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3D object detection and segmentation inside an RI map

Application Note by Nanolive SA

Abstract

Nanolive's 3D Cell Explorer allows for the creation of very powerful 3D images of living cells with very high spatio-temporal resolution (x,y:180nm; z:400nm; t:1.7sec). Being able to detect objects in such images and segment them is key in leading-edge biological research based on imaging techniques. This is a rather complex task that requires specific knowledge and set of competences. In this application note we will start with a simple task and describe how to properly segment the nuclei of mammalian cells present in a 3D refractive index map using FIJI.

1. Introduction

The 3D Cell Explorer allows to acquire a three-dimensional map of the refractive index (RI) composing your objects of interest (Cotte et al., 2013). The finesse of the device allows to observe differences of RI as small as 0.001, by far the best sensitivity on the market, thus providing a fine view of the different elements composing a sample, such as the different compartments of a cell for example. Consequently, even a non-expert would be able to identify by eye the nucleus of a mammalian cell in an RI map. However, at the age of quantitative cell biology (Langen et al., 2015; Li et al., 2007) where seeing, feeling and manual image analysis is no more the norm, it is crucial that the computer crunches your image to extract meaningful object features organized in space, time and object identity (Frechin et al., 2015). The goal of this note is to provide you with computational tools and some knowledge required to analyze your images. We will use a simple example, the 3D segmentation of HeLa cells nuclei (**Figure 1**). This will require the software FIJI (Schindelin et al., 2012; Schmid, Schindelin, Cardona, Longair, & Heisenberg, 2010; Schneider, Rasband, & Eliceiri, 2012) as an addition to the acquisition software STEVE. We will then see how to export a decent digital staining (D-stain) which is acquired using STEVE and improve it in FIJI, to finally make a good 3D nuclei segmentation. **You should then be able to redo the process for any object of interest of which you have a D-stain.** Please keep in mind that the parameters showed here are no rule of thumb and should be carefully evaluated for your specific problem.
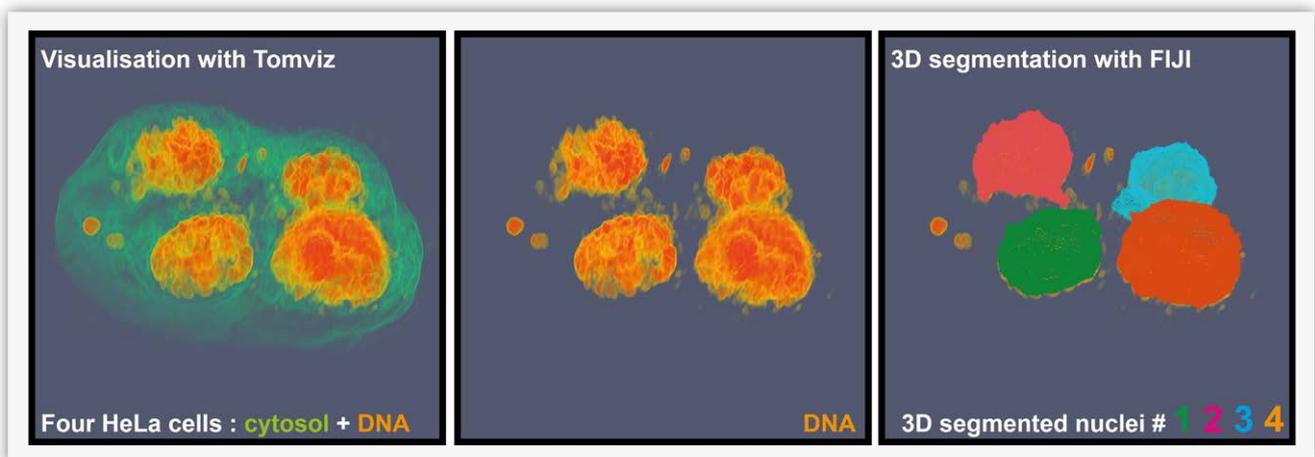
Figure 1: FIJI allows to do efficient 3D object segmentation

2. Prerequisite

First, you will need the latest version of STEVE, the software that allows to control your 3D Cell Explorer, and second, the **latest version of FIJI** (Schindelin et al., 2012; Schneider et al., 2012) (fiji.sc) with the **3D ImageJ suite** (**Figure 2**). Update FIJI to get the 3D ImageJ suite, click **Help > Update...** (**Figure 2, steps 1-2**) press **manage update** (**Figure 2, step 3**). It could be that FIJI asks you for a couple of update rounds. Go through them, restart as many times as necessary (usually just once) until you can go through the process by ticking on the **3D ImageJ suite** option (**Figure 2, step 4**). Now you can close (**Figure 2, step 5**), restart FIJI and check that the **3D** and **3D viewer** are present in the **Plugin** menu (**Figure 2, step 6**).
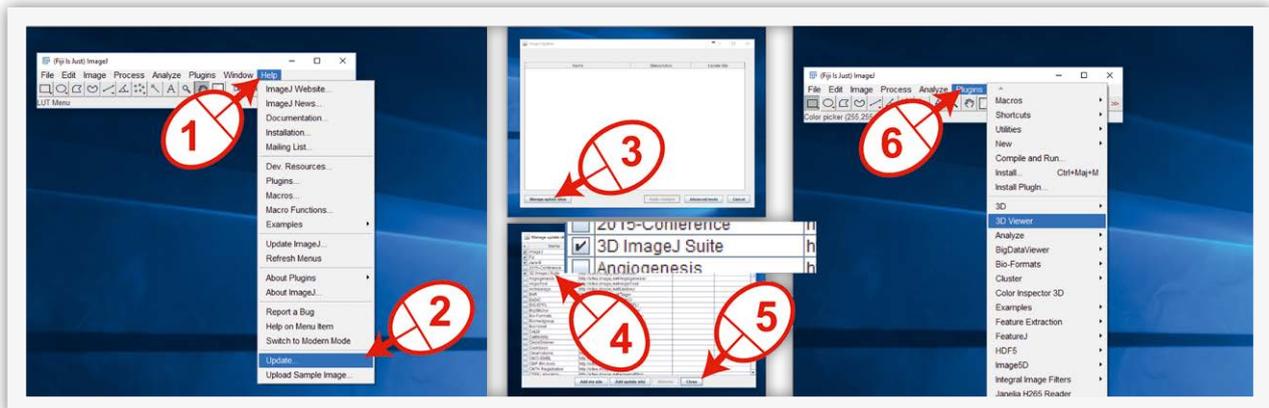
Figure 2: Installation of the 3D ImageJ suite

Finally, you will need a nice acquisition file generated with the 3D Cell Explorer. We must insist on the obvious, everything starts with having excellent acquisitions. If you are not sure to be in optimal imaging conditions, please follow our guidelines (http://nanolive.ch/supporting-material/) or contact us (http://nanolive.ch/contact/), we will answer you with great pleasure. For the sake of this presentation we will take a simple example of four HeLa cells that have been growing in a 3D gel matrix. They are at an early stage and just starting to divide.

3. Exporting D-stain

The first step after obtaining a suitable dataset acquisition, is to export the 3D refractive index (RI) map and the D-stain with the .tiff format. Use the exporting tool of STEVE to export your dataset as an **anyname.tiff** stack. After your acquisition, when your dataset is loaded into STEVE (if not load it with File > Load), do **File > Export** (**Figure 3, steps 1-2**) and in the panel that opens choose first RI volume (**Figure 3, step 3**) and change the format to Tiff (**Figure 3, step 4**). Provide a location and a name to your file and export it (**Figure 3, steps 5-6**). Redo the same process (**Figure 3, steps 7-12**) for exporting the digital staining (D-stain). The results of these exports contain 96 slices, covering the acquired Z-dimension. It might well be that not all slices contain useful information on your sample. Such slices will only generate noise in your 3D visualization and need to be removed from the **anyname.tiff** file before opening it in a 3D visualization tool. Doing so is easy and you will find all necessary information in the application note **"Visualizing three-dimensional data produced by the 3D Cell Explorer" at chapter 4 "Removing unwanted Z-slices"**.
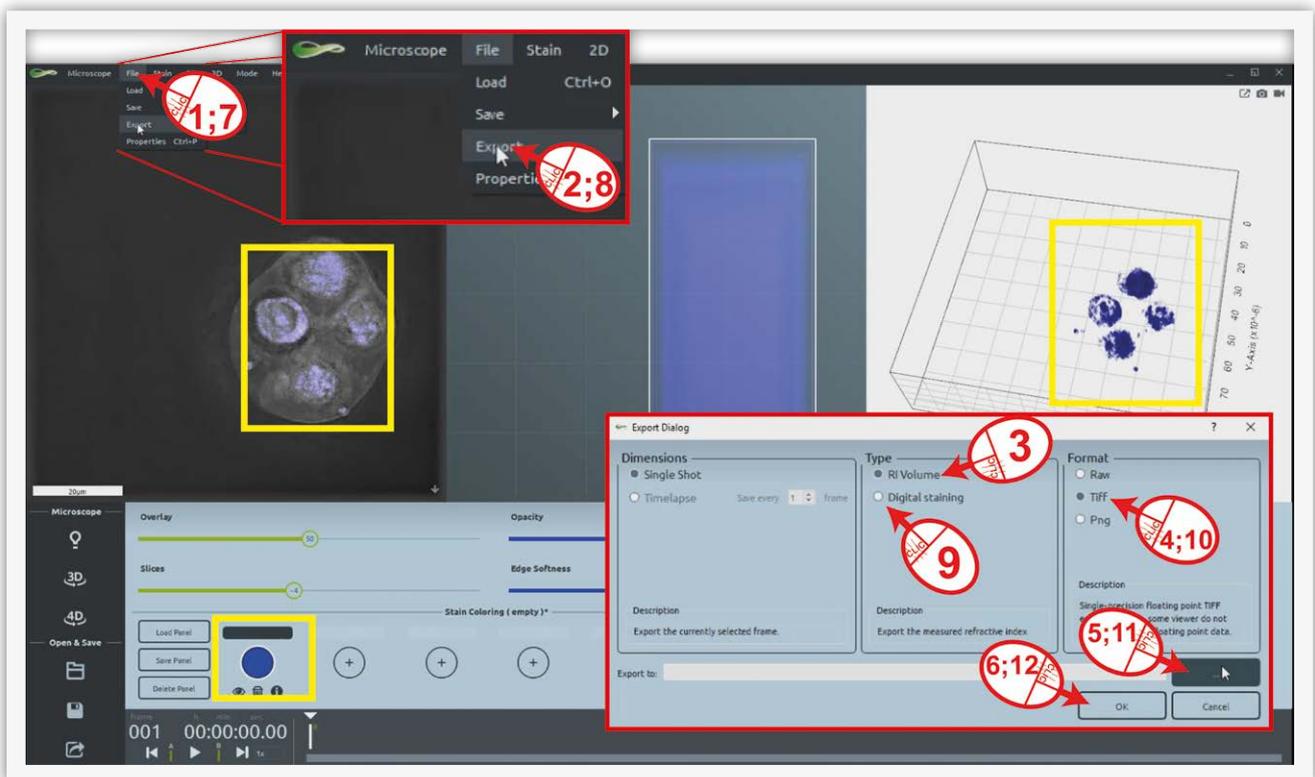
Figure 3: Export of D-stain and refractive index map from STEVE to .tiff

4. Preparing the data for object filtering and nuclei segmentation

Once the D-stain stack (**Ds_anyname.tiff**) has been reduced to the slices containing information it is necessary to transform it into an 8-bit grayscale image. To do so in FIJI, go to **Image > Type > 8-bit** and a new stack appears (**Figure 4, steps 1-2**). To reduce the effect of noise, we propose to apply a smooth filtering on the data. Since your data is three-dimensional, a special 3D filtering is needed. No worries, do **Plugins > 3D > 3D Edge and Symmetry Filters** (**Figure 4, steps 3-5**) and enter your values in the 3D filter panels. For our D-stains, the values indicated in the figure (**Figure 4, step 6**) should generally fit well, however if you have small objects you might consider skipping this filtering step or apply tiny filter sizes. Your **Ds_anyname.tiff** stack is now ready for object detection.
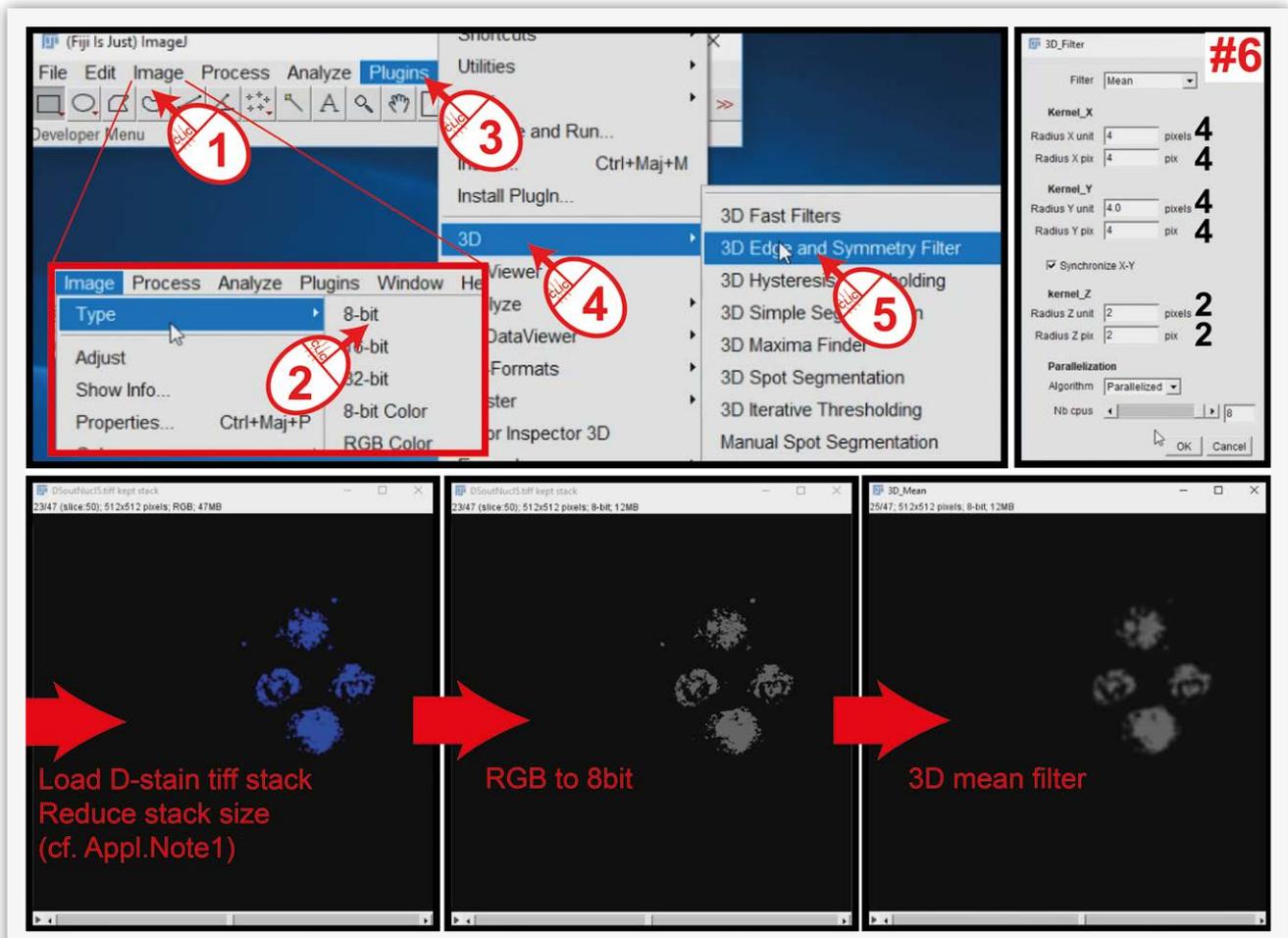
Figure 4: Loading and preparation of the D-stain .tiff stack for further object detection

5. Using the 3D plugin of FIJI to detect nuclei

The signal contained in your **Ds_anyname.tiff** stack will now be analyzed for voxel connectivity, which will detect the connected components that form your objects. Our eyes identify clearly four nuclei; however, few voxel blocks are clearly not nuclei even if they have been labeled as nuclei parts in the D-stain process. We need to filter them out. Do **Plugins > 3D > 3D Simple Segmentation** (**Figure 5, steps 1-3**). A window opens (**Figure 5, step 4**); you must now enter three parameters (do not tick the lowest option!): **low threshold** defines the signal over which a voxel is considered as part of an object. **Min size** and **Max size** defines the minimal and maximal volumes in voxels that an object needs to have to be retained during the segmentation process. **Be careful!** Before pressing **OK**, take some time to define properly those values as they will determine the quality of your segmentation. The four nuclei objects that have been retained are almost ready for visualization.
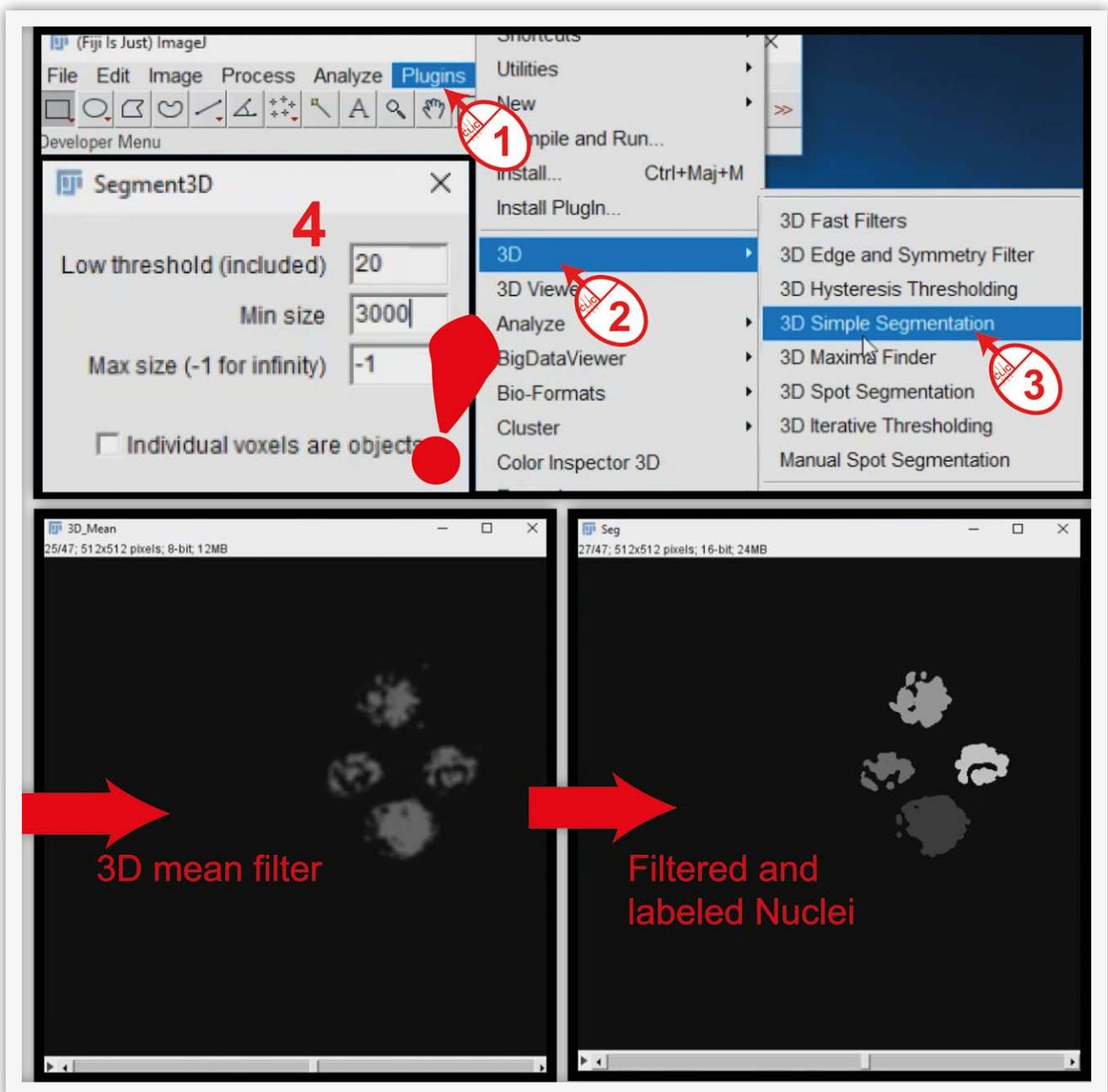
Figure 5: Objects filtering and 3D segmentation of Nuclei

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We will now see how to take care of the imperfections of the D-stain we made in STEVE. These imperfections lead to holes in the shape of the detected objects (**Figure 5**), let's fill them up. Do **Plugins > 3D > 3D Binary Close Labels** (**Figure 6, steps 1-3**). Again, a window opens (**Figure 6, steps 4**). You must now enter two parameters: **RadiusXY** and **RadiusZ**. Define up to what distance a gap can be filled. This is case specific and the indicated values are no rule of thumb. Press OK and a new stack describing the nuclei segmentation in X,Y and Z appears, ready for visualization.
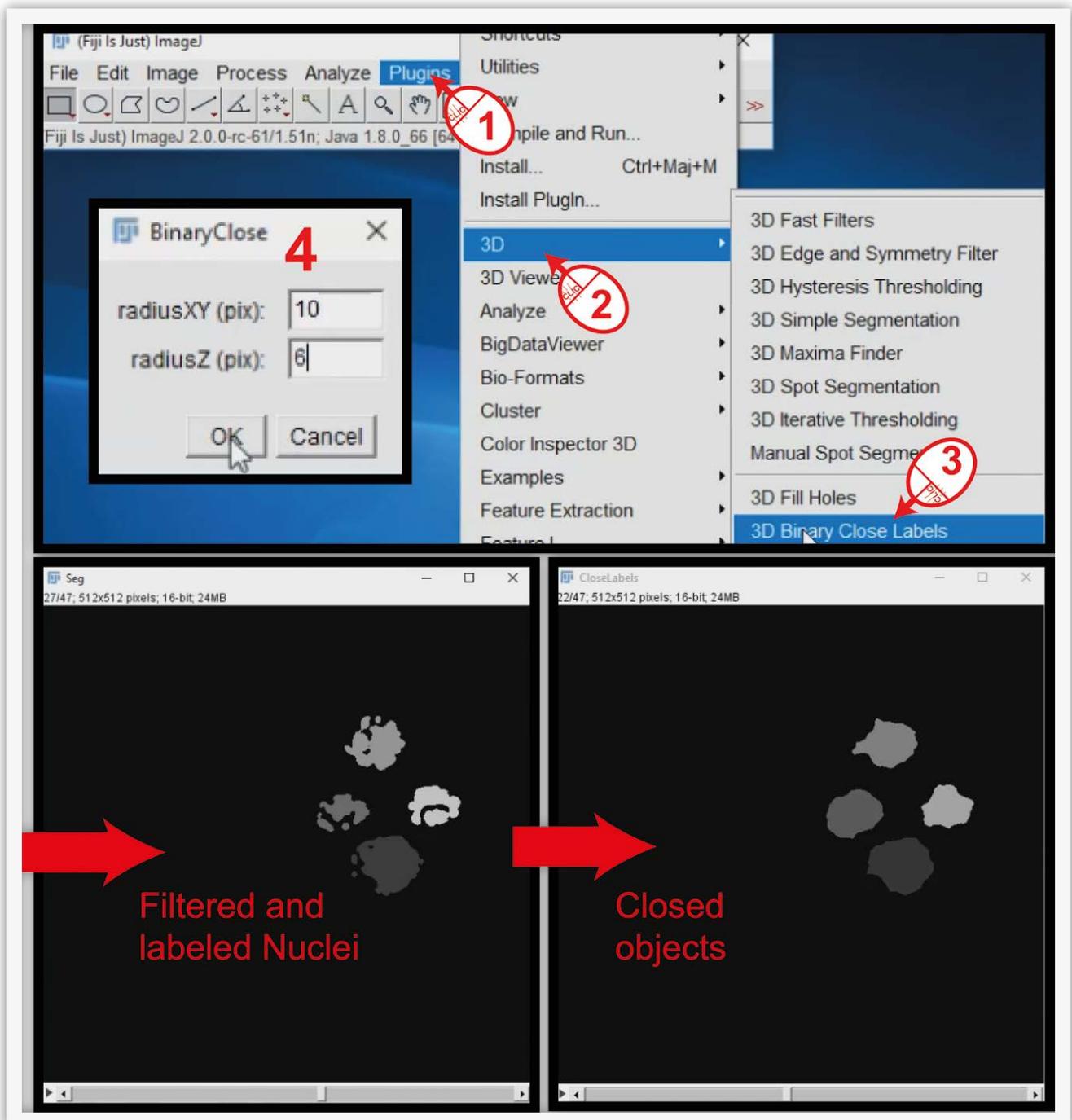
Figure 6: Closing unwanted holes in 3D objects

Finally, the 3D nuclei segmentation can be merged with the RI volume of the sample of interest for better evaluation of the quality of the segmentation and eventually adapt the parameters for another more refined iteration of the process. First, load the RI stack in FIJI previously optimized by removing z-slices that do not have proper signal. At this point you should have the 3D nuclei volume

and the RI volume open in FIJI (**Figure 7**). Do **Image > Color > Merge channels… (Figure 7, steps 1-3**). For the third time, a window opens (**Figure 7, steps 4**), you must now select which volume is attributed to which color. We link the segmentation to the red channel (**red star**) and the RI volume to the gray channel (**gray star**) to keep the RI image in grayscale. Tick "Create composite" and press ok. You can now watch the result.
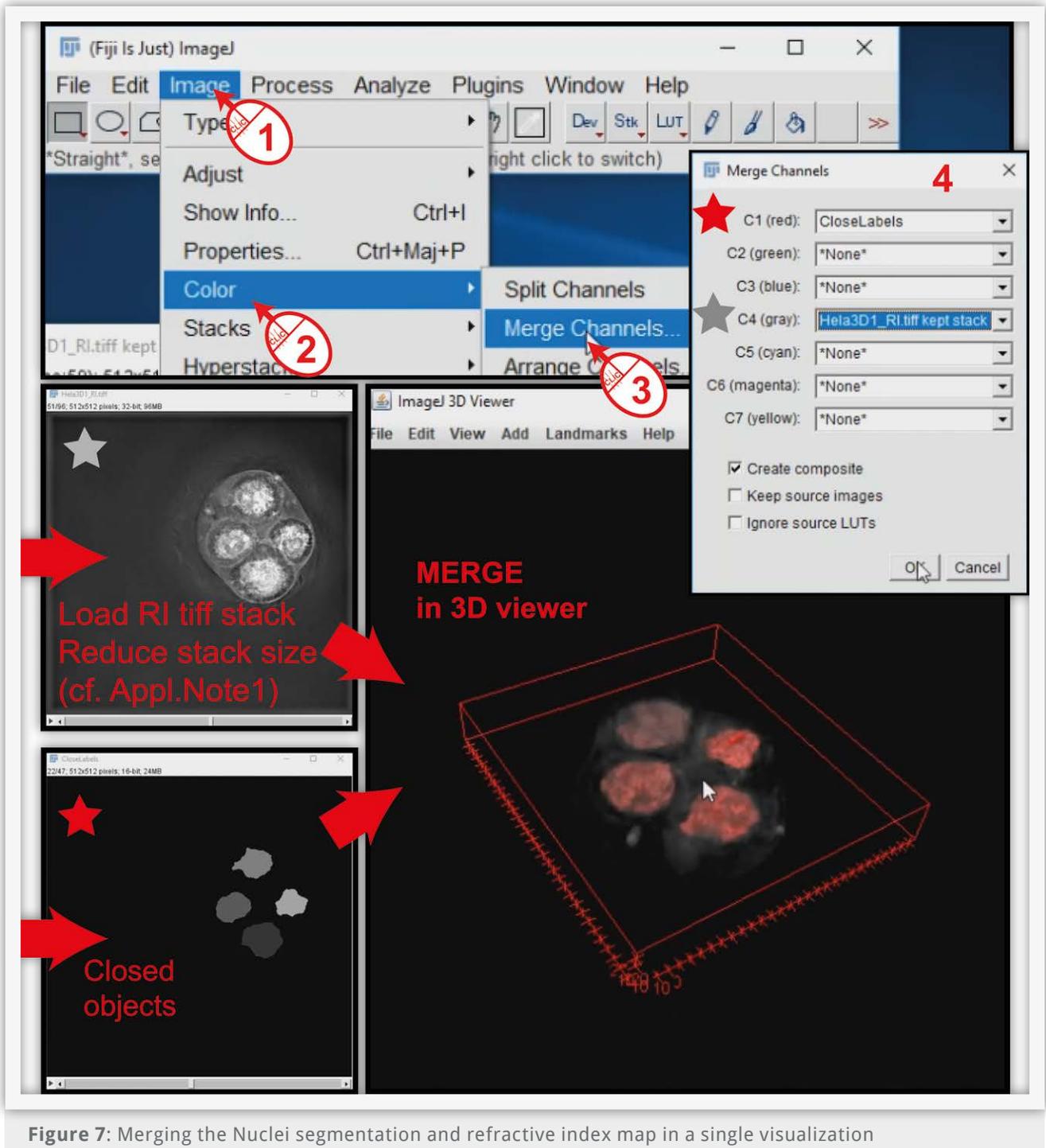
Figure 7: Merging the Nuclei segmentation and refractive index map in a single visualization

With this application note and the previous one explaining how to use the visualization tool Tomviz, you should now be able to produce a visualization equivalent to the one shown in **Figure 1.**

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