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SOFTWARE TOOL ARTICLE

SAPHIR: a Shiny application to analyze tissue section images

[version 1; peer review: 2 approved with reservations]

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Abstract

Study of cell populations in tissues using immunofluorescence is a powerful method for both basic and medical research. Image acquisitions performed by confocal microscopy notably allow excellent lateral resolution and more than 10 parameter measurement when using spectral or multiplex imaging. Analysis of such complex images can be very challenging and easily lead to bias and misinterpretation. Here, we have developed the Shiny Analytical Plot of Histological Image Results (SAPHIR), an R shiny application for histo-cytometry using scatterplot representation of data extracted by segmentation. It offers many features, such as filtering of spurious data points, selection of cell subsets on scatterplot, visualization of scatterplot selections back into the image, statistics of selected data and data annotation. Our application allows to quickly characterize labeled cells, from their phenotype to their number and location in the tissue, as well as their interaction with other cells. SAPHIR is available from: <https://github.com/elodiegermani/SAPHIR>

Keywords

Tissue cellular quantification, spatial cellular profiling, cell-cell interactions, scatterplot, histo-cytometry, image cytometry

This article is included in the **Bioinformatics** gateway.

This article is included in the **NEUBIAS - the Bioimage Analysts Network** gateway.

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Any reports and responses or comments on the article can be found at the end of the article.

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Author roles: **Germani E:** Software, Writing – Original Draft Preparation; **Lelouard H:** Conceptualization, Project Administration, Resources, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; **Fallet M:** Conceptualization, Project Administration, Resources, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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Introduction

The identification, localization and quantification of cell subsets in tissue is a difficult but essential task for biologists to understand spatial cellular organization in different settings (e.g. homeostasis vs inflammatory diseases or cancer). Advances in optical microscopy allow image acquisitions with more than 10 channel measurements using spectral fluorescence imaging or multiplex imaging combined with z-axis optical slices of tissue sections ranging from 10 μm to more than 200 μm when clearing methods are used¹⁻⁴. Analysis of such complex images is very challenging due to the size and complexity of data. It requires image segmentation in 3D that can be further improved using deep learning-based segmentation⁵⁻⁷. Then, like flow cytometry, complex image analysis can benefit from scatterplot representations that allow to gate cells of interest^{2,8}. Surprisingly, in existing software, this scatterplot representation is rarely interactive with the image itself, although this would allow to locate selection results back into the image but also to filter or correct results and fine-tune the gates defining cell populations to obtain in return a better visualization of them into the image.

To this end, we developed the Shiny Analytical Plot of Histological Image Results (SAPHIR), an R/Shiny application for the quantitative analysis of tissue section images. Since image segmentation is a complex task in continuous development and highly dependent on image quality and information, the integration of a single type of segmentation method in an image analysis application is not necessarily recommended. Therefore, we decided to separate segmentation from SAPHIR, but we provided two Fiji macro examples with associated images, which can be used to perform this task before running SAPHIR. SAPHIR offers many features such as interactive scatterplots with the image and data filtering and correction as described below.

Methods

Data requirement

The segmentation required to use the application SAPHIR has been carried out under Fiji⁹, with custom-made macros, which are available on GitHub in the [Demonstration Files](#). Cell nucleus detection was used for segmentation and fluorescence intensity signal of each channel within a circular ring around nuclei was determined to define cell of interest (COI) phenotype. In addition, regions of interest were defined using DBSCAN (optional)¹⁰. The segmentation process of the image to be analyzed should create a csv result file containing the channel intensity values (ranging from 0 to 255) and, if required, the positioning (x, y, z) linked to each COI. Another file, termed ROI.zip, should contain the image contour of each COI. Importantly, many other morphological measurements like area, roundness, or solidity as well as other information (COI belonging to a region of interest, COI interaction with other cells, COI centroid position) can be added to the segmentation result file. Finally, the minimal requirements to run the application are the image in TIFF with multiple channels and optionally slices, the segmentation result file with COI identity in csv and the roi.zip file containing contours of each COI. A legend file with all channel information can also be provided (optional).

Operation

SAPHIR is built in the R programming language, version 4.0.2, and uses many packages available on CRAN (e.g. shinydashboard, ijttiff, magick, ggplot2, plotly) and one package (EBImage) available on Bioconductor. It has been tested on macOS and Windows 10. A user flowchart from image acquisition to obtention of quantitative data of COI with SAPHIR is shown in [Figure 1](#).

Implementation

The first step of SAPHIR is either to run a segmentation program to obtain appropriate files or to load the three required files (image.tif, roi.zip, results.csv) and if needed the legend (csv file) in the “select your results” menu of the application. Here, result, region of interest (ROI) and legend files were obtained with an in-house-made segmentation macro developed under Fiji.

The first tab of the “Plot to Image” menu is shown in [Figure 2](#) and allows the users to filter their data depending on one or two parameters displayed on histogram or scatterplot, respectively. This can be used to remove cells that have been badly segmented. Indeed, such cells display an aberrant area or volume (doublets or aggregates in red in [Figure 2](#)). Here, only cells within the Gaussian curve were retained (in blue in [Figure 2](#)). This can also be used to work only on given subpopulations filtered based on their location, interaction with other cells or other criteria (see use case section below). Then, filtered cells can be separated into subsets thanks to a two-parameter (most often channel intensities) scatterplot ([Figure 3A](#)). In addition, users can add a third parameter that is displayed on the scatterplot through a change of symbol shape for each COI beyond a threshold defined by the users for a given parameter value ([Figure 3A](#)).

Gating can be made easily with quadrants, rectangles and lassos in single or multiple selection mode. Interactivity between scatterplot and image is optional to avoid slow interaction when high-resolution images are used. In the latter case, we recommend users to perform their gating before allowing their selection to be shown in the image by ticking the appropriate box. Users can easily change the slice and the displayed channels with buttons to monitor selected COI spatial distribution and fluorescence intensity in the image ([Figure 3B](#)). Moreover, several options are available such as cell identity display or channel overlay ([Figure 3B](#)). Finally, statistics of the gated COI are downloadable, and gates are saveable for further analyses.

The SAPHIR menu “image to plot” allows to select a region in the image and to display cells of this region on a two-parameter scatterplot ([Figure 4](#)).

Finally, the menu “Annotation” allows, based on the previous analyses, correction of the data from the result csv file but also from the scatterplot-gated cells when saved in the “plot to image” menu. For each selected cell, a cropped image of this cell is displayed, and users can change its parameters if necessary ([Figure 5](#)).

Figure 1. Flow chart of tissue image analysis from image acquisition and segmentation (left side) to extracted data analysis with SAPHIR (right side).

Figure 2. Menu "Plot to Image" of SAPHIR application. The data-filtering tab allows the selection of cells to be analyzed based on one or two parameters. Based on the cell area parameter, only cells that displayed a conventional cell area (in blue) were selected. Badly segmented cells (large area in red) were discarded.

Figure 3. Menu “Plot to Image” of SAPHIR application. (A) Scatterplot of two channel intensity parameters used to gate COI. User can add a third parameter that is displayed on the scatterplot through a change of symbol shape for COI beyond a threshold defined by the user. (B) Visualization of selected cells in the image. Some of the available options are shown (displayed channel(s), channel overlay, cell identity number, brightness).

Figure 4. Menu “Image to Plot” of SAPHIR application. Selection of one region in the image (left) and visualization of COI of this region in a corresponding scatterplot where two parameters are displayed (right).

Figure 5. Menu “Annotate your data” of SAPHIR application. The results to annotate can be selected based on the Plot to Image scatterplot gated cells that have been previously saved (left panel). Each cell of the selection can be visualized in the image (right panel) with the possibility to change image size (magnification), the displayed channel(s), the slice (z optical section) and the brightness. Based on the different analysis, results for the selected COI can be modified and final results saved and exported.

Use case

To show the usefulness of our application, we used it on a project that aimed to characterize the interaction between phagocytes and proliferative immune effector cells in murine Peyer’s patches (PP), i.e. B and T cells. PP are immune inductive sites distributed along the small intestine in charge of sampling noxious antigens and mounting an immune response against them. In PP, antigens are taken up by phagocytes that, upon stimulation, migrate in the T cell zone and its periphery to interact with and prime naïve T cells. The periphery of the PP T cell zone is indeed an area of intense proliferation of immune effector cells after stimulation, suggesting that this region is a privileged site for their activation¹¹. We therefore decided to examine the evolution of proliferative cell number, to determine their identity (B or T cells) and to analyze their interaction with migratory phagocytes during the course of the stimulation.

We used SAPHIR application and two ImageJ macros, which are available on GitHub in the [Demonstration Files](#). The first macro allows the counting of proliferative cells through segmentation of Ki-67+ nuclei, provides their identity through analysis of T and B cell staining of Ki-67+ cell membrane, and analyses their interaction with phagocytes. The second macro

identify the area of high density in proliferative cells as ROI, thanks to the DBSCAN algorithm and determine whether previously analysed cells belong to this ROI.

Then, SAPHIR application allowed us to integrate these data and provided scatter plots and statistical analyses. Thus, the filtering tab was used to select only proliferative cells belonging to the ROI (Figure 6, upper left). Then, ROI-proliferative cells were split into B and T cells using the scatter plot (Figure 6, lower left). Finally, we used the “symbol shape change parameter” option to highlight ROI-proliferative cells that interacted with phagocytes. This clearly showed that in the ROI, there were more proliferative T cells than B cells that interacted with phagocytes. Exportable statistical tables confirmed this observation. Selecting these T cells, we could localize these cells interacting with phagocytes directly into the image (Figure 6, right).

Conclusion

The SAPHIR application provides a simple and user-friendly interface to obtain quantitative data from tissue images as well as COI positioning in the tissue. It is based on the interactivity between quantitative data and image to simplify the analysis

Figure 6. Use case: analysis of proliferative immune effector cells interacting with phagocytes in the T cell priming region of murine Peyer's patches. Upper left: Cells belonging to the proliferative area of the PP T cell zone were selected using the filtering tab. Lower left: ROI proliferative cells were split into T and B cells on the scatterplot and phagocyte-interacting cells highlighted thanks to the symbol shape change parameter (circle). Right: T cells selected in the scatter plot in the lower left (orange) are visualized in the image (magenta contour).

and limit bias. Further developments of SAPHIR will include use of pyramidal images to minimize loading and analysis time and unsupervised clustering methods for scatterplot generation¹².

Data and availability

SAPHIR is provided with segmentation data from two demo pictures (<https://github.com/elodiegermani/SAPHIR/tree/master/Demonstration%20files>): one with only two intensity channels to test the application in a very easy and quickly way, and a more complex one with 6 intensity channels, 12 slices and two additional parameters (belonging to a ROI and cell-cell interaction) for advanced testing of the application.

These data are parts of several projects conducted in the Center of Immunology of Marseille-Luminy (France). Acquisition of the images was made with spectral confocal microscopy and analyzed with Fiji with the provided macro.

Software availability

Source code available from: www.github.com/elodiegermani/SAPHIR

Archived source code as at time of publication: <http://doi.org/10.5281/zenodo.4088899>¹³

License: GNU General Public License v3.0

Acknowledgments

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Seth Winfree

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SAPHIR is an R based tool for image cytometry. SAPHIR provides a novel R based Shiny application for analyzing an existing segmentation, an associated image and measured features of the segmented objects. The tool is intended to meet an unmet need for a flow cytometry-like exploration tool that enables 1) the highlighting of cells-of-interest in the original image volume defined by cells gated on staining intensity or morphological features and 2) the plotting of cells to a scatterplot of staining intensity or morphological features based on an image region-of-interest (ROI).

Major criticisms:

1. Rationale for tool development: A number of tools previously described or referenced in this manuscript include functionality similar to or overlapping with SAPHIR albeit with different implementations. Although the rationale presented by the authors is clearly explained, it is unclear what the rationale is for a new implementation of these functionalities in R (versus CellAnalyst in Python and CodexMAV and Volumetric Tissue Exploration and Analysis in Java). A tool in R could prove very useful especially in light of major R-based projects in transcriptomic analyses and data visualization and the platform's unrivaled strength in statistical analysis, but this is not addressed. Furthermore, R's strengths and weaknesses in imaging (versus other platforms) are not considered or addressed. These points require further justification and discussion.
2. Rationale for tool development: It is unclear if this tool is designed as a generalizable solution or intended only for ImageJ/FIJI users as it is tied to ImageJ/FIJI functionality to generate a number of input files-especially the segmentation which is based on the ImageJ ROI class. Although there may be a path or utility for using ROIs from Icy, CellProfiler, Ilastik or other common segmentation tools, this is not discussed and needs to be clarified.
3. Interpretation of output results and tool functionality: The authors mention the importance of supporting multiplexed fluorescence images in the introduction, "more than 10 channel

measurements”, but in neither the results nor supporting material is it clear to what degree SAPHIR supports these kinds of datasets (e.g. Lelouard *et al.* the manuscript). Furthermore, the support for 3D datasets is unclear, as ImageJ ROIs are limited to 2D and the link referenced in the supporting material for the “Complex Image Analysis”, https://zenodo.org/record/4056001#.X8HVRC2ZM_W, suggests the scripts used to generate the input files only consider a single optical slice. Please clarify these functionalities.

4. Tool performance: The claim of the application’s quickness is hard to assess. Although, SAPHIR appears to function well with the demonstration datasets the authors also qualify their quickness claim: “Interactivity between scatterplot and image is optional to avoid slow interaction when high-resolution images are used. In the latter case, we recommend users to perform their gating before allowing their selection to be shown in the image by ticking the appropriate box.” This apparent contradiction is unqualified, beyond “high-resolution”, the claim of quickness is poorly clarified. Please elaborate, and demonstrate if necessary, this limitation-for instance with larger or more complex datasets.
5. Manuscript: Figures are difficult to interpret due to size and resolution, especially figure 6 in which the “orange” color could not be easily identified nor the “magenta contour”. This is critical to demonstrating the behaviors of SAPHIR. Please adjust to facilitate the interpretation of the results.

Minor criticisms:

1. Tool performance: One of the major goals appears to be addressing “bias and misinterpretation” but this is not directly discussed in the results. It is unclear in what way(s) SAPHIR directly addresses bias.
2. Tool performance: The title and introduction suggest that large or mesoscale datasets that can be generated by modern imaging modalities are a unique challenge due to their size and complexity. It is unclear to what degree SAPHIR is able to analyze datasets with a large number of cells. This is an important qualification that is not directly addressed.

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

Partly

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?

Yes

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: I authored a article demonstrating a tool I developed with similar functionality.

Reviewer Expertise: light microscopy, multispectral microscopy, image analysis, tissue cytometry, kidney immunology, neutrophil biology

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

Author Response 23 Mar 2021

Elodie GERMANI

Dear reviewer, we would like to thank you for all your comments on our app and article that helped us improve them.

We studied every remark and wanted to give you an answer :

Major criticisms:

1. Rationale for tool development: A number of tools previously described or referenced in this manuscript include functionality similar to or overlapping with SAPHIR albeit with different implementations. Although the rationale presented by the authors is clearly explained, it is unclear what the rationale is for a new implementation of these functionalities in R (versus CellAnalyst in Python and CodexMAV and Volumetric Tissue Exploration and Analysis in Java). A tool in R could prove very useful especially in light of major R-based projects in transcriptomic analyses and data visualization and the platform's unrivaled strength in statistical analysis, but this is not addressed. Furthermore, R's strengths and weaknesses in imaging (versus other platforms) are not considered or addressed. These points require further justification and discussion.

Due to the short format chosen for our manuscript (allowed initial length of 1000 words), it was not possible to include a discussion on the advantage/disadvantage of each software and its environment. CodexMAV is a commercial software but available for free as an ImageJ plugin. It needs FCS files to run. The software is very powerful to do spatial analysis (t-SNE) of cell populations in tissues, but we didn't test it in our datasets, yet. CellProfiler (Analyst) is very interesting in term of segmentation and analysis but as far as we know, scatterplots and images are not interconnected. VTEA is also a great and easy application to use, as it is an ImageJ plugin. However, at the time we needed quantitative analysis to be done, segmentation was included in VTEA without all the flexibility that was required for the segmentation of our datasets. We therefore asked the VTEA developer to add the possibility to load ROIs from ImageJ, which is done now. However, the strong interconnection between the image and the scatterplot that we wanted to have to control and correct cells identity was lacking. We have added a sentence in section Operation to explain why we have chosen

R for developing SAPHIR application.

2. Rationale for tool development: It is unclear if this tool is designed as a generalizable solution or intended only for ImageJ/FIJI users as it is tied to ImageJ/FIJI functionality to generate a number of input files-especially the segmentation which is based on the ImageJ ROI class. Although there may be a path or utility for using ROIs from Icy, CellProfiler, Ilastik or other common segmentation tools, this is not discussed and needs to be clarified.

Following reviewer recommendation, we have now added a sentence in the data requirement section to help users with the conversion of COI obtained from other software into the Fiji .roi format.

3. Interpretation of output results and tool functionality: The authors mention the importance of supporting multiplexed fluorescence images in the introduction, “more than 10 channel measurements”, but in neither the results nor supporting material is it clear to what degree SAPHIR supports these kinds of datasets (e.g. Lelouard *et al.* the manuscript). Furthermore, the support for 3D datasets is unclear, as ImageJ ROIs are limited to 2D and the link referenced in the supporting material for the “Complex Image Analysis”, https://zenodo.org/record/4056001#.X8HVRC2ZM_W, suggests the scripts used to generate the input files only consider a single optical slice. Please clarify these functionalities.

SAPHIR is supporting multichannel images and Z stack image (ijtiff is used to load the image). We indeed used 3D images but following 3D segmentation only the main COI corresponding to the largest nucleus area was kept for calculating the intensity of other channels. In Fiji, the ROI are linked to specific slices allowing this operation. Thus, we used 2D ROI of 3D images for SAPHIR analysis. This is now explained in the data requirement section.

Of note, it is now possible to obtain 3D ROI in Fiji using a Plugin from Thomas Boudier (https://imagejdocu.tudor.lu/plugin/stacks/3d_roi_manager/start#d_roi_manager).

4. Tool performance: The claim of the application’s quickness is hard to assess. Although, SAPHIR appears to function well with the demonstration datasets the authors also qualify their quickness claim: “Interactivity between scatterplot and image is optional to avoid slow interaction when high-resolution images are used. In the latter case, we recommend users to perform their gating before allowing their selection to be shown in the image by ticking the appropriate box.” This apparent contradiction is unqualified, beyond “high-resolution”, the claim of quickness is poorly clarified. Please elaborate, and demonstrate if necessary, this limitation-for instance with larger or more complex datasets.

As the application could be slowed down with images >300MB, we decided to remove the term “quickly” from the abstract of the manuscript. Moreover, the limitation of 300MB is now specified in the implementation section. Big images (with multiple slices and channels) have to be stored on SSD drive to limit slow interaction. Since our application was mainly used for tissue section < 20 µm, we did not test datasets > 300MB, yet.

5. Manuscript: Figures are difficult to interpret due to size and resolution, especially figure 6 in which the “orange” color could not be easily identified nor the “magenta contour”. This is critical to demonstrating the behaviors of SAPHIR. Please adjust to facilitate the interpretation of the results.

The resolution of the figures has been improved and Figure 6 has been modified to allow better visualization of specific parts of the panels. Moreover, the app tools now include options to adjust the cell contour thickness in the image and the spot size in the scatterplot.

Minor criticisms:

1. Tool performance: One of the major goals appears to be addressing “bias and misinterpretation” but this is not directly discussed in the results. It is unclear in what way(s) SAPHIR directly addresses bias.

By allowing to analyze for each cell of the scatterplot its position and its characteristics in the image, SAPHIR allows to check that the parameters obtained automatically with the segmentation and other analysis algorithms fit with the observation of the selected cells in the image. Then, the annotation menu allows to correct any bad analysis (e.g., cell identity). We have now extended the use case section to better describe SAPHIR use.

2. Tool performance: The title and introduction suggest that large or mesoscale datasets that can be generated by modern imaging modalities are a unique challenge due to their size and complexity. It is unclear to what degree SAPHIR is able to analyze datasets with a large number of cells. This is an important qualification that is not directly addressed.

Our aim was clearly not to analyze mesoscale datasets. We apologize if that is the impression given by our title or introduction, although we see no allusion to it in the title and only a small reference in the introduction (i.e., “10 μm to more than 200 μm when clearing methods are used”) but not related to the description of the application. Actually, as mentioned in the introduction our main goal was to obtain an interconnection between the representation of image parameters in scatterplot and the image itself in order to analyze positioning of scatterplot-selected cells in the image and to check and correct any analysis errors by direct observation of the selected cells.

We hope these responses and the new version of our article will satisfy you.

Competing Interests: No competing interests were disclosed.

Reviewer Report 11 November 2020

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In this article, the authors describe a shiny app to load and explore the segmentation output of fluorescence images from ImageJ/Fiji. The app provides an interactive interface to filter, select, and annotate the regions of interest as defined by the ImageJ/Fiji macros. Besides, the app allows for performing statistical testing on the selected regions. The manuscript describes the motivation for developing the app, the basic functionality, and a use case. In the use case, the app was used to filter proliferative T and B cells based on cell membranes staining and determine that more T cells interacted with phagocytes in Peyer's patches images.

The article is written in clear and concise language. The motivation for developing the app and the description of the operation is sound. A few issues remain:

Major Issues

1. The implementation section focuses on describing the interface but doesn't explain much about the inner working of the app or the development process. (related, question 3).
2. Four types of files are needed to use the app: Images, Data files, ROIs Files, and Legend files. Each file needs to be processed and prepared to match the formats and requirements of the app. The user has to perform many steps, including using ImageJ and R, to process the files to be able to use SAPHIR. Besides, if the user wants to combine 2 images, he or she has to prepare 4 files for each image. These complicated steps could limit the replication of the analysis by others. It is better if the providers could explain the steps and the tools that they have used to prepare their files. Or if there is an option in SAPHIR that we can use to directly prepare the files.
3. The article explains well using the app to perform different filtering and selection on an example image. However, it doesn't say much about the interpretation of the outputs, or the context in which these decisions could be made. (related, question 4)
4. The use case is very brief and it seems like many of the analysis steps were done outside of the app using custom macros. This makes it hard to evaluate the use case or determining how the app contributed to the final calculations. The authors could provide a more complete description of the use case starting from the raw images and describe how the macros work. It would be helpful to provide links to the data and the tools they used as well.
5. The two examples provided in the article use a single image. To be able to make any claims on the performance (related, question 5), the authors might need to perform testing with

multiple images or provide a comparison with existing tools.

Minor Issues

1. Several screenshots from the app were included in the manuscript as figures. It might be helpful to change the size/resolution of the images for clarity. Also, highlighting the important parts of the image would make it easier to understand the interface.
2. The app depends on ImageJ so it should be bundled with the app or explain how to obtain and add to the app.
3. Related. Trying to run the app using rstudio server on rocker/verse failed. A dependency libpoppler-cpp-dev was required but not declared.
4. Provide a viable demo, for example using shinyapps.io or rstudio.cloud would make it easier to evaluate the tool and lowers the entry cost for users.
5. The app is written as one long R script. This makes it very difficult to examine, modify, or extend the code. Factoring the code or making it more modular would be helpful.
6. After installing the most recent versions of the required packages, trying to run the app in a fresh rstudio session through an error related to shinyjs::extendShinyjs. I was able to get it to run by adding functions = 'winprint' as an argument. [Source](#)

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

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: image-analysis, genomics, adipocyte-differentiation, autophagy

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.

Author Response 23 Mar 2021

Elodie GERMANI

Dear reviewer, we would like to thank you for all your comments on our app and article that helped us improve them.

We studied every remark and wanted to give you an answer :

Major Issues

1. The implementation section focuses on describing the interface but doesn't explain much about the inner working of the app or the development process. (related, question 3).

Following the recommendation of the reviewer we have now explain the development process of the app in the operation section.

2. Four types of files are needed to use the app: Images, Data files, ROIs Files, and Legend files. Each file needs to be processed and prepared to match the formats and requirements of the app. The user has to perform many steps, including using ImageJ and R, to process the files to be able to use SAPHIR. Besides, if the user wants to combine 2 images, he or she has to prepare 4 files for each image. These complicated steps could limit the replication of the analysis by others. It is better if the providers could explain the steps and the tools that they have used to prepare their files. Or if there is an option in SAPHIR that we can use to directly prepare the files.

We thank the reviewer for this suggestion. We have now added in the data requirement section a description of the segmentation process. A segmentation menu to is available in the application where Fiji and the two macros used in the manuscript can be launched.

3. The article explains well using the app to perform different filtering and selection on an example image. However, it doesn't say much about the interpretation of the outputs, or the context in which these decisions could be made. (related, question 4)

We agree with the reviewer and based on the use case we now describe the results we could obtain and the conclusions we were able to draw using SAPHIR on a specific application, which is the immune response initiation in murine Peyer's patches. We also show how useful the filtering tool is to focus on a given population of cells (i.e., in our case proliferative cells of the ROI) and how interaction between the image and the scatter plot can be used to focus on a given cell of interest and to control that analysis is correct.

4. The use case is very brief and it seems like many of the analysis steps were done outside of the app using custom macros. This makes it hard to evaluate the use case or determining how the app contributed to the final calculations. The authors could provide a more complete description of the use case starting from the raw images and

describe how the macros work. It would be helpful to provide links to the data and the tools they used as well.

We followed the recommendation of the reviewer and have extended the section dedicated to the use case (see answer to point 3). However, since the segmentation process is now described in detail in the data requirement section, we only briefly described this step to rather focus on the contribution of SAPHIR to quantitative image analysis. As explained in the main text, all data and macros are available on GitHub in the Demonstration Files.

5. The two examples provided in the article use a single image. To be able to make any claims on the performance (related, question 5), the authors might need to perform testing with multiple images or provide a comparison with existing tools.

The main goal of this manuscript was to provide a brief description of the application and its usefulness using two types of image as demos. It is now applied in the lab to study biological processes using dozens of images to validate statistics and its use will be included in several studies, but it was not the aim of this manuscript to already provide this kind of analysis. We indeed tested other tools before developing SAPHIR, such as VTEA and FlowJo, but none was really adapted to our needs as we especially wanted to have a strong interconnection between images and scatterplots.

Minor Issues

1. Several screenshots from the app were included in the manuscript as figures. It might be helpful to change the size/resolution of the images for clarity. Also, highlighting the important parts of the image would make it easier to understand the interface.

The resolution of the figures has been improved and Figure 6 has been modified to allow better visualization of specific parts of the panels. Moreover, the app tools now include options to adjust the cell contour thickness in the image and the size of the dots in the scatterplots.

2. The app depends on ImageJ so it should be bundled with the app or explain how to obtain and add to the app.

Link to Fiji is now provided in the segmentation menu of the app, where Fiji and the two macros used in the manuscript can be launched and applied to any image.

3. Related. Trying to run the app using rstudio server on rocker/verse failed. A dependency libpoppler-cpp-dev was required but not declared.

The dependency to libpoppler-cpp-dev is due to the use of the magick package, which is required to use the application. We also had this problem when trying to deploy our app on shinyapps.io. This problem can be solved using a Unix operating system instead of Windows. We have created a Docker file to use the app without this kind of issues.

4. Provide a viable demo, for example using shinyapps.io or rstudio.cloud would make it easier to evaluate the tool and lowers the entry cost for users.

We are soon going to deploy a demo version on shinyapps.io. However, it will only be operable on our demo images and the user will not be able to change the images to use.

5. The app is written as one long R script. This makes it very difficult to examine, modify, or extend the code. Factoring the code or making it more modular would be helpful.

We agree with the reviewer and we are going to modularize the code in the following months.

6. After installing the most recent versions of the required packages, trying to run the app in a fresh rstudio session through an error related to shinyjs::extendShinyjs. I was able to get it to run by adding functions = 'winprint' as an argument. [Source](#)

We modified the script in order to correct this. Indeed, the package shinyjs was recently updated and a new argument in the function "extendShinyjs" was made mandatory. We added this modification to the latest version of our script.

We hope these responses and the new version of our article will satisfy you.

Competing Interests: No competing interests were disclosed.

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