

**Circulating Tumor Cells TheRapeutic APheresis: a  
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## **SOP.8. Automated Analysis**

### **Scoring CTC with ICY**

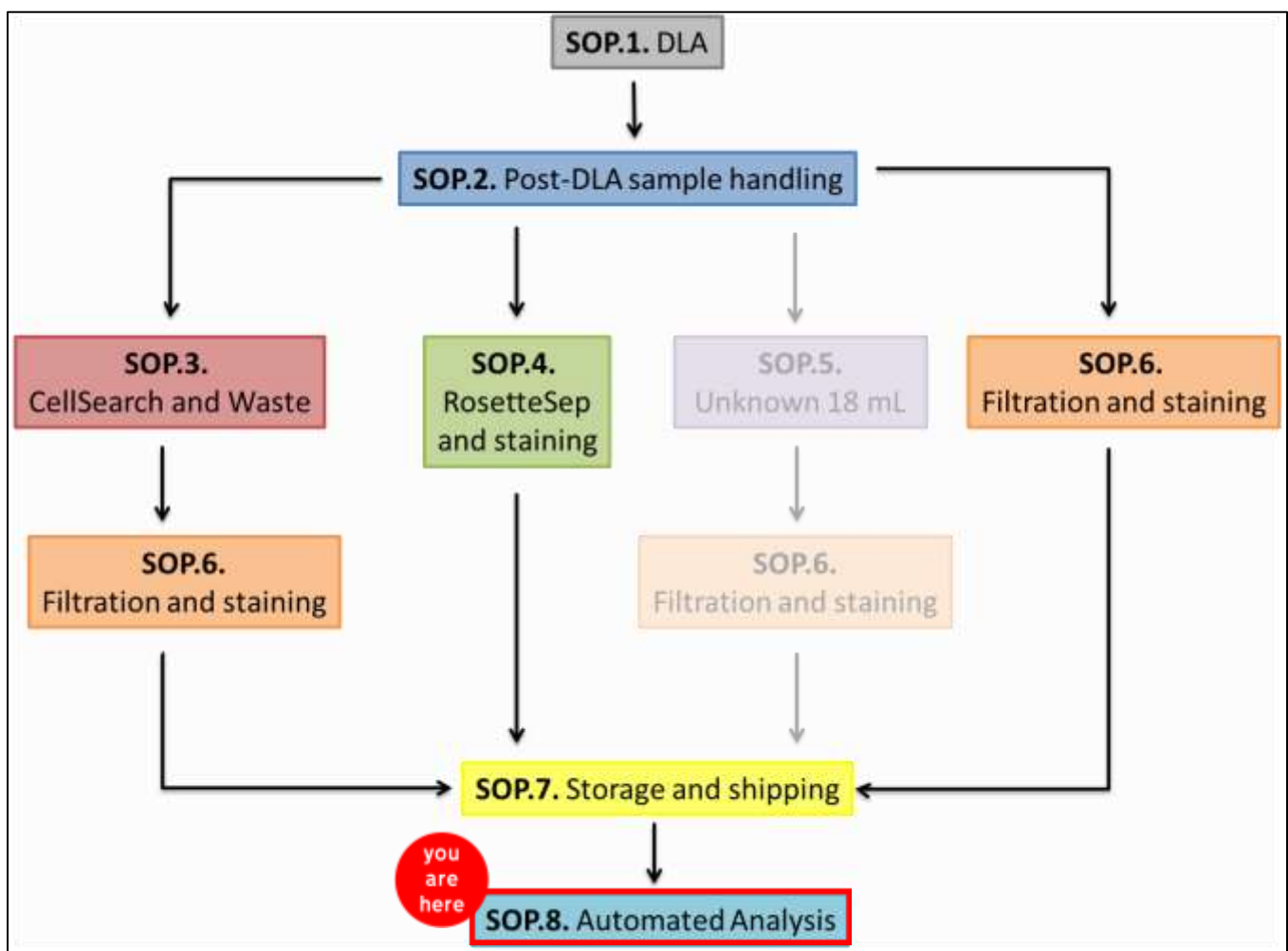


## Introduction

This Standard Operating Procedure (SOP) describes the scoring of CTC with the program ICY. This is adapted to the images that are acquired after filtration and staining of a sample (DLA, CellSearch Waste, CTCTrap eluate or RosetteSep), which are described in **SOP.4.** and **SOP.6.**. This SOP is the end of the workflow of the procedures in the CTCTrap Program.

*This is SOP.8. Automated Analysis; version 1.0-092015*

### Workflow of procedures in the CTCTrap program



# SOP.8. Automated Analysis

## Scoring CTC with ICY

To evaluate the performance of our algorithm we need datasets that were scored manually by trained reviewers. The data sets need to be scored according to the following manual. For scoring, the open-source image analysis software ICY is used.

The SOP.8. is split in the following parts:

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# 1. Installation of ICY and the required plugin

## 1.1 Install ICY

ICY can be downloaded from the website <http://icy.bioimageanalysis.org/download>. Some installation instructions can be found on this website, too. When installing ICY, make sure that you locate the ICY folder somewhere where you have writing permission. For Windows, this means that you cannot place the folder under 'Program Files'. Otherwise, the automatic update process won't work. For Mac, you can place the folder under 'Applications'. When first opening ICY on a Mac, use a right click on the icy symbol and then choose open. To use ICY, java needs to be installed on your computer. If it's not already installed on your computer, a java runtime environment (JRE) can be downloaded from <http://www.oracle.com/technetwork/java/javase/downloads/jre8-downloads-2133155.html>. Make sure that you choose the version corresponding to your operating system (for a 32bit Windows system this is the Windows x86 JRE).

## 1.2 Install Plugin

The plugin for CTC scoring can be downloaded from

[https://www.dropbox.com/sh/7qm0ourwte9ihld/AABjkWrml9aAf15\\_kdKAponga?dl=0](https://www.dropbox.com/sh/7qm0ourwte9ihld/AABjkWrml9aAf15_kdKAponga?dl=0)

Then, go to the ICY folder and open the subfolder 'plugins'. Copy the folder containing the CTCCounting.jar file into the plugins folder of ICY. When opening ICY you can find the plugin in the toolbar under 'Plugins' → 'Other Plugins' → 'ctcanalysis' → 'ctccounting' → 'ctccounting'.

## 2. Loading and Preprocessing of data

### 2.1 Loading Data and merging of image channels

For importing your images into ICY go to 'File & ROI tools' → 'Open' and open the folder containing your data. Select all images simultaneously and then click 'Open' (see Figure 1). If your images are labeled with sequential

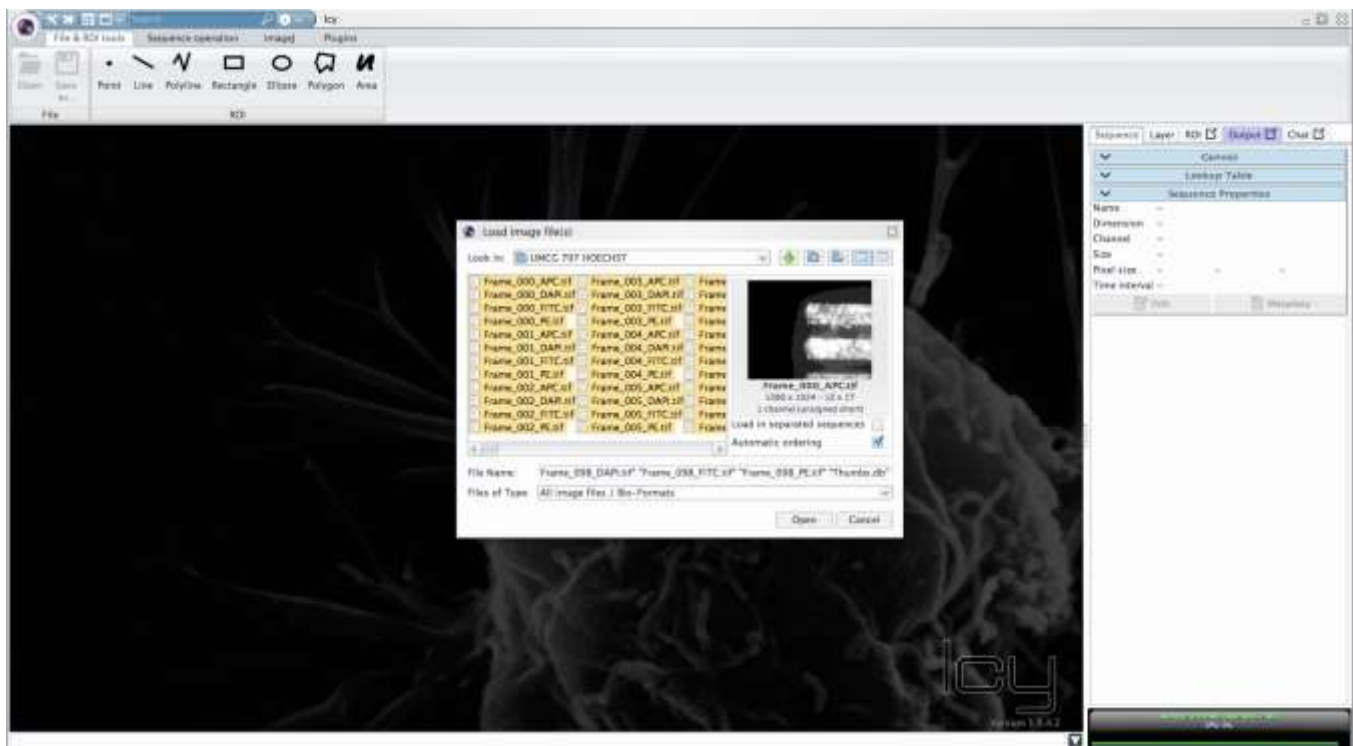


Figure 1 Opening of all images simultaneously.

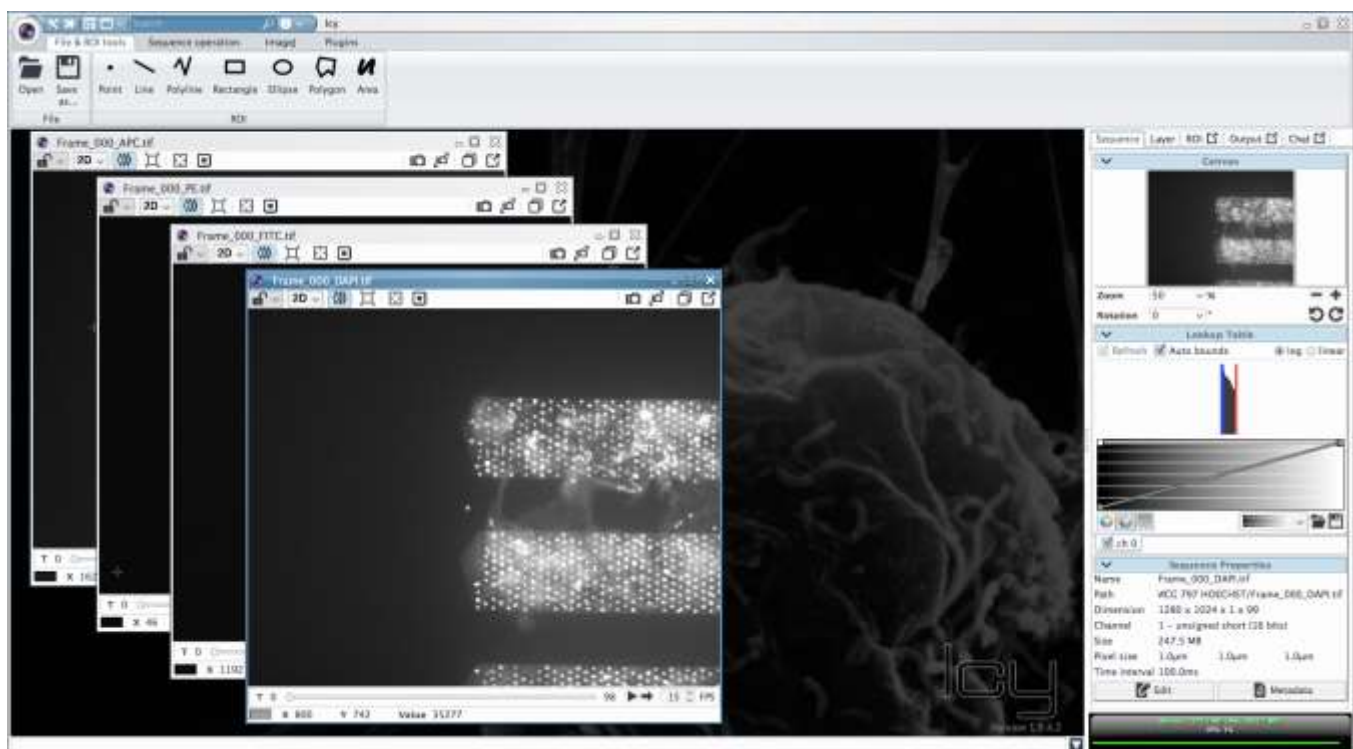


Figure 2 Time series data per channel.

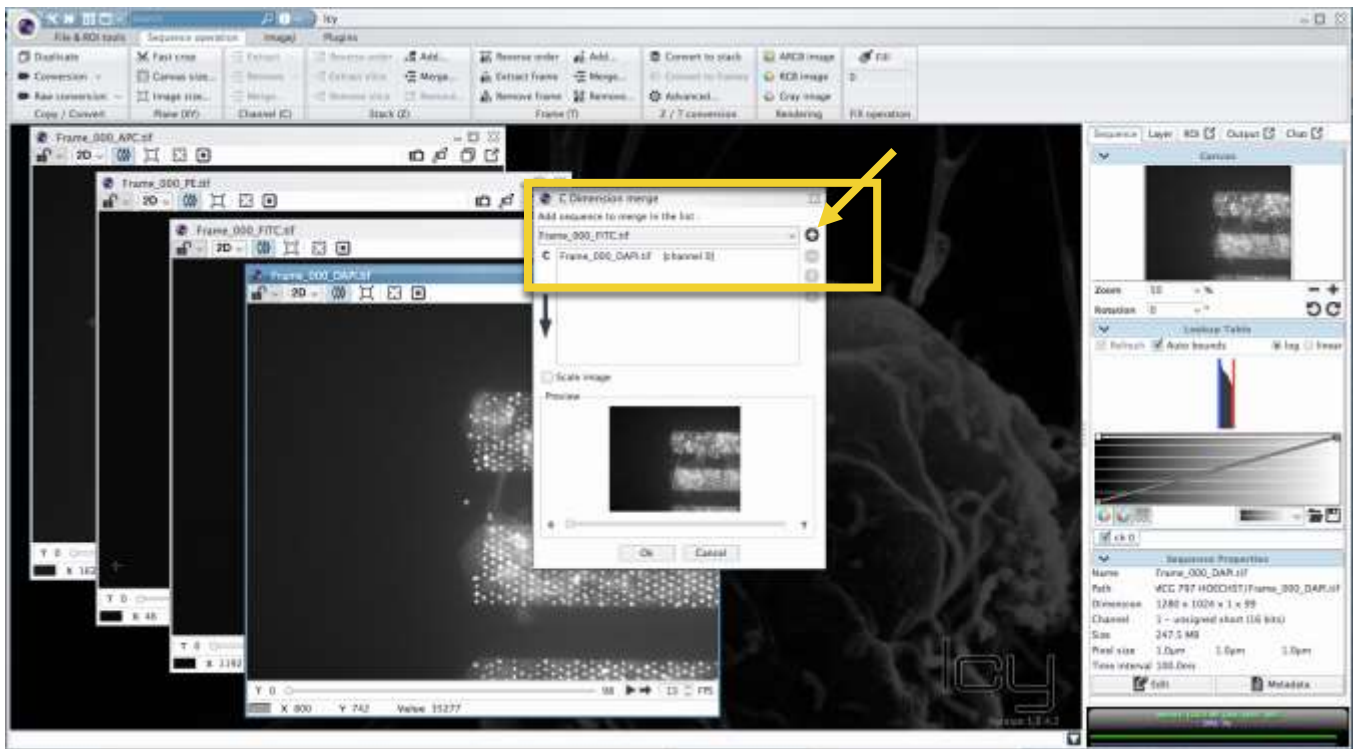


Figure 3 Merging of all channels.

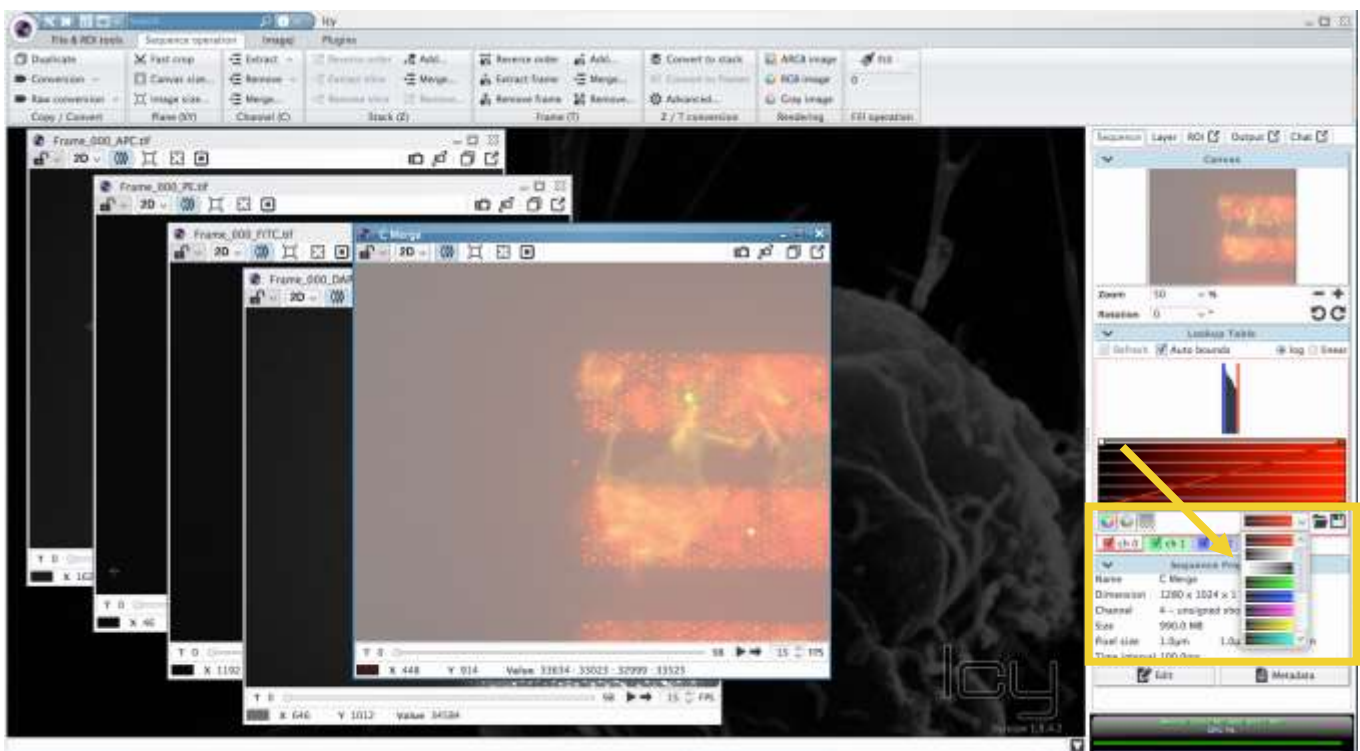


Figure 4 Adjusting the channel colors.

numbers and the labels indicate to which channel the images belong, ICY should directly create separated series of time frames of images for each channel (see Figure 2). Note, that this might only work if every image is saved separately for each channel and not as a multipage tiff file. Dependent on your operating system it could be that for multipage tiff files, ICY opens them all separately instead of a time series of images. If this happens, see the instructions below. Next, go to 'Sequence operation' → 'Channel (C)' → 'Merge...'. Successively add the channel series that you want to merge (see Figure 3) and then click 'Ok'. ICY will open a new series of images that is a merge of the different channels.

If your images are not saved for each channel individually, but as a multipage tiff file and ICY opens them all separately, it might be advantageous to convert them beforehand using Fiji ([www.fiji.sc](http://www.fiji.sc)). After opening Fiji go to



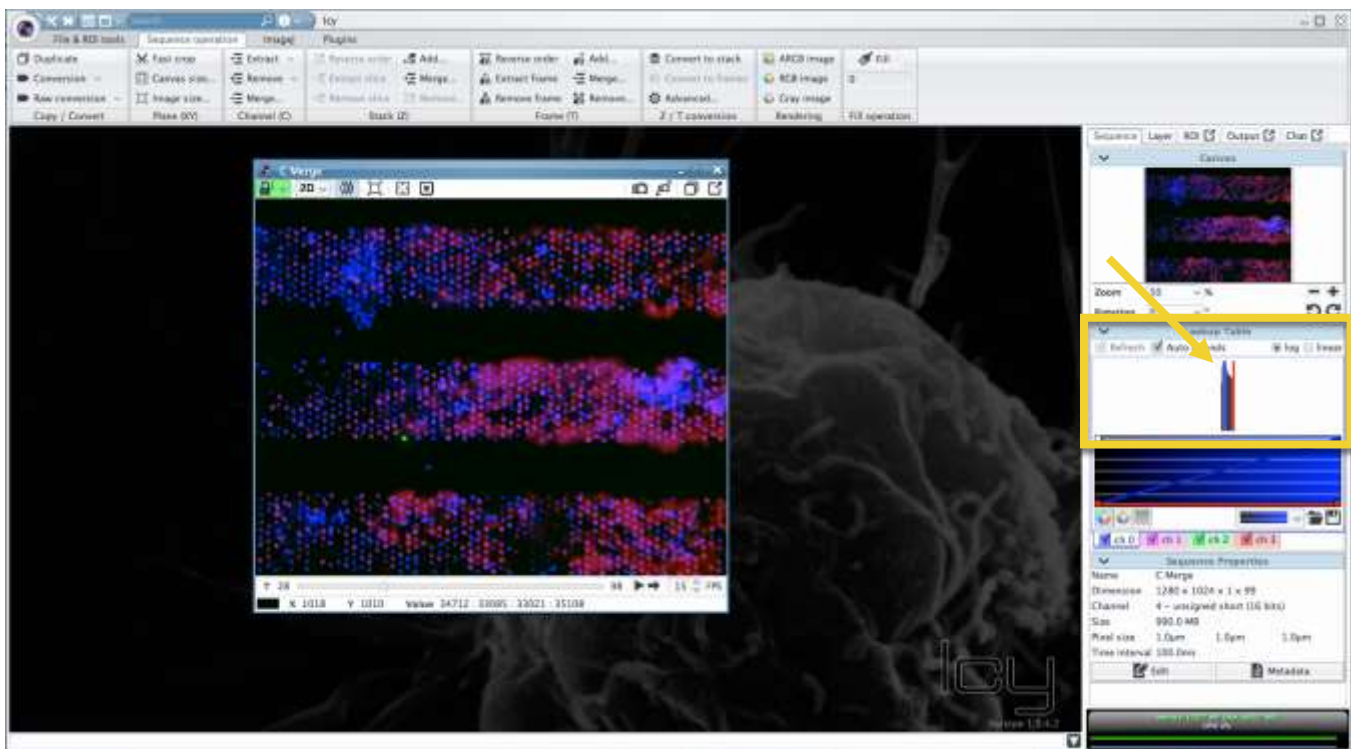


Figure 5 Adjusting the color coding (histogram bounds).

'File' → 'Import' → 'Image Sequence' and choose the folder containing your sequence. Next, go to 'Plugins' → 'Image5D' → 'Stack to Image5D'. Set the 3rd dimension to 'ch' and the 4th dimension to 't' and adjust the sizes below. Then, save the image and import the saved image into ICY.

Before scoring the images, always check if ICY or Fiji loaded the images in the correct order.

## 2.2 Image Adjustments

Prior to labelling your images, you might want to adjust the color of each channel or the color coding. The color of each channel can be adjusted on the right site in ICY. Click on the channel which color you want to adjust and then choose the corresponding colormap in the drop-down menu (see Figure 4).

By default, ICY adjusts the color coding in the images in the way that the darkest color is associated with the lowest value present in the images while the brightest one is associated with the highest value. Nevertheless, you can adjust the histogram bounds (blue and red vertical bar in the histogram on the right site (see Figure 5) to change the appearance of your images. This is done for each channel separately. Note, that hereby you are **not** modifying the data but only the view of the data. If you move the histogram bounds closer together you get a better contrast and therefore more details are visible then before. This can help, especially in the case when some intensity outliers (pixels with very high intensity compared to all other pixels) are present in the image. All other pixels appear very dim making it more complicated to see details in your images. In this case, it makes sense to exclude these outliers and move the upper boundary closer to the smaller intensity values. In general, there is no "correct" way of adapting your histogram bounds and partly depends on your own preferences. Nevertheless, there are some rules that you should follow. Since the decision if a cell is a CTC or not depends to a large extend on the information if the cell is "active" or "not active" in a certain channel, your histogram adjustments have a strong influence on your final decision. Therefore, it is import that:

- the intensity in the channel that you use as an **inclusion criteria** should **not be too dim**
- the intensity in the channel that you use as in **exclusion criteria** should **not be too bright**.

If the inclusion criteria is too dim you might consider a cell as not active even if it is active and with a too bright exclusion criteria you might exclude cells that were of interest. For the ground truth data set that we need for the development of our algorithm it is always better to include a cell which might not be informative in the end rather than excluding one that is informative. Therefore, always make sure that you set your histogram bounds according to the above mentioned rules when labelling CTC.

## 3. Labeling CTC

### 3.1 Image Representation

Once the image view is adjusted in the way you prefer it, you can start labelling CTC. To scroll through all the images you can either move the button on the timeline below your image (see Figure 6 ) or you can just use the left and right arrow keys on your keyboard. If you want to zoom in, you can either use the plus sign in the toolbar on the right hand side or use the scroll wheel of your mouse. If you want to zoom out, again you can use the scroll wheel or the minus sign on the right. If you want to zoom back to the full view of the image, you can also click the rectangle above your image (see Figure 7).

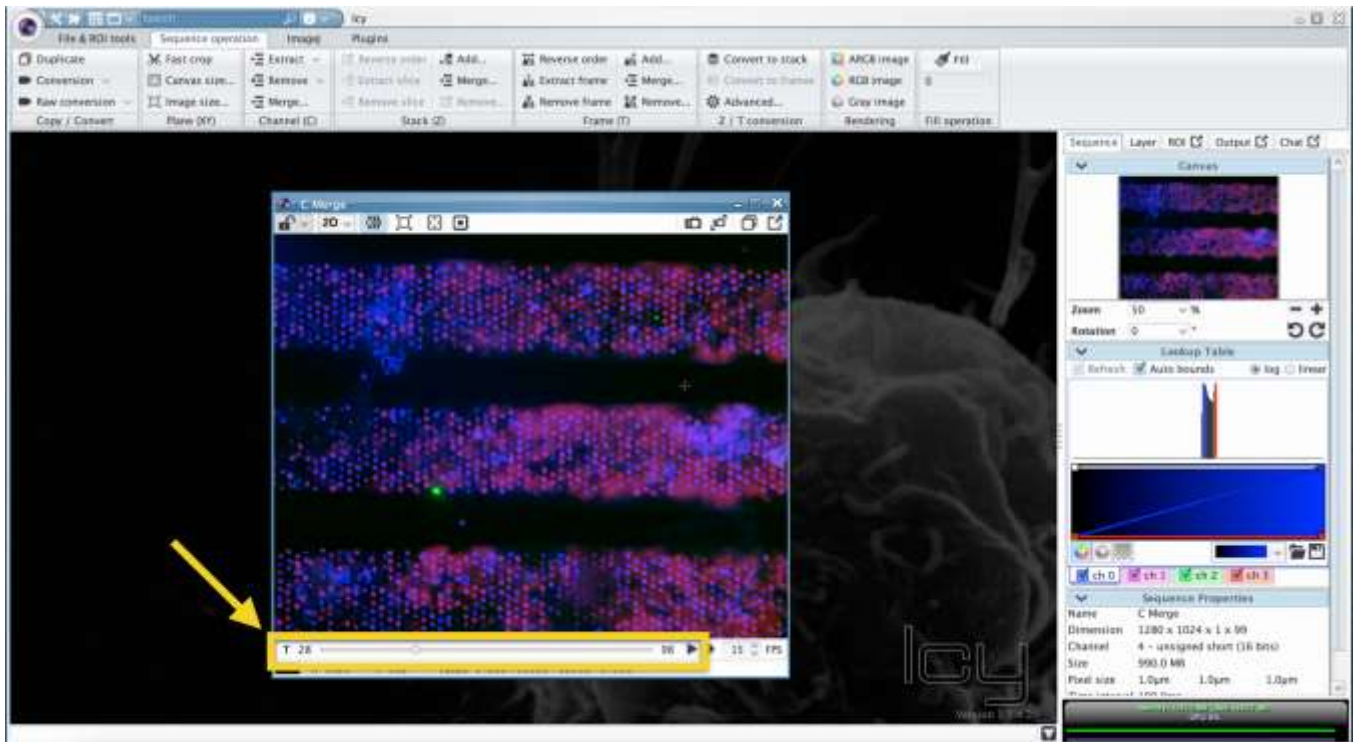


Figure 6 Scrolling through all images.

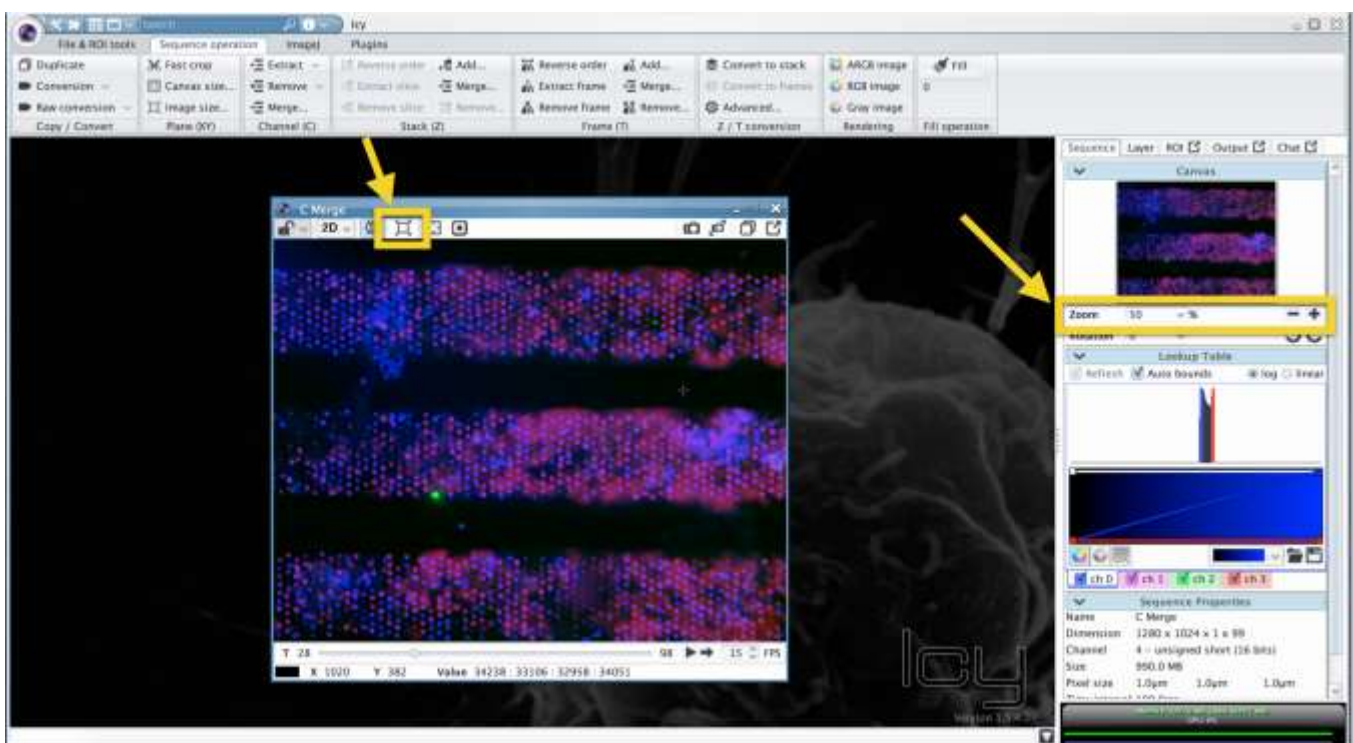


Figure 7 Zooming in and out.



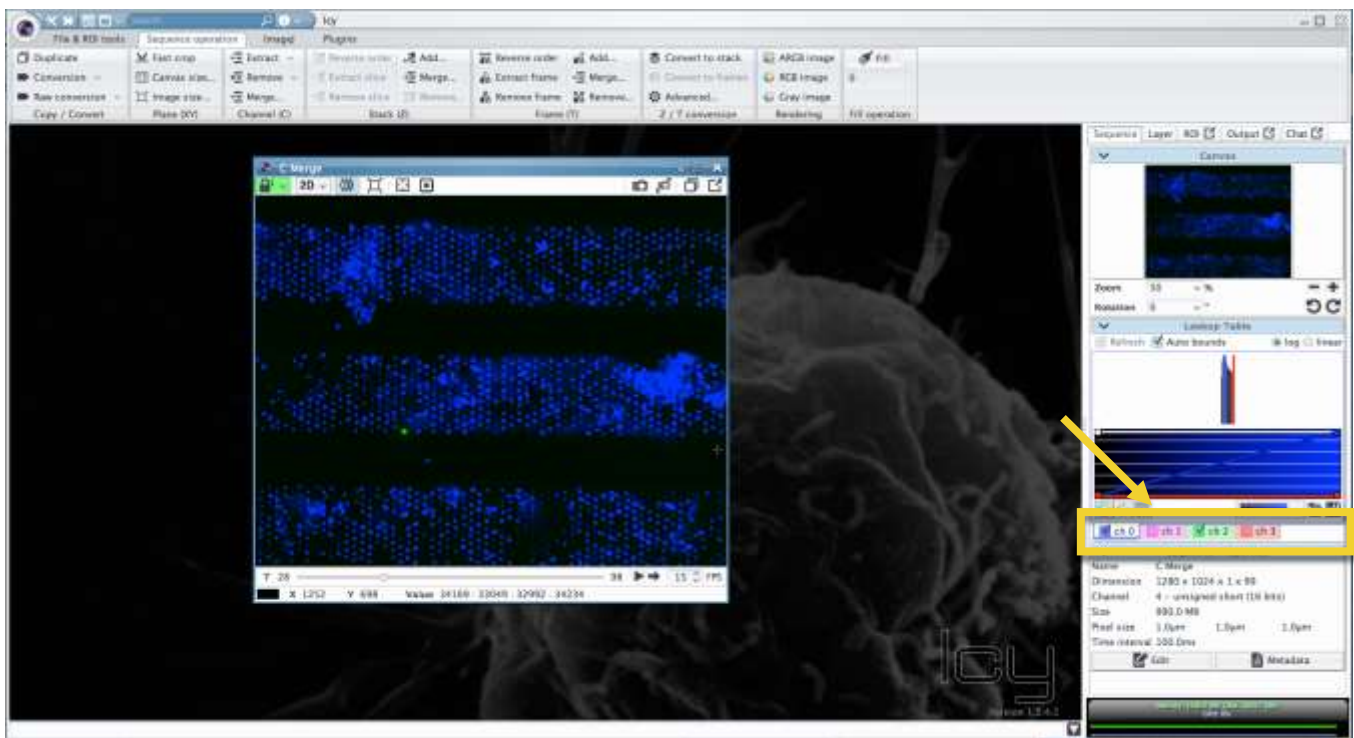


Figure 8 (De-)activation of channels in the merged image.

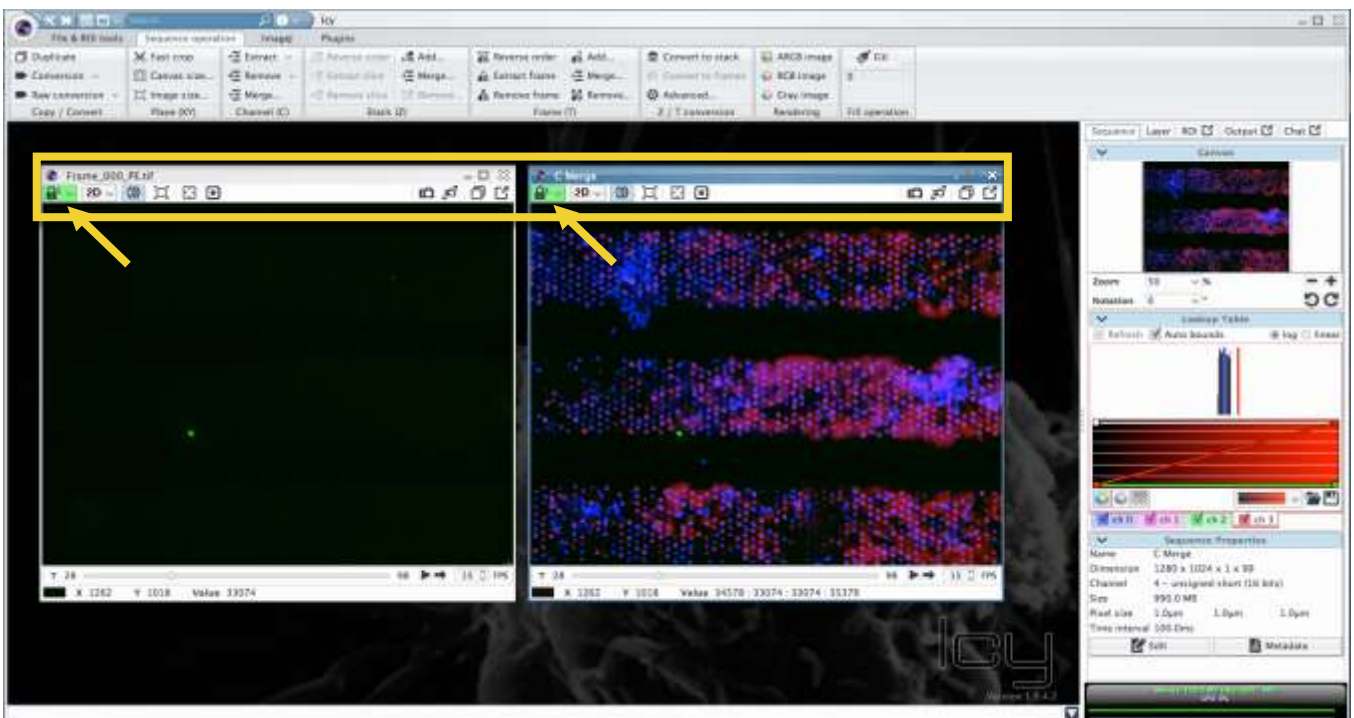


Figure 9 Synchronisation of multiple frames.

Depending on whether you prefer to look at the individual channels separately or at the merge of the images, you can activate and deactivate which channels should be shown in the merged image. This is done by ticking the channels on the right side in ICY that you want to be included in the merged image. If you are only interested in some of the channels, deactivate all the other ones (see Figure 8).

If you want to look at the merged image but simultaneously also on one (or more) individual channels, you can synchronise multiple frames. This is done using the lock on the right top of a frame. By default, this lock is open but by using the drop-down menu you can choose one of the closed locks. Choose the same lock in all the frames you want to be synchronised (see Figure 9). Then, the view in all of the locked frames is the same. That means, all actions that you perform (like zooming in and out, scrolling through all the images, etc.) are simultaneously performed in all frames that are locked. Also, your mouse cursor is now present in all frames.

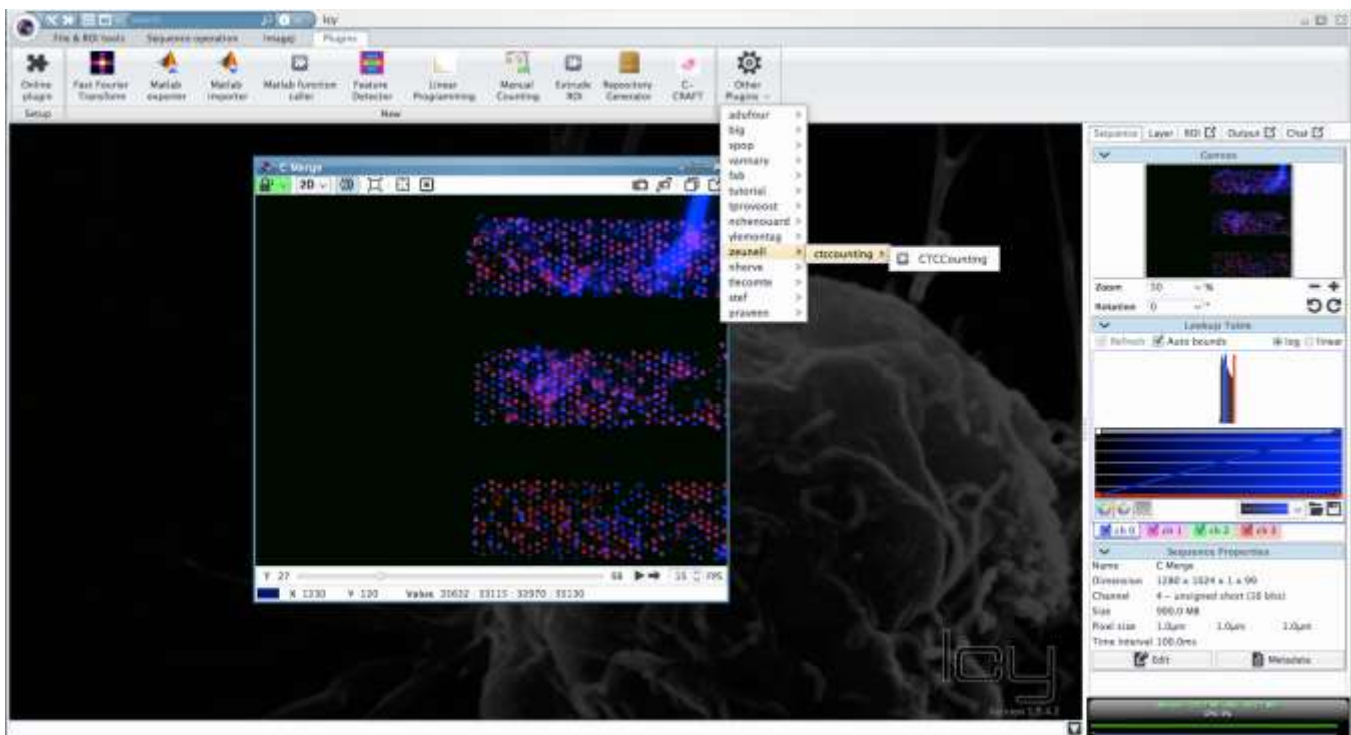


Figure 10 Open the Plugin.

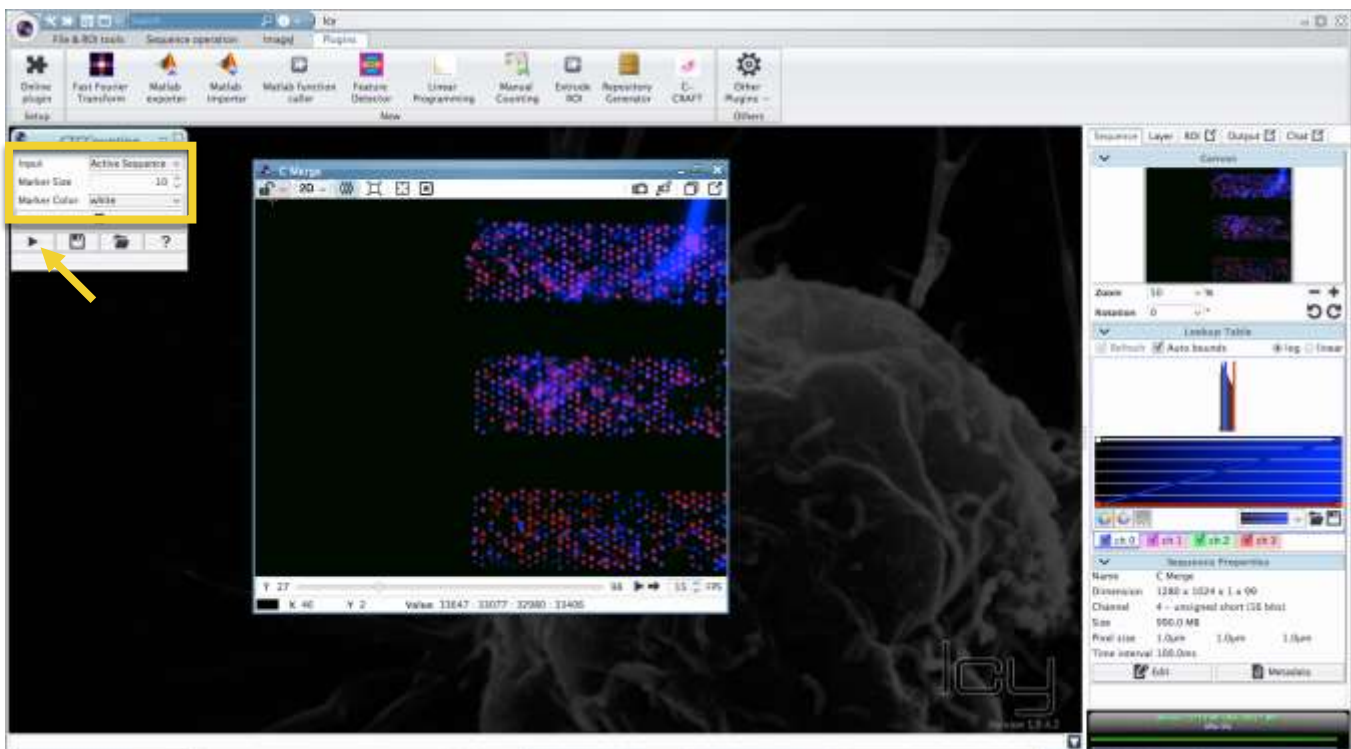


Figure 11 Adapting the parameters and start Plugin.

When you are using the synchronisation tool, make sure that the frames you want to synchronise have the same x-,y- and t-dimension.

### 3.2 Start the Plugin and Adjust the Parameters

To mark the CTC candidates in the images open the plugin as described before (see Figure 10). A GUI is opened where you can choose in which sequence you want to label CTC and the size and color of your marker (Figure 11). By default, the marker size is 10 but can be changed to any integer between 1 and 30. The marker color is white by default but can be changed to green, red, blue and yellow. Under 'Input' there is a dropdown menu containing all open sequences. By default, the plugin always uses the active sequence (can be identified by its blue instead of gray frame) as input, but you can choose another one if you want. After adjusting all input

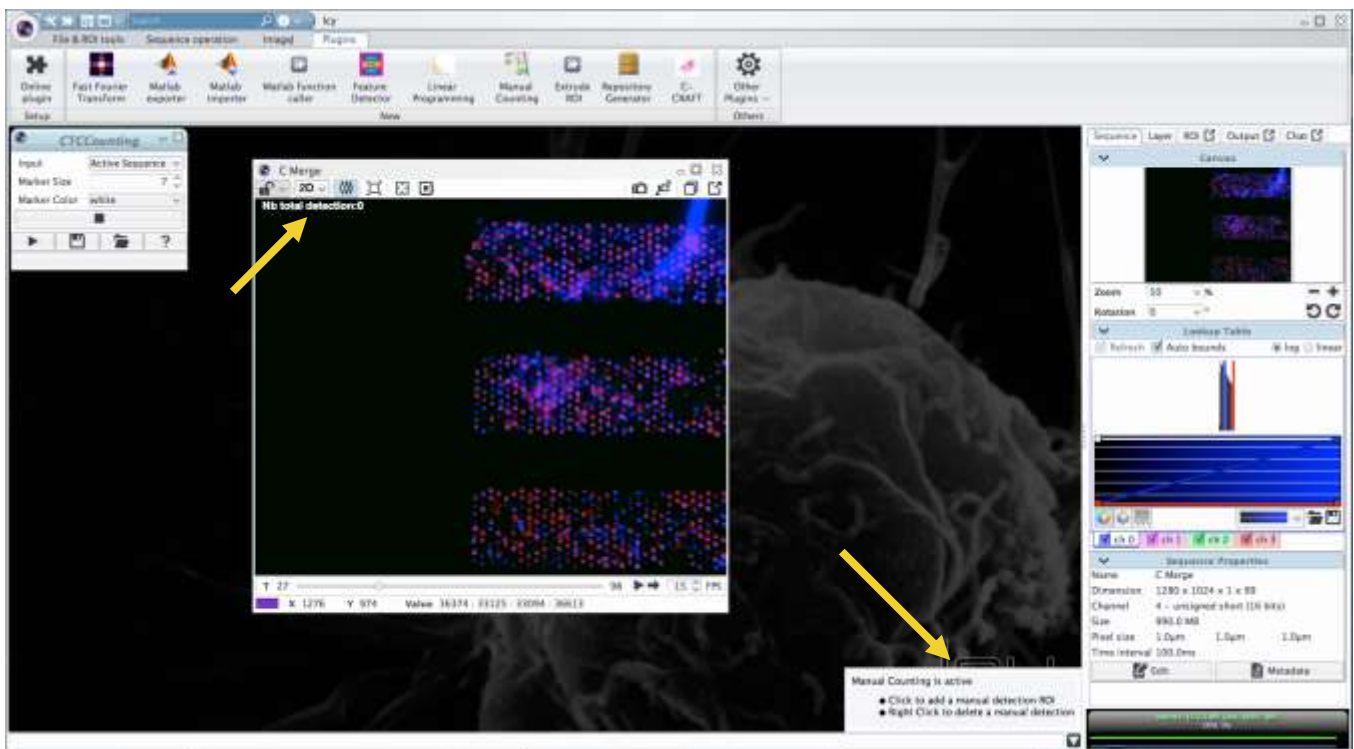


Figure 12 Start screen of the Plugin.

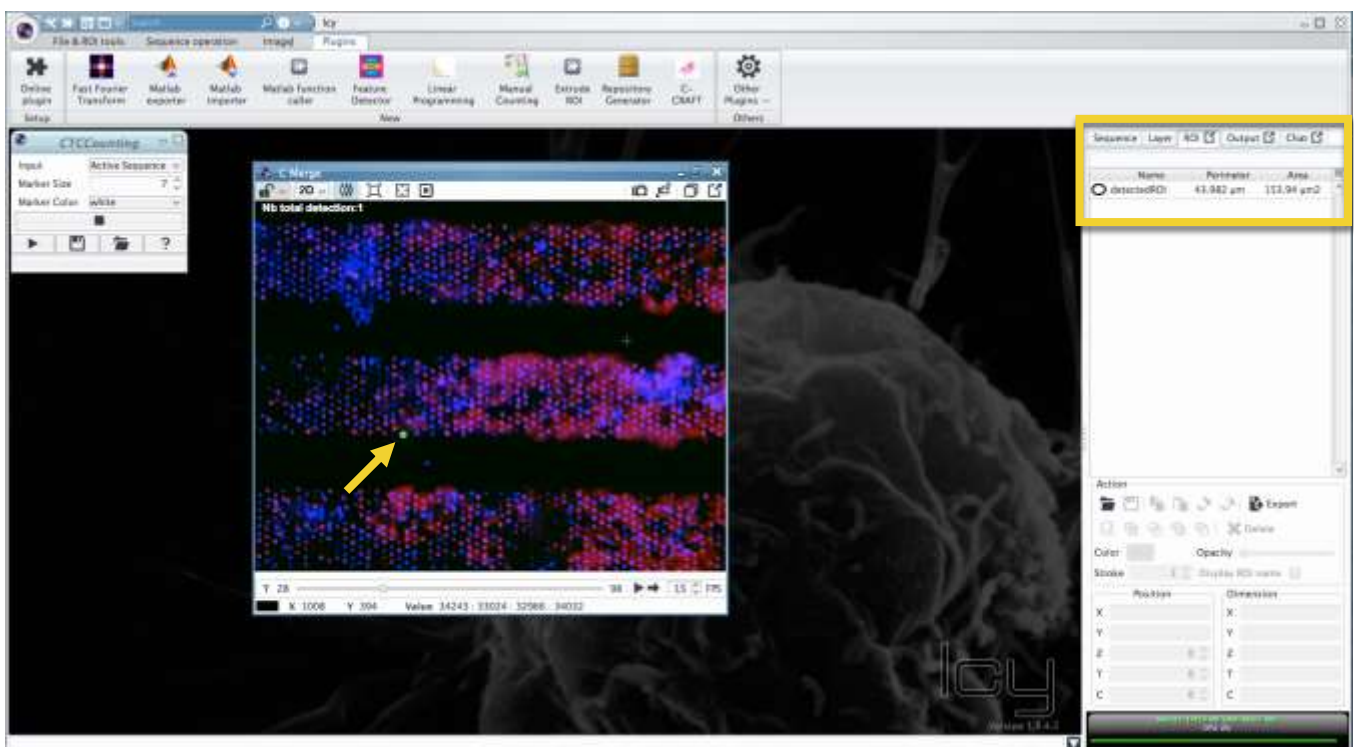


Figure 13 Labelling a cell.

parameters, click on the run button to start the plugin. Note, that every time you make adjustments to this input parameters (i.e. change the marker size/color or choose another sequence for labelling), you have to click the run button again to load the parameters. When the plugin is started you can see some explanations at the bottom right side of the window. At the top left side of your image you see the number of cells that you have already scored (see Figure 12).

### 3.3 Mark Region-of-Interest (ROI) in Images

If you found a CTC, you can label it by double-clicking on the cell in your image. The labeled cell is then marked with a circle. Click in the middle of the cell so that the cell is within the circle (this does not have to be 100%



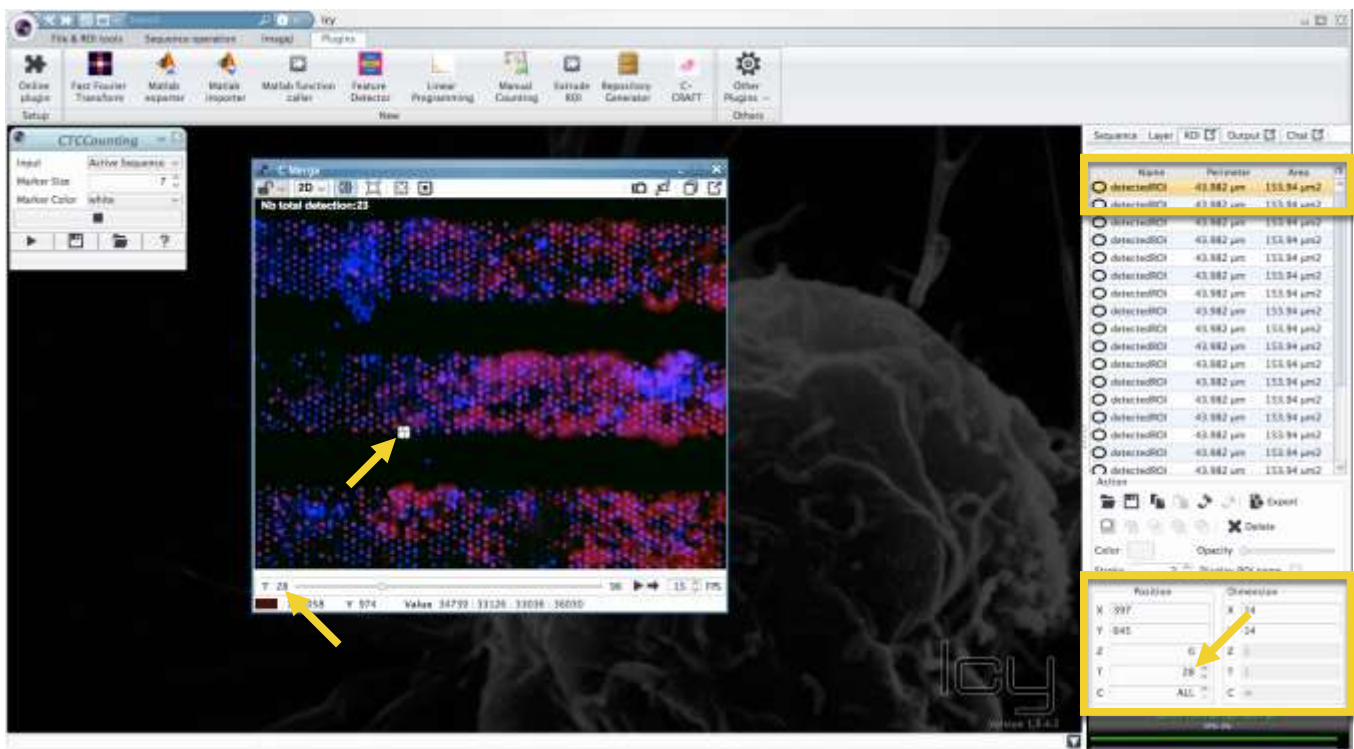


Figure 14 See ROI details.

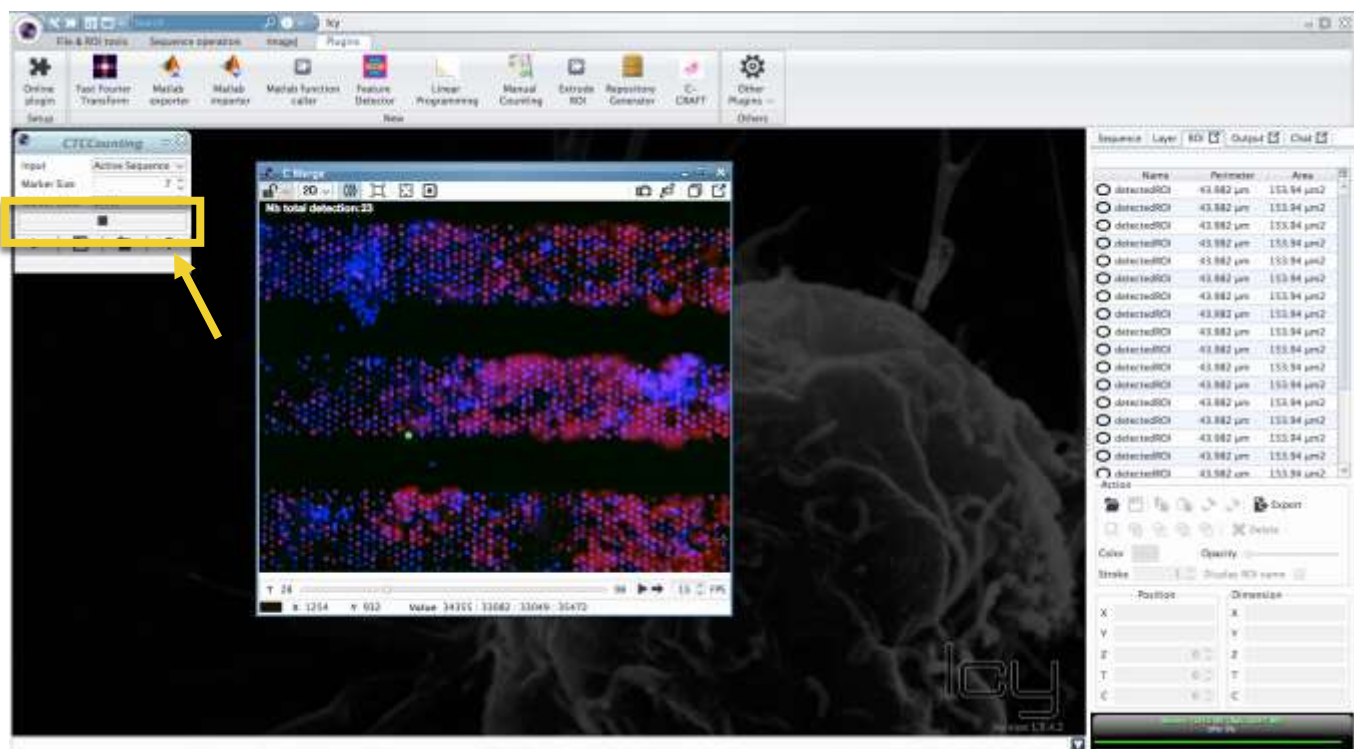


Figure 15 Stop the Plugin.

precise). If the size of the circle is too small or if you want to change the marker color, adjust the corresponding parameters in the GUI and click the run button again. If you click on the 'ROI' tab in the tab menu on the right, there is a list of ROIs that were already labeled (see Figure 13). Each entry consists of the position and dimension of this ROI and some additional measurements. By clicking on one ROI entry in the list you can see some details of this region such as x- and y- position and the frame number/t-position (see Figure 14). The cell corresponding to this entry is marked in the image.

If you want to remove a label, either use a right click on the cell to remove the label or remove the corresponding entry in the list of ROIs on the right. There is automatically a ROI called 'default ROI'. Please do not remove this ROI from the list.

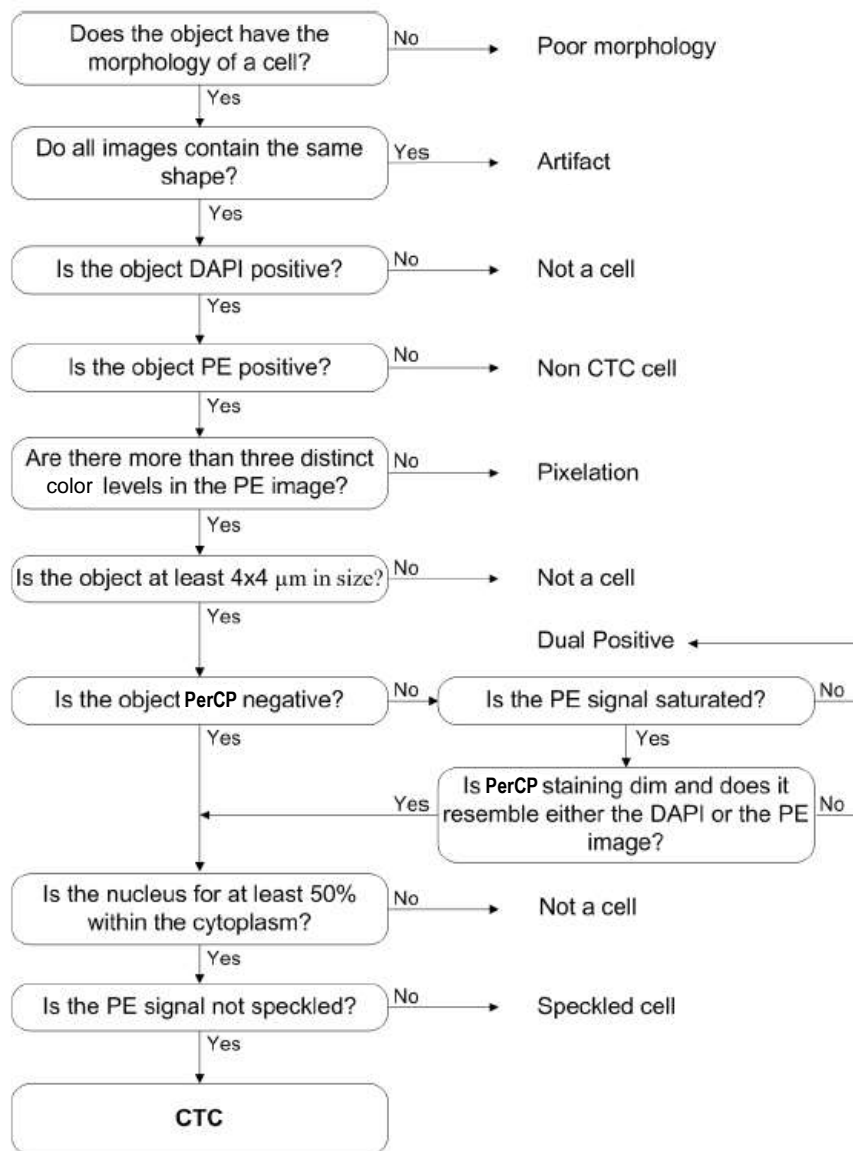


Figure 16 Decision tree showing how trained reviewers classify whether or not an object is a CTC.

The scoring of CTC should be according the criteria used for the scoring of CTC in the CellTracks system. The flow diagram for decision making if the object is a CTC, see Figure 16.

### 3.4 Export results

Once all CTC are labeled, close the plugin by clicking on the stop button in the GUI (see Figure 15). If the plugin is stopped, no cells can be labeled or removed anymore by a double-click or a right click, respectively. The counter on the top left in the image is not shown in the image anymore but the labeled regions remain labeled. The results can then be exported by selecting all entries of the ROI list in the 'ROI' tab on the right and then clicking the 'Export' button below (see Figure 17). All measurements are saved as an .xls file that needs to be uploaded together with the corresponding data set. Make sure that you add the extension .xls if you change the name of the results file.

In case there are no cells of interest in your sample, please export the list anyway (in this case only the details of the 'default ROI' will be exported). In this way we can see that you've processed this sample but couldn't find any events of interest.



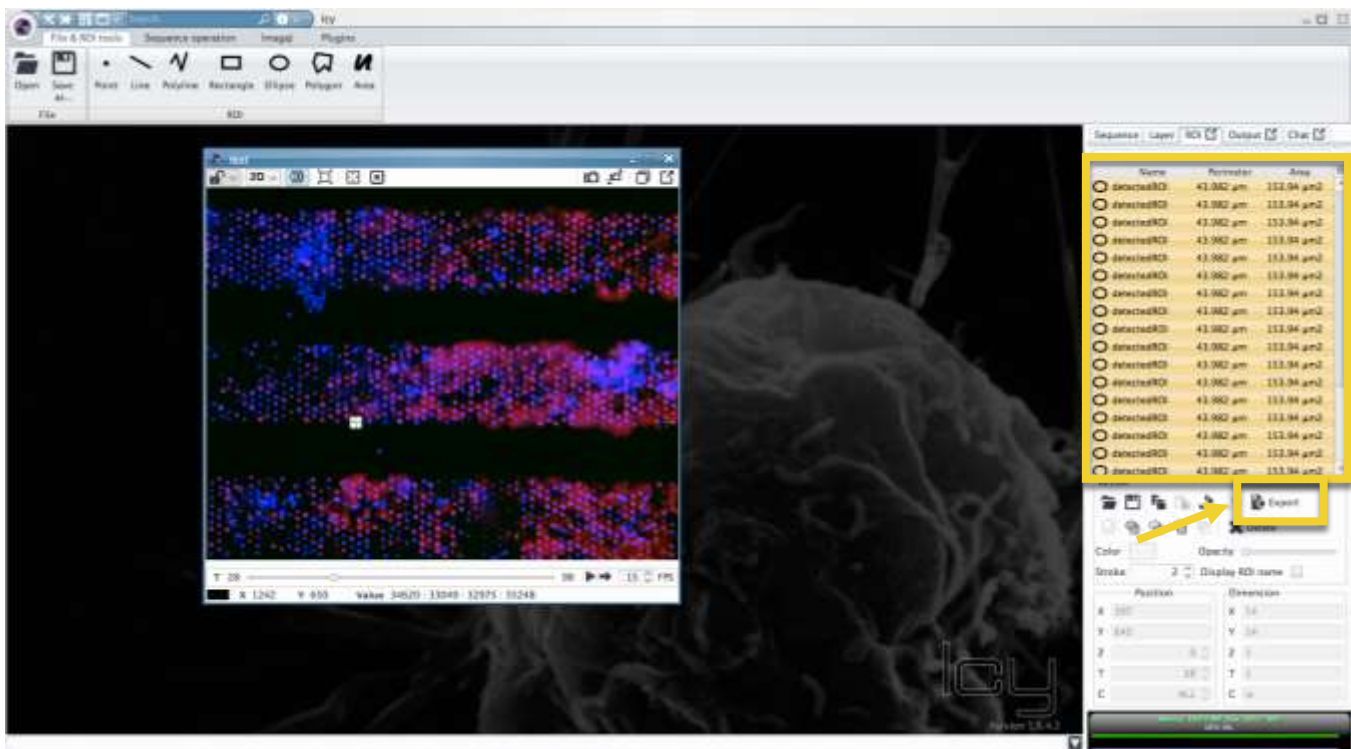


Figure 17 Export the results.

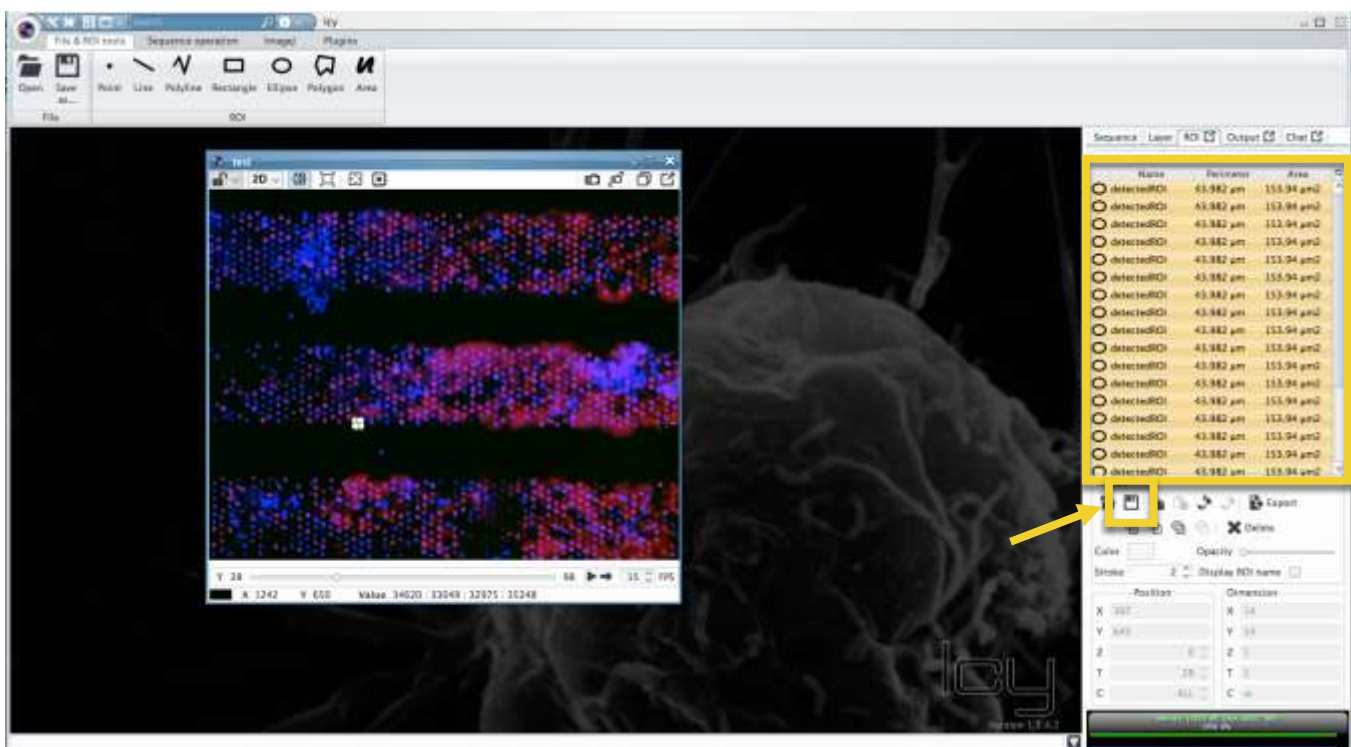


Figure 18 Export ROIs.

To export the regions that you have labeled so that you can import them later on, select all entries in the ROI list and then click on the disk symbol below the list (Figure 18). The regions are saved in an .xml file. Choose where you want to save the file and rename it if desired. Make sure that you add the .xml ending to the name. You can then import these regions later on, by opening your sequence again, going to the ROI list and clicking the folder symbol below the list (see Figure 19). By choosing the .xml file that you have saved before, the labeled regions are imported again.

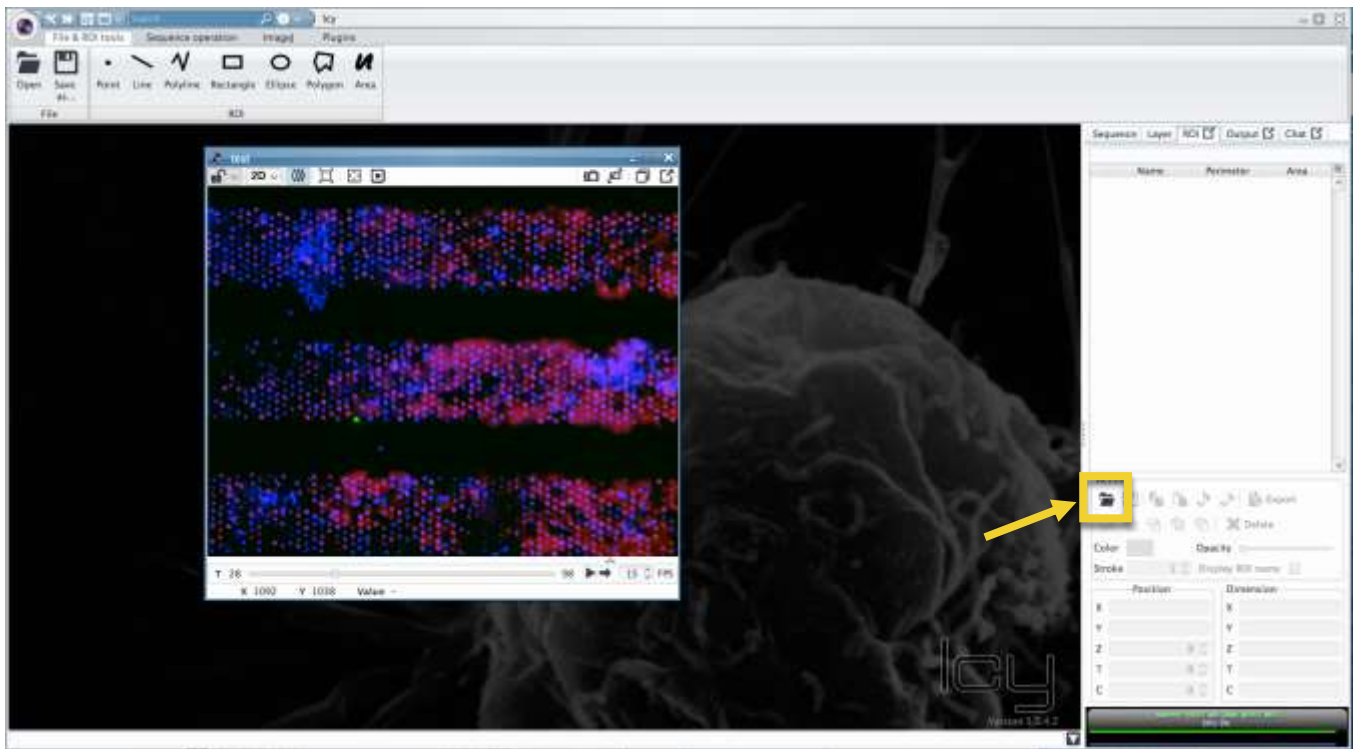


Figure 19 Import ROIs.

## 4. Uploading images

Scan all the manually filled-in checklists from the sample you have now processed (**SOP.1.** through **SOP.8.**). Upload all the images and these scans of checklists to the MCBP server in the corresponding folder.

Contact Leonie Zeune (University of Twente) ([l.l.zeune@utwente.nl](mailto:l.l.zeune@utwente.nl)) if you have any questions or problems concerning the usage of this plugin, or if you have questions about the uploading to the server.

## 5. Checklist SOP.8.

Sample name	
Operator name	
Draw date	Clinical site
Prep date	Clinical site
Microsieve scan date	Clinical site
Analysis date	Clinical site
Were all the channels and frames of the scan opened?	<input type="checkbox"/> Yes <input type="checkbox"/> No
Were all CTC scored with the plugin running?	<input type="checkbox"/> Yes <input type="checkbox"/> No
Were the CTC scores exported to the .xls file?	<input type="checkbox"/> Yes <input type="checkbox"/> No
Were the CTC scores saved to the .xml file?	<input type="checkbox"/> Yes <input type="checkbox"/> No
Did you upload the images to the server?	<input type="checkbox"/> Yes <input type="checkbox"/> No
Did you upload a scan of all processed checklists to the server?	<input type="checkbox"/> Yes <input type="checkbox"/> No
Notes:	