



Manual for Suiker:

a program for e.g. evaluating cellular incorporation of sugars

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1. Installing Suiker

There is no need for any registration or special rights to install and use the program. Simply copy the Suiker.exe file from the Suiker.zip file onto your hard disk and it will run. Alternatively, you can use the “extract” function to do this.

2. A few words of warning!

This program was custom-made for solving the specific problems in our laboratory. It was programmed for using the formats we use (especially in the project visualizing sugars on neurites), on our computer setup and with our aims in mind.

3. Technical background in brief

a. programming background

Suiker was written in Delphi XE5 (Embarcadero Technologies, Inc.) for the use on Windows 32bit. It will run on all Windows versions later than Windows98, regardless whether it is a 32 bit or a 64 bit version. Suiker will not run naturally on Mac or Linux. If you have Wine installed there is a very good chance it will run on the latter two OSes. However this has **NOT** been checked by us.

b. types and resolution of images used

Suiker supports a range of image formats, including JPEG, PNG and TIFF. A resolution of 512 x 512 pixels or higher is recommended but not necessary. In principle, any image which can be split to a maximum of three channels can be used.

c. our experiments used to establish Suiker

For all our experiments, in which we evaluate the incorporation of unnatural sugars, we use 8-well glass bottom slides from Ibidi.

To analyze the results we use three fluorescent channels. The blue channel is used to visualize the nucleus with H-33342. The total cytoplasm is detected in the green channel with the use of Calcein-AM. Finally, the metabolically labeled sialic acids are monitored in the red channel. However, the program is capable to switch channels, see page 6.

Pictures are taken on a Zeiss LSM 880 confocal laser-scanning microscope, with a 40 x oil immersion objective.

The freeware program “Fiji” is used to convert the Zeiss images, representing a slice with a total thickness of four microns, into one picture. Fiji uses up to 9 focal planes 0.5 microns apart to generate one with maximum intensity by z-projection. This is done for all three channels used and a final picture of 512 by 512 pixels is exported in PNG format, ready to be used by Suiker.

d. batch processing

Usually, all the final pictures of experiments that belong together (like controls, different concentrations etc.) are saved in one folder. Suiker is capable of assaying all these in one single sweep using the same settings, thereby shortening assay duration enormously.

e. output

The output can be exported directly to Microsoft Excel - this program has to be installed on the computer in order to use this feature.

4. Running Suiker

a. opening your first file

The first thing to do is, to load one of the pictures you made.

As you can see, there is just one menu item (File) on the menu bar. Clicking it will reveal the only command: Open. This command will open the standard input window specific for your Windows version.

As soon as you load one file from a folder, all other files in this folder will be checked and their names will be listed on the right panel of Suiker. The file names will be equivalent to the name of the images you opened.

Not only other files will be listed but your whole screen is now filled with graphics and text (Fig. 1).

On the left side you will see the picture you just opened in four copies: three of them show the information on one of the channels. Only the fourth, the right hand lower corner, shows the original picture. It might not look as it would look in another program, but we will get to that later.

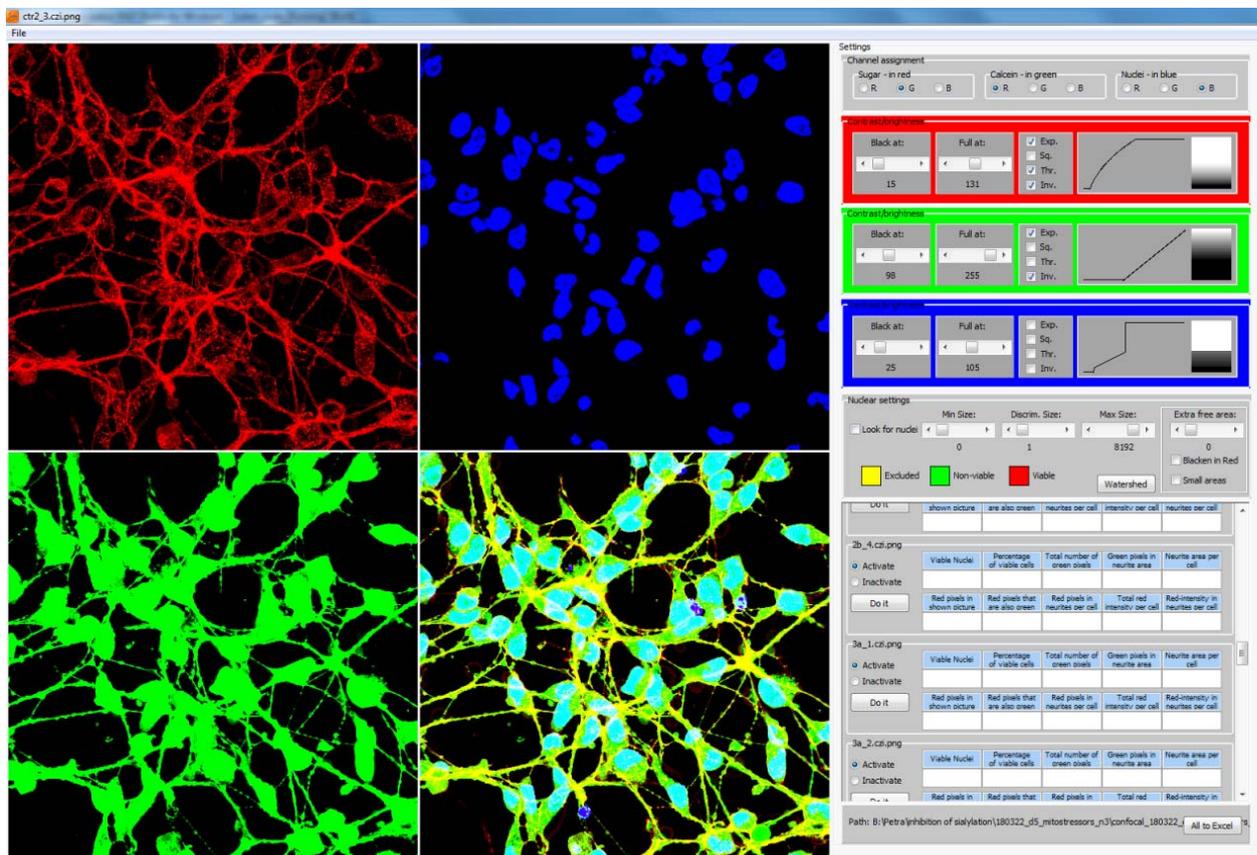


Fig. 1 Suiker's graphical interface, control bars and results panel.

b. channel assignment

Let's start with the right part of the screen, containing a lot of text in colorful bars. However, the one at the very top is in a dull gray, see Fig. 2. This panel allows you to choose the color channels to be used in Suiker. The program always shows the information in the same way: the nucleus in blue, the cytoplasm in green and the sugar moieties in red. But there is no rule to make the pictures only with these settings. You can do it differently. Say, for instance, your sugar is in green and your



cytoplasm is in red, then just click the “G(reen)” radiobutton in the box labelled “Sugar - in red”. It will now look exactly as depicted in Fig.2. Secondly click on the “R(ed)” button in the “Calcein - in green” box. As you do this you will see that the pictures on the left hand side change accordingly.

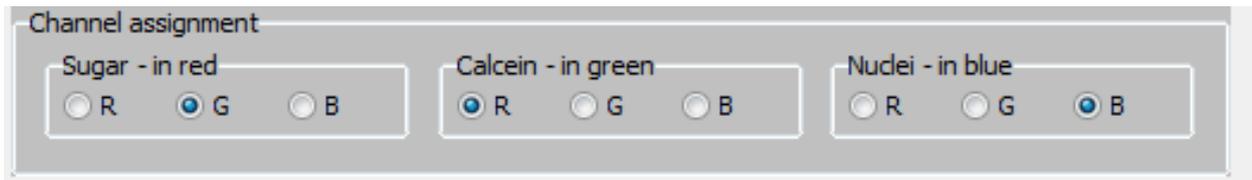


Fig. 2 Channel assignment.

This is true for all changes you make in Suiker. You will see the results in real-time. Which brings us to the three very colorful panels directly underneath the previous one.

c. channel settings

All the settings you change in the next few panels will change the appearance of the pictures, sometimes dramatically. But remember: all these changes are just made to visualize small changes more clearly; they will not change the outcome of the calculations. Most calculations (e.g. on the sugars and calcein) will be done on the original data. Exceptions to this rule are the calculations depending on the background subtractions and on the nuclei (see further down).

As an example, the top - red channel is shown in Fig. 3.

All the settings are identical for the three different colors. Therefore, the various possibilities will be described here only once.

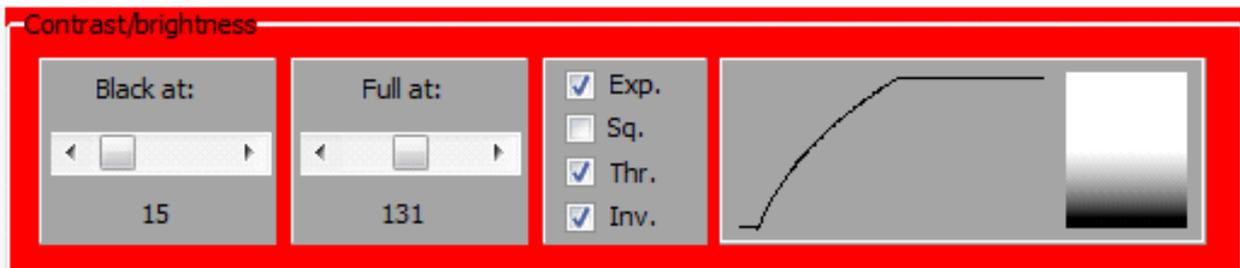


Fig. 3 The color adjustment panel - red version.

The leftmost subpanel is labelled “Black at:” To explain what it does, consider that a lot of pictures you make with a microscope do not have a truly black background. Most, if not all backgrounds, are in reality just a very dark gray. For a computer, the color black is represented by three times the number “0” (meaning no Red light, no Green light and no Blue light - RGB colors).

As there is almost always is a little light in any dark room, most backgrounds that look black are rather something like 5-7-13 in RGB. Here is your chance to correct this: just move the slider to a higher position and the background for the corresponding channel will be forced to zero.

The next slider to the right does something similar as the first, but now with the color white instead of black. In RGB, white is - 255-255-255. With this slider you can turn all bright grays into pure white.

The final parts of the panels control exactly how different shades of gray of the original picture are depicted on your screen. This process is controlled by the four checkboxes shown in Fig. 4A.

There are three basic shapes for the transformation of your gray colors. If both, Sq. and Thr. are not checked, the shape will be linear. Without any settings of the “Black at” or the “White at” the transformation is one to one = no change. This is depicted by the two graphs that show this on the right. Horizontally the original gray scale values are plotted, vertically the newly calculated values are shown. This is depicted in Fig. 4B.

The distribution of the various grays is shown continuously in the box on the far right. As soon as the user changes the setting the graphs will change along.

In Fig. 4C it is obvious that all original values up to the value set in “Black at” are transformed into 0, - the beginning horizontal part of the line. Then there is a linear increase up to the setting of “White at” after which all values are put to 255. The box on the right shows this as a black bar on the bottom, the gray band in the middle and finally as the white bar at the top. The gray band contains all grays squeezed into the space between the black and white bars.

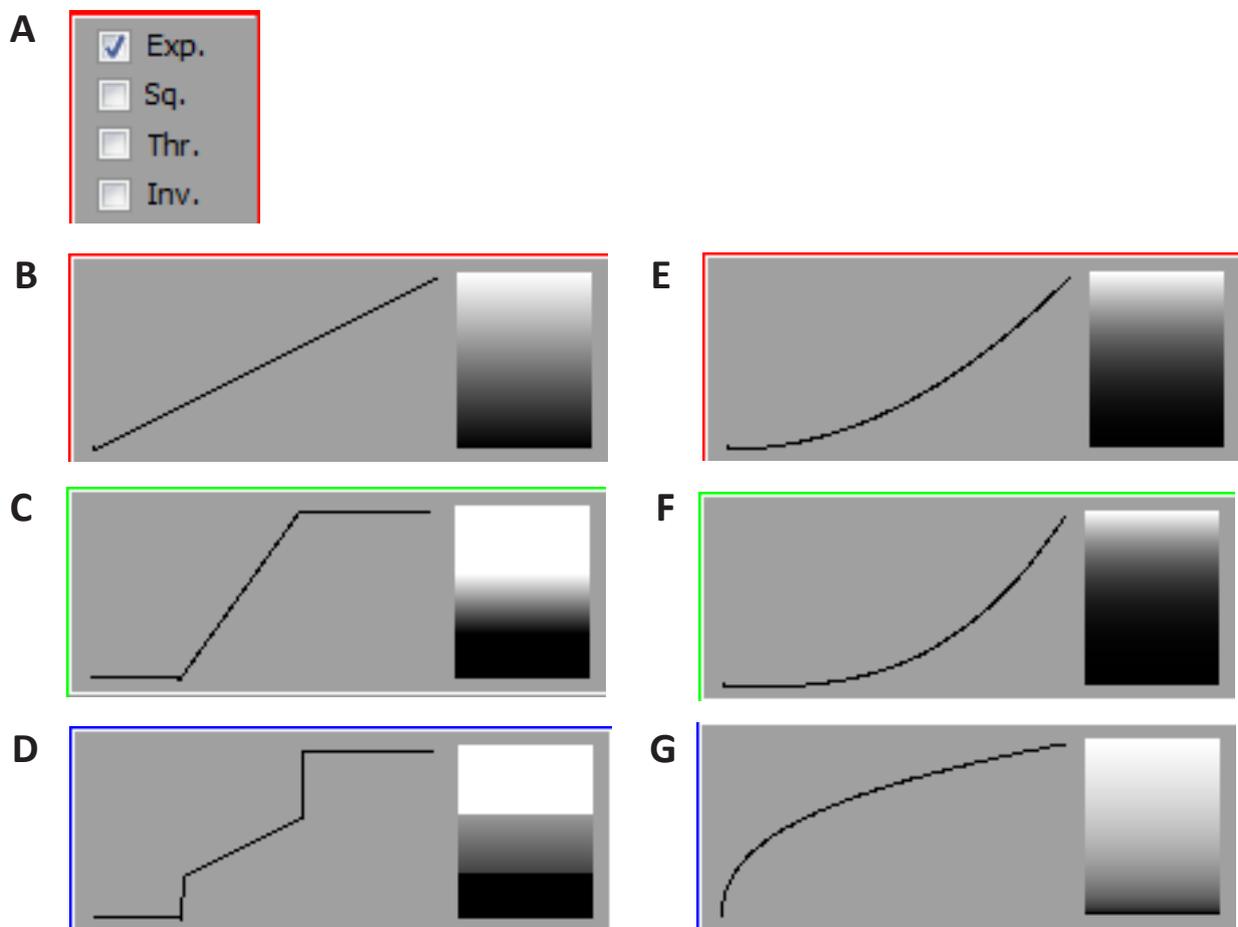


Fig. 4 The control of shades of gray.

- A) The central four checkboxes, controlling the basic shape (linear, squared, to the third power and inversed)
- B) Linear, no correction for black or white
- C) Linear with black at 65, white at 155, with expansion (Exp. checked)
- D) Linear with black at 65, white at 155, no expansion
- E) Squared
- F) To the third power
- G) To the third power, inversed



In Fig. 4D the results are shown for the same settings but with the Exp.-checkbox unchecked. The lowest values are put to black, the highest to white but now those in between are not squeezed in. These values are left at their original values. You try the use of the Exp. checkbox, it will make a big difference in a lot of your pictures.

The relation between the original value and its latter counterpart does not have to be linear. Just click on “Sq.” or “Thr” and you will see the difference. You will see differences between shades that your natural vision can not distinguish. If you want to see differences in another range, combine “Sq” or “Thr” with “Inv”.

Of course all these settings can be combined freely with “Black at”, “White at” and “Exp.”. With these combinations, in all three channels, you can not only make everything visible but even clearly obvious.

d. finding the nuclei

The settings of the blue channel are also used for finding the nuclei. Together with the “Nuclear settings” (Fig. 5A) the program will decide what is and what is not a nucleus and visualize this in a color scheme. You will have to switch this on by checking the “Look for nuclei” checkbox on the far left in the gray “Nuclear settings” panel. This will activate the subroutines to detect the nuclei.

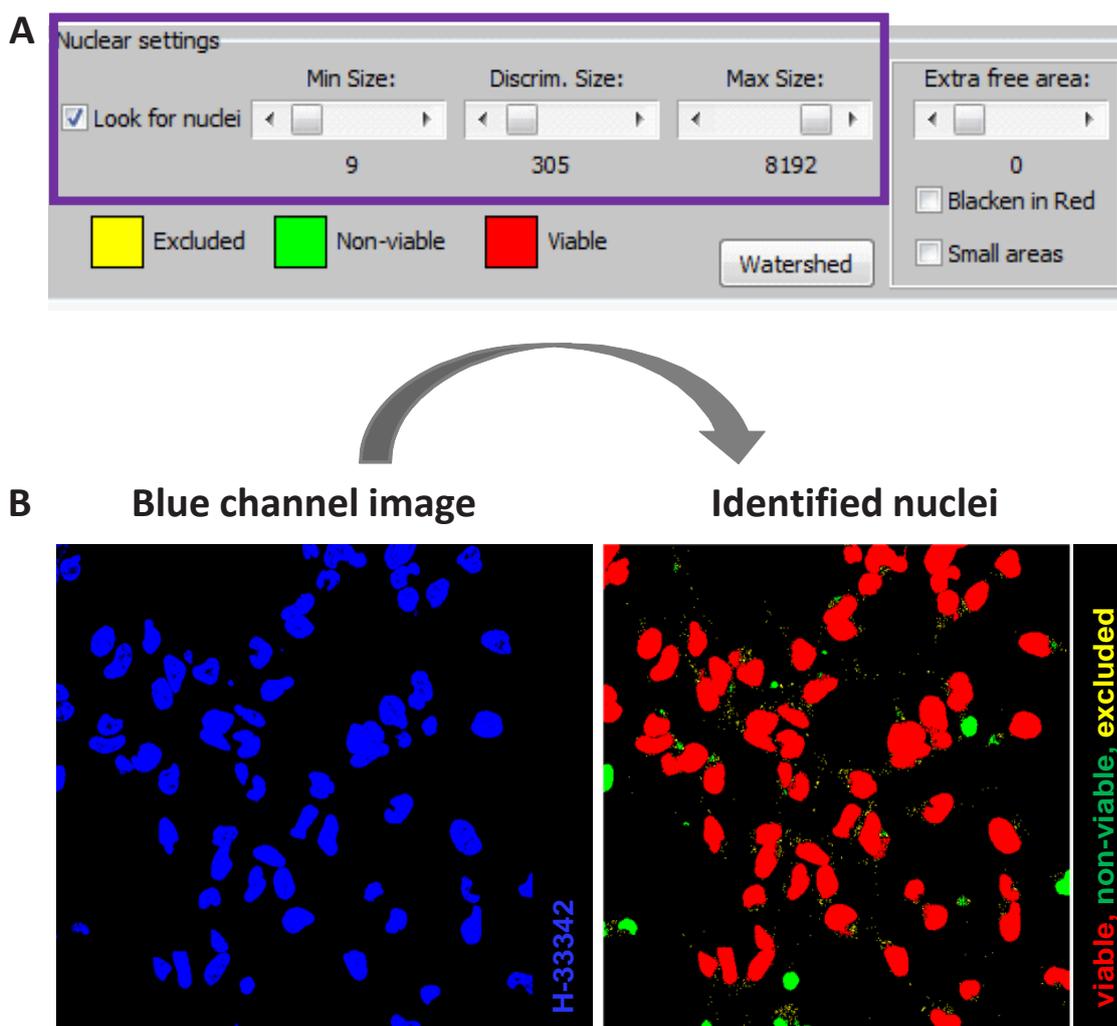


Fig. 5 Finding the nuclei.

The three sliders, labeled “Min. Size”, “Max. Size” and “Discrim. Size” should be rather obvious in function. Use them to define the size criteria for identifying nuclei. In principle, nuclei that do not fit your defined ‘viable’ criteria size are changed to green. Those that do fit the criteria are changed to red. The remaining background pixels will be depicted as yellow (Fig. 5B).

You will notice that the settings for the blue channel influence the detection of the nuclei. As all light emitting objects have a slight halo around them it is possible to influence the apparent size of an object by changing the “Black at” settings in the blue channel.

e. defining neurite area and neurital glycans (or other superimpositions)

The right part of the panel is much more interesting, it enables you to distinguish between the nuclear area and the cytoplasmic/neuronal area. After you ‘Looked for nuclei’ by adjusting the nuclear size parameters, use the slider marked “Extra free area” (Fig. 6A). This will shift the border of where the nucleus ends and the surrounding start. You can expand this area by as much as you need in order to cover that part of the cell (in our case, everything except of the neurites) which you would like to exclude from the analysis.

Small areas can also be subtracted by ticking the “small areas” box in the Nuclear Settings panel (Fig. 6A). Small areas are e.g. those nuclei that you considered too small and therefore fragmented and non-viable (depicted as green in Fig. 5B). As non-viable neurons still possess (parts of) neurites, we also expand the “Extra free area” for the “Small areas”.

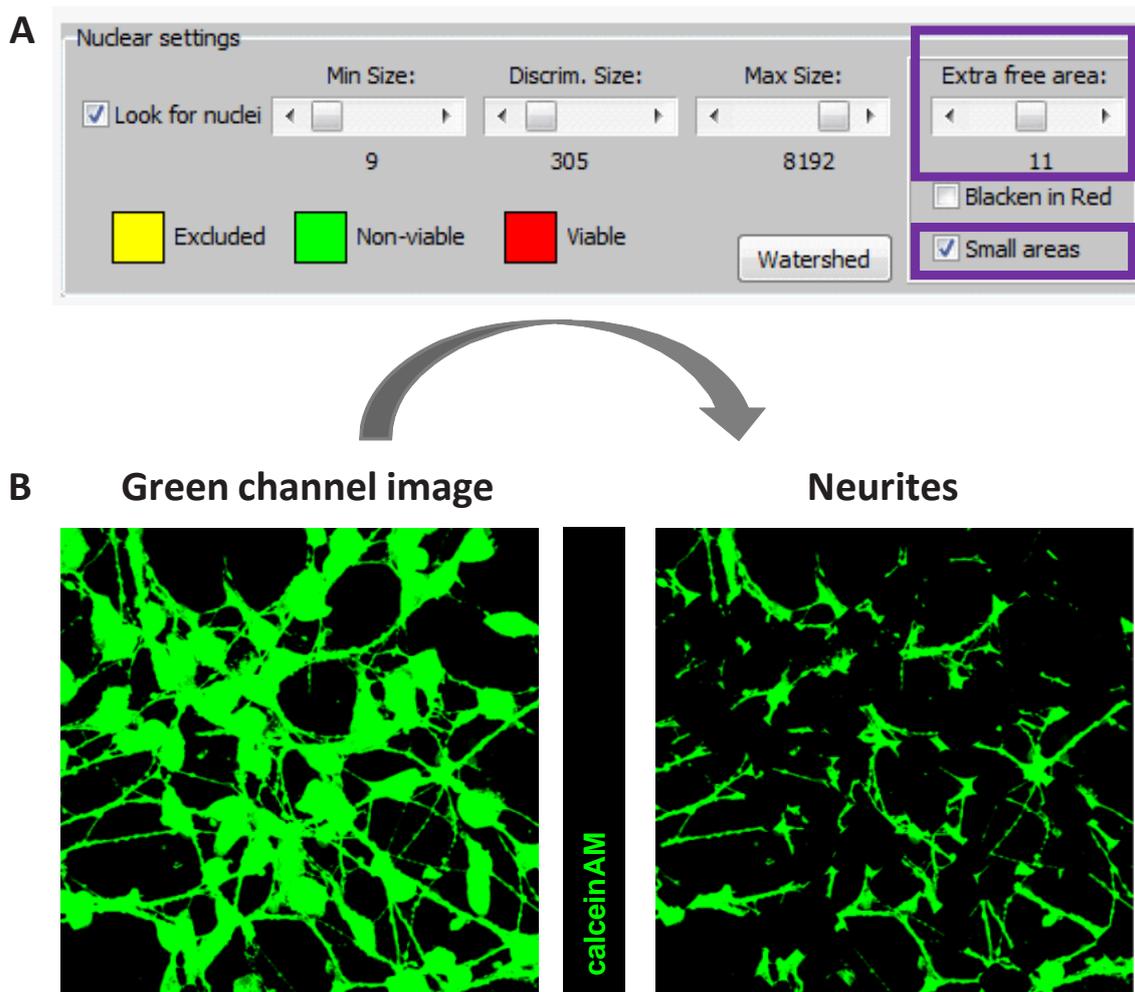


Fig. 6 Defining neurite area.

By now, you managed to identify the nuclei from the blue channel and the neurites by subtracting the expanded nuclear area from the green channel. It is time to look for the intensity of the red (sugar) fluorescent pixels that colocalize with the (green) neurites. By selecting the “Blackened in Red” option in the Nuclear Settings panel (Fig. 7A), the expanded nuclear area will also be subtracted from the red (sugar) channel. Now, the red (sugar) staining within the neurites only will be displayed and analyzed (Fig. 7B).

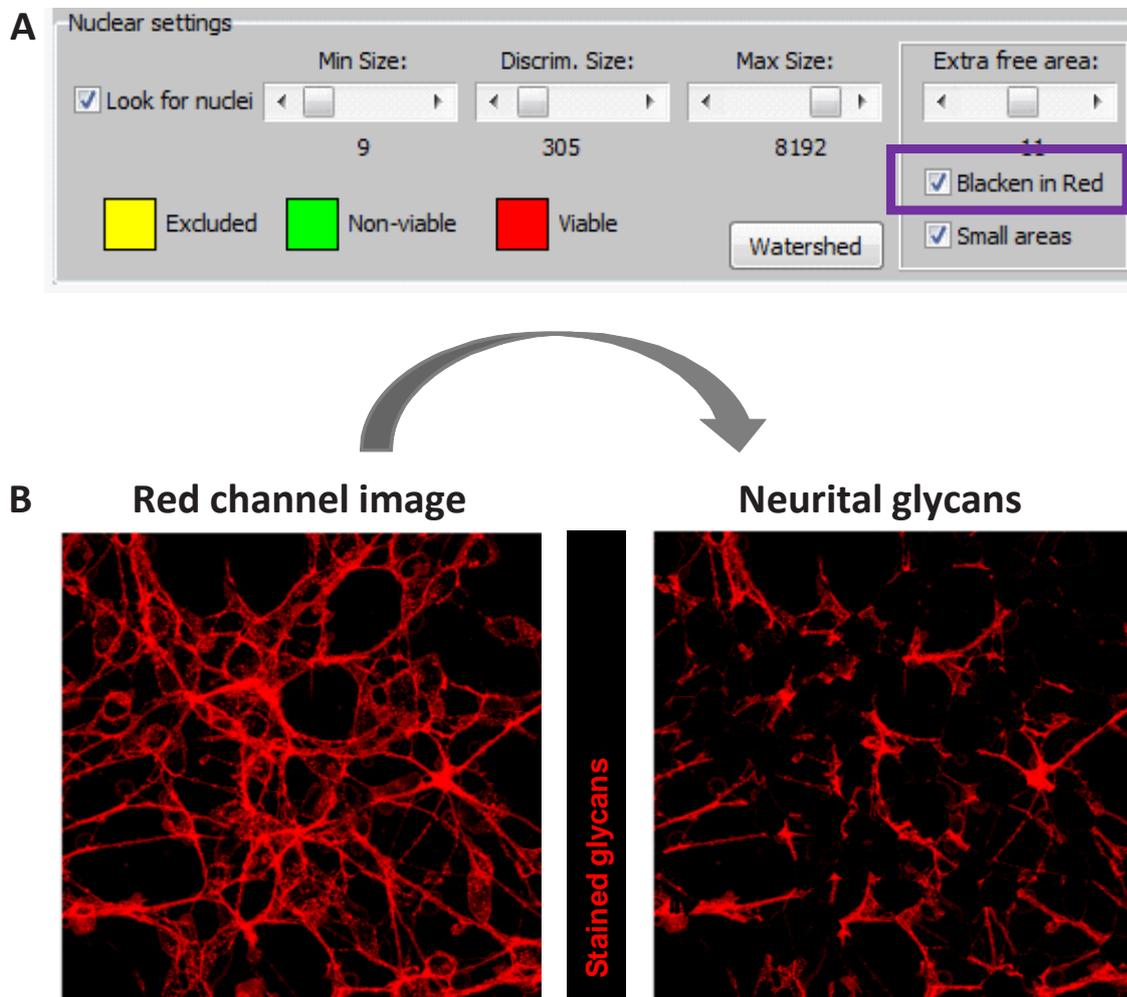


Fig. 7 Defining neurital glycans.

There is only one more button in this part of the panel named “Watershed”. This is an optional additional function of Suiker (currently not used for the ‘Visualizing sugars on neurites’ project). When clicked, the program will draw lines in the composite picture (bottom - right). These indicate the probable borders of neighboring cells. This data is for your information only, it has no further influence on any calculation.

The watershed function has a great potential for being used with cells other than neurons for e.g. colocalization of proteins in single cells or fluorescence and colocalization studies where segmentation of cells is necessary. However, with neuronal cells where the neurites are interconnected and often overlapping, a proper segmentation is not possible (Fig. 8), explaining why we analyze the entire image first, before normalizing to cell number as further explained in the ‘Results’ section.

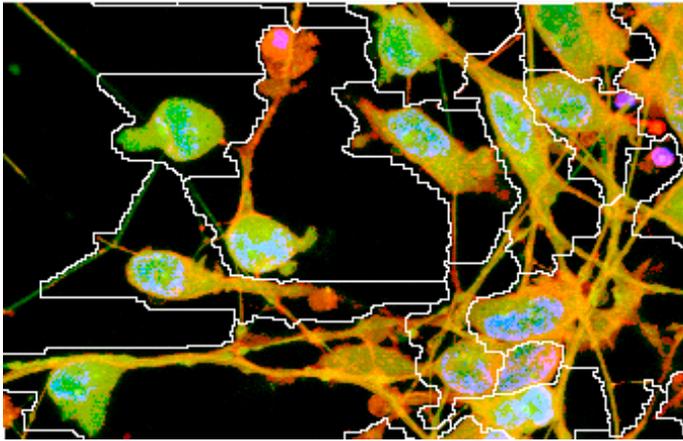


Fig. 8 The result of watershed, showing the cellular areas.

f. evaluation

Once you defined all the above mentioned parameters, they will be used to calculate a number of different things, to be displayed in the 'Results tables'. There will be as many of these tables as there are images in the folder from which you opened the original file. The name of this folder and the complete path are shown at the very bottom right of Suiker (Fig. 1). If some of the tables are not shown directly, you can scroll down to see those corresponding to other files.

There are some controls on the left, next to the table proper. The activate/inactivate radiobuttons will exclude/include the corresponding file in the en masse evaluation. This feature will be discussed later (page 12, under Export to Excel). The single "Do it" button will use the settings you have at this moment and apply them to the file whose button you clicked. The file will be loaded and displayed at the same time.

5. The results

As can be seen in Fig. 9 there are 10 different values Suiker will calculate. The first field of the table contains the number of **Viable Nuclei** counted. This number is automatically corrected for nuclei that appear "fused" in the blue channel. As it is important to be able to compare several pictures, some of the values will be expressed as: per cell.

The next field is the **Percentage of viable cells**. This calculation is based on the number of nuclei and the number of nuclei you set as too small (non-viable). The number of the small nuclei is shown as the second number between the brackets.

ctr2_3.czi.png

Activate
 Inactivate

Do it

Viable Nuclei	Percentage of viable cells	Total number of green pixels	Green pixels in neurite area	Neurite area per cell
61	$51/(61+27)*1$	111572	33527	550
Red pixels in shown picture	Red pixels that are also green	Red pixels in neurites per cell	Total red intensity per cell	Red-intensity in neurites per cell
55147	32449	532	50435	39876

Fig. 9 The results tables.

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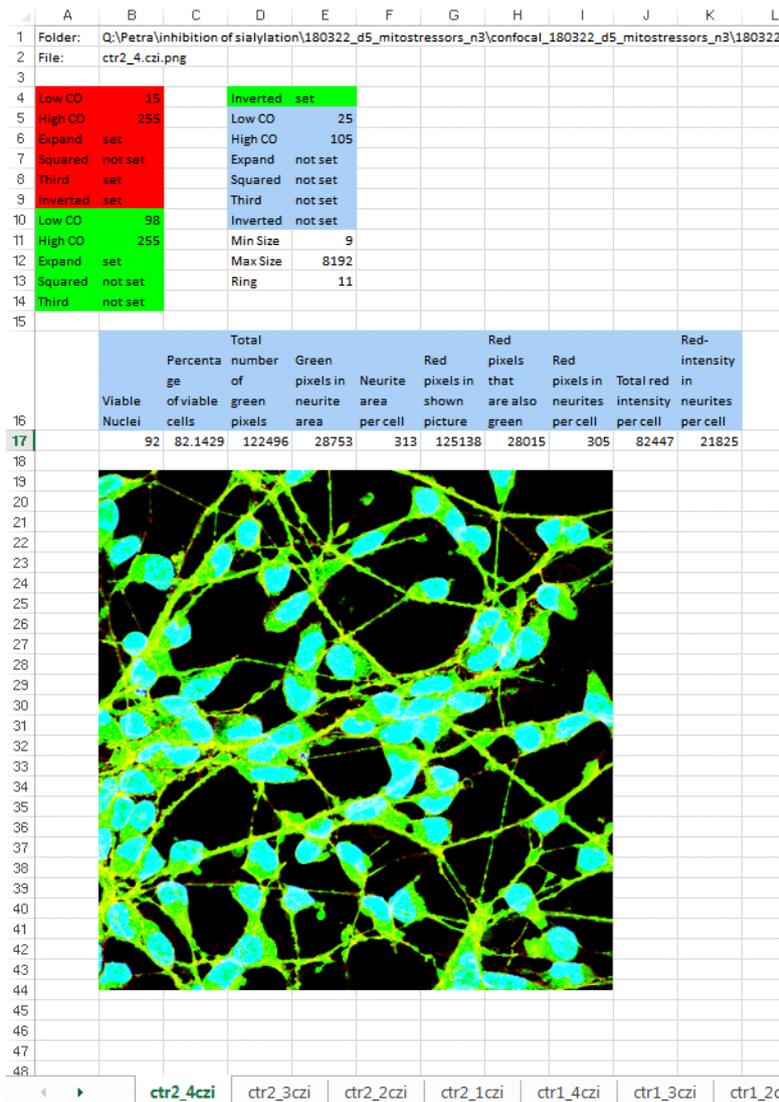


Fig. 10 Results Excel sheets.

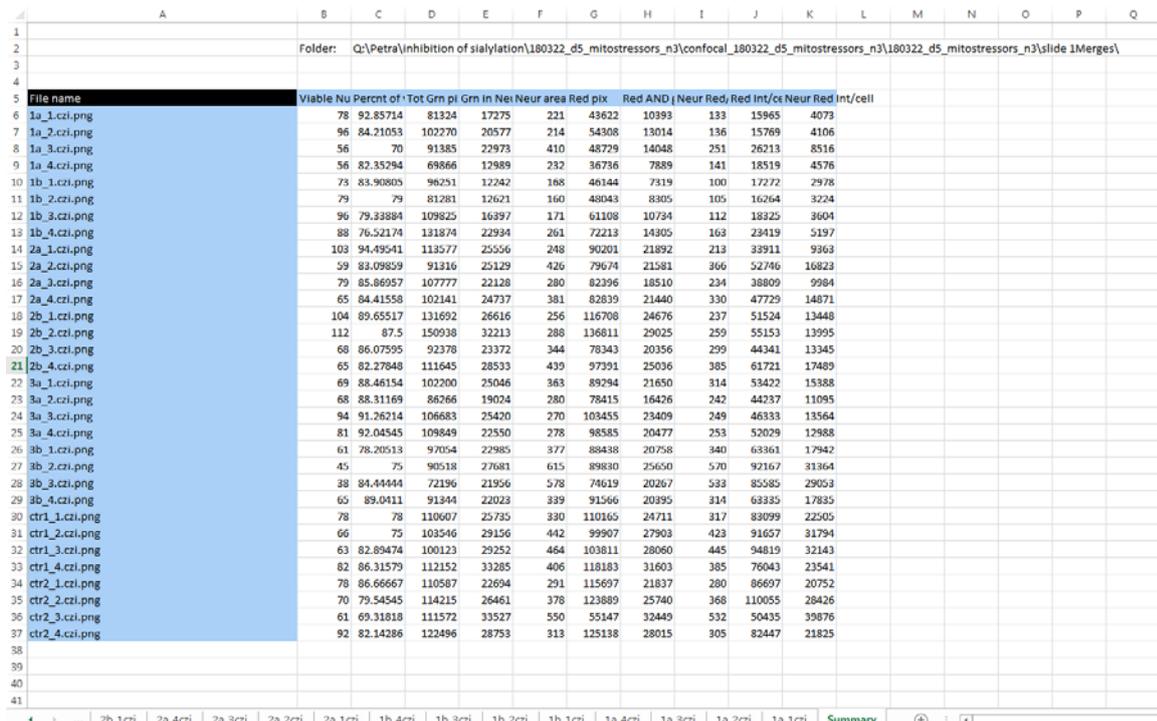


Fig. 11 Summary sheet.

