmentation module a default black and white threshold supports cell segmentation. The ROIs are outlined and artefacts are discarded by the command "Analyse Particles". The mean fluorescence intensity (MFI) is then calculated as the average of each ROI (Fig. 2).

For FISHji1, optimization treatments are not applied. In the FISHji2 and FISHji3, a background subtraction based on the "rolling ball" algorithm (radius = 80 pixels) is performed to the green channel in order to reduce background heterogeneity and the presence of artefacts [25]. In the FISHji2 method, the obtained image is segmented and further analysed to determine the MFI of the image. In FISHji3, the brightness and contrast features of the corrected image are manually adjusted using the "B/C Adjustment" (Brightness and Contrast) tool to further enhance the foreground. Subsequently, the adjusted image is thresholded and a binary mask is created. The ROIs present in the mask are redirected to the corrected image and MFI is quantified by the ROI manager interface.

In the automatic FISHji4 and FISHji5 methods, the corrected image is convoluted using a Laplacian of Gaussian (LoG) filter (9×9 kernel) [26]. In the FISHji5 method, an automatic B/C adjustment of the corrected image is performed before the convolution. First, a B/C adjustment based on the image's histogram is automatically applied and then the convolution step was performed. In addition, the binary operation "Fill Holes" is applied to fill intra-aggregate spaces and maintain ROI integrity, providing an optimal binary mask.These optimization steps (except "Rolling Ball") change the real pixel values of the image, including the ROIs. So, a duplicated image is taken from the corrected image in order to allow enhancement of foreground pixels without compromising MFI quantification. After segmentation, the ROIs defined by the binary mask are redirected to the duplicated image, and only then the MFI is calculated. Thus, the MFI is determined from the real pixel values of the Corrected Image, outlined by the binary mask overlay.

All FISHji methods were implemented as macros in ImageJ (version 1.49o or higher). The analysis parameters were set as 100–600 pixels for the minimum and maximum valid object size (i.e. pixel$\hat{2}$) and 0.1–0.7 for the range of circularity i.e. $4 \times \text{pi} \times (\text{area}/\text{perimeter}\hat{2})$. These values were established by experimental trials in *H. pylori*, and taking into account the real size and shape of the bacterium. Images were not calibrated since all had the same resolution (1392×1040 cm).

FISHji4 and FISHji5 macro can be found on the webpage http://paginas.fe.up.pt/~nazevedo/.

2.3. Overlap index

Overlap index Ω is a measure of activating regions' spatial overlap and symmetry. This value was determined using two different edges (image processing technique for finding the boundaries of cells within images detecting discontinuities in brightness): the defined by the ImageJ method and the manual one as the ground truth [27]. After obtaining the binary images of each ROI using ImageJ, a Matlab® script (data not shown) was developed to overlap the images and subsequently to calculate the overlap index Ω according to the following equation [28]:

$$\Omega = \frac{\text{TP}}{\text{TP} + \text{FP} + \text{FN}} \quad (1)$$

where TP is the true positive area, FP is the false positive area (FP) of the ROI and FN is the false negative area or background of the ROI.

2.4. Oligonucleotide probe design and synthesis

Fluorescein-labeled locked nucleic acids oligonucleotides DNA/LNA and LNA/2′-OMe RNA chimeras with phosphates (PO)