Manual for Suiker:

a program for evaluating cellular incorporation of sugars

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Juiker

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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 backround

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.

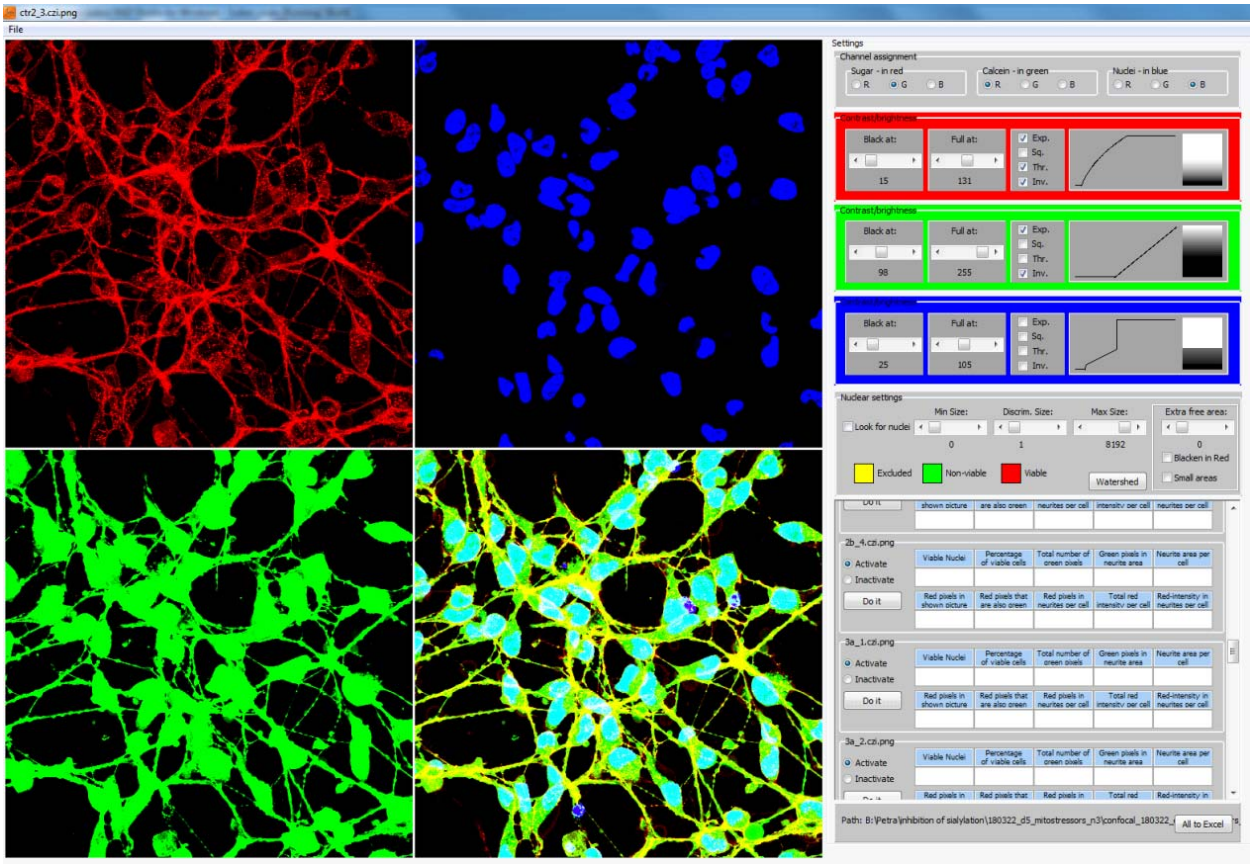


Fig. 1 *Suiker's graphical interface, control bars and results panel.*

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 moities 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.

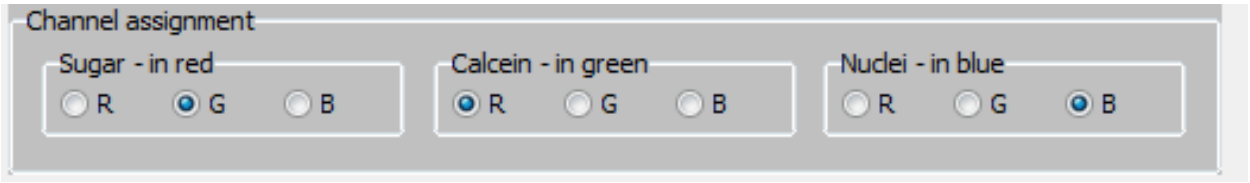


Fig. 2 *Channel assignment.*

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.

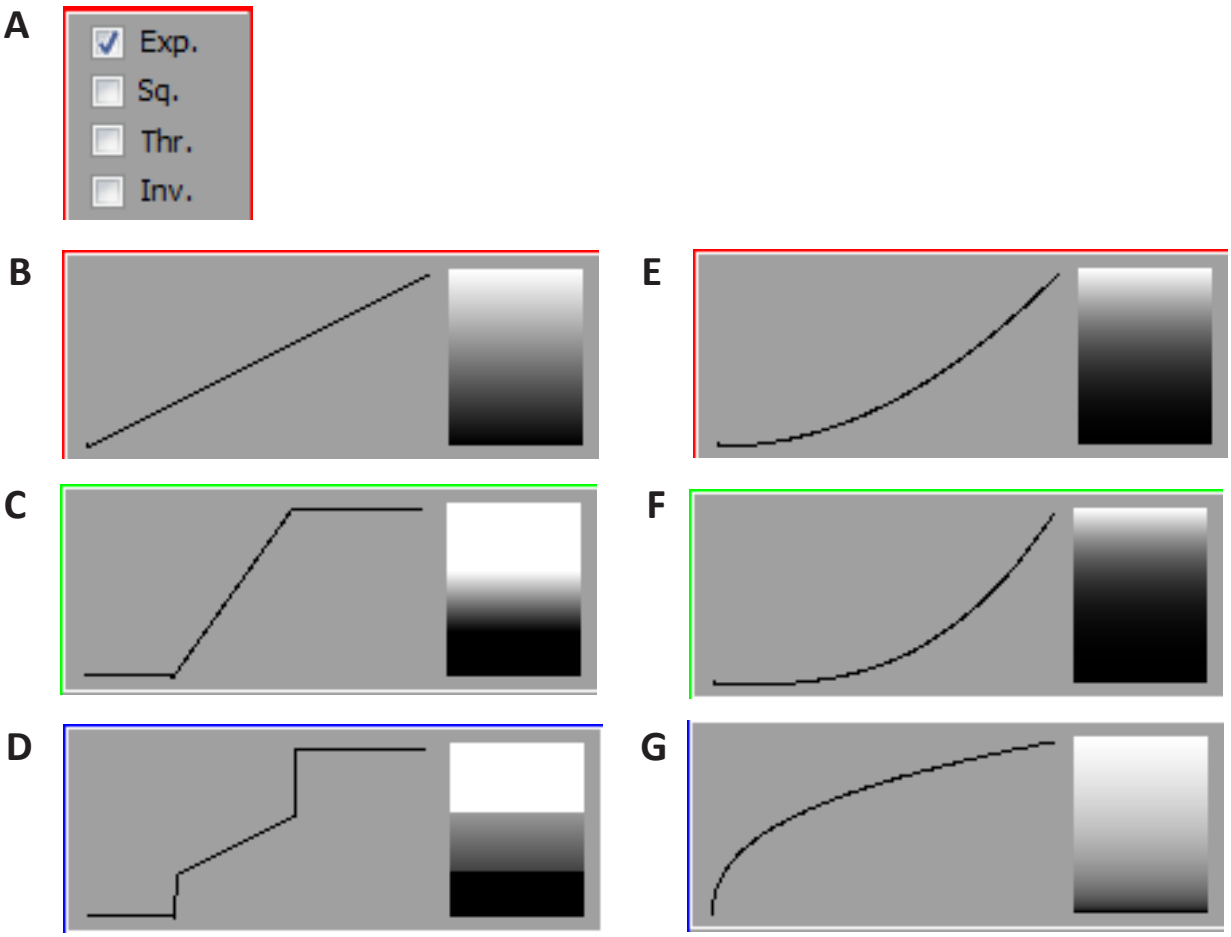


Fig. 4 *The control of shades of gray.*

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.

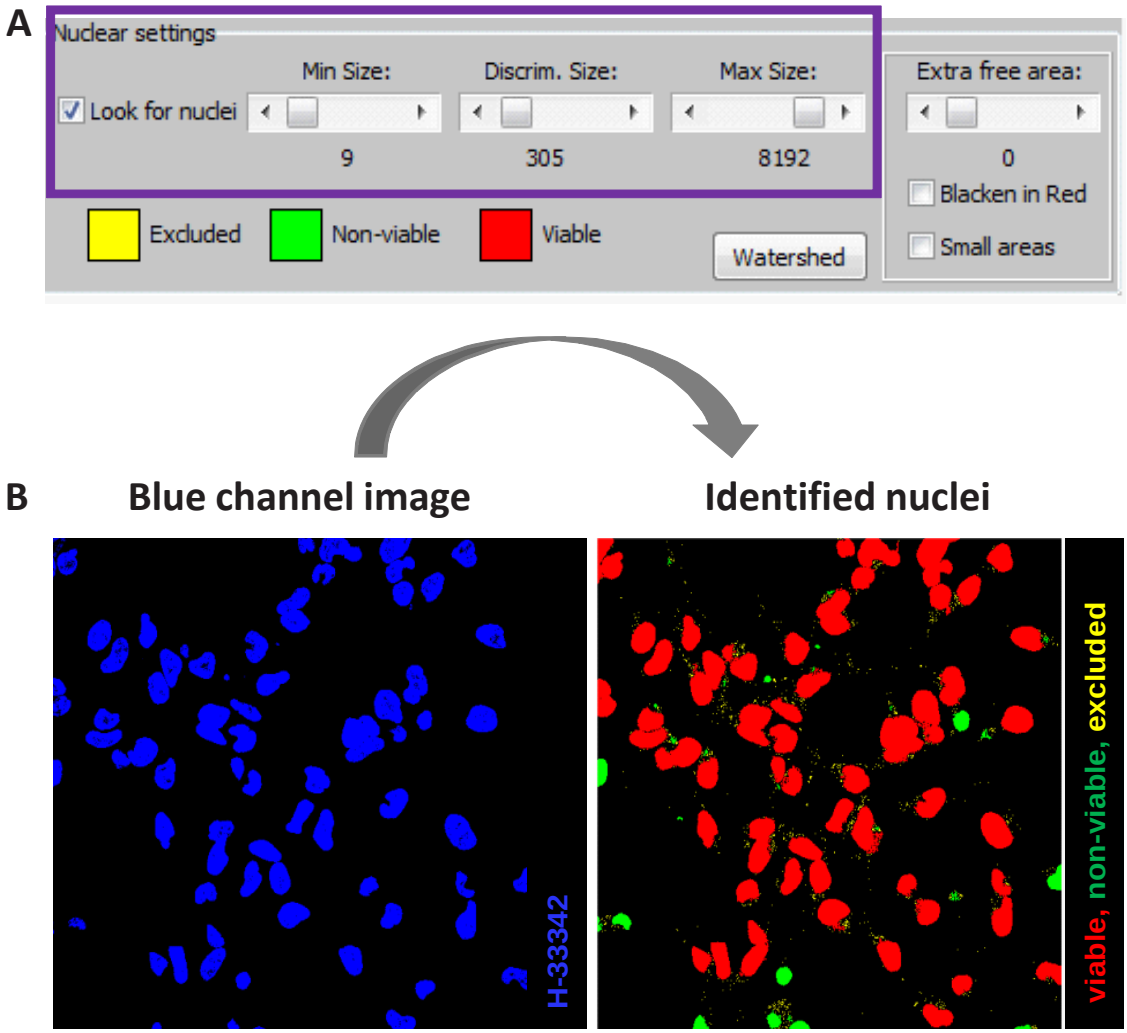


Fig. 5 *Finding the nuclei.*

The three slidebars, 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’’.

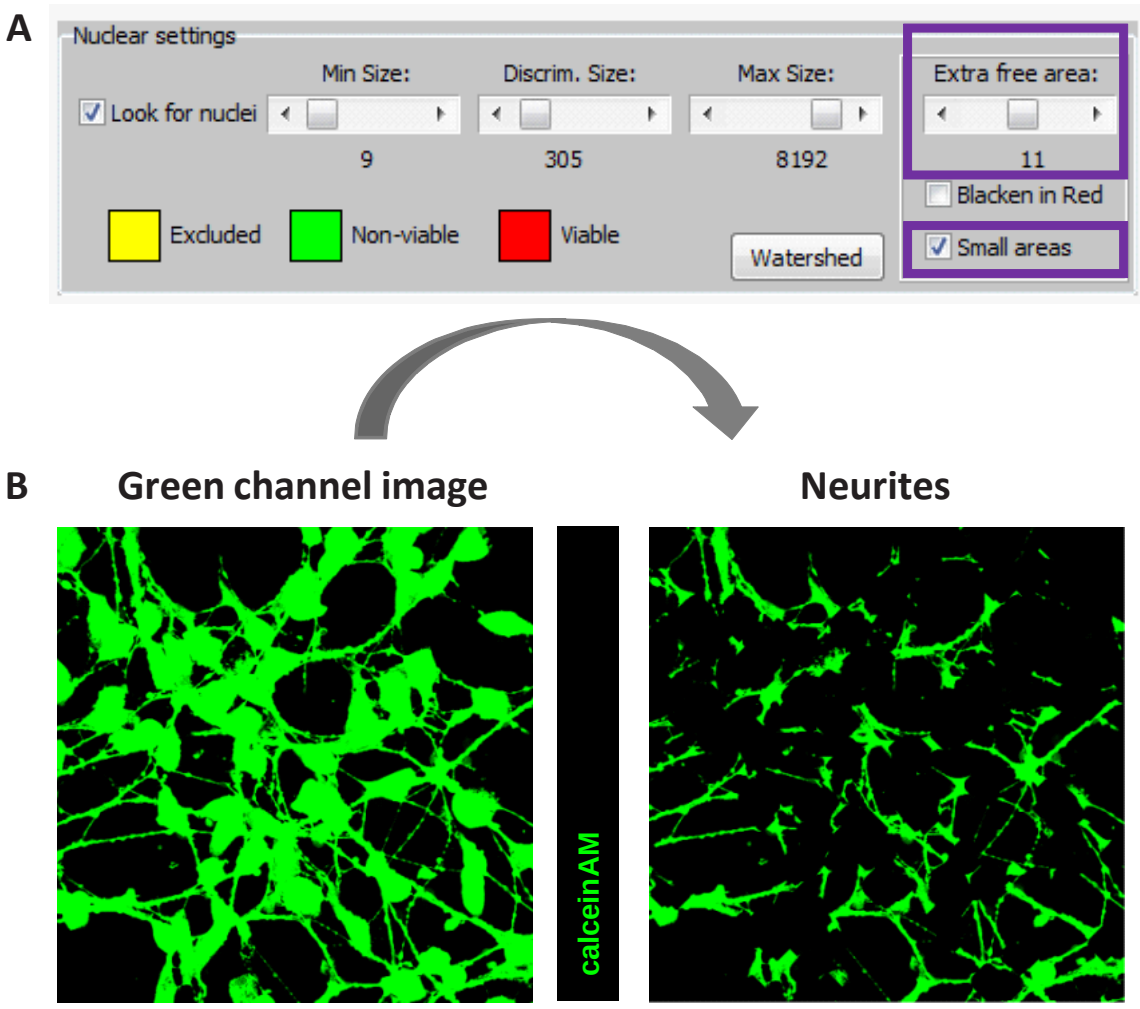


Fig. 6 *Defining neurite area.*

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 ''Blacked 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).

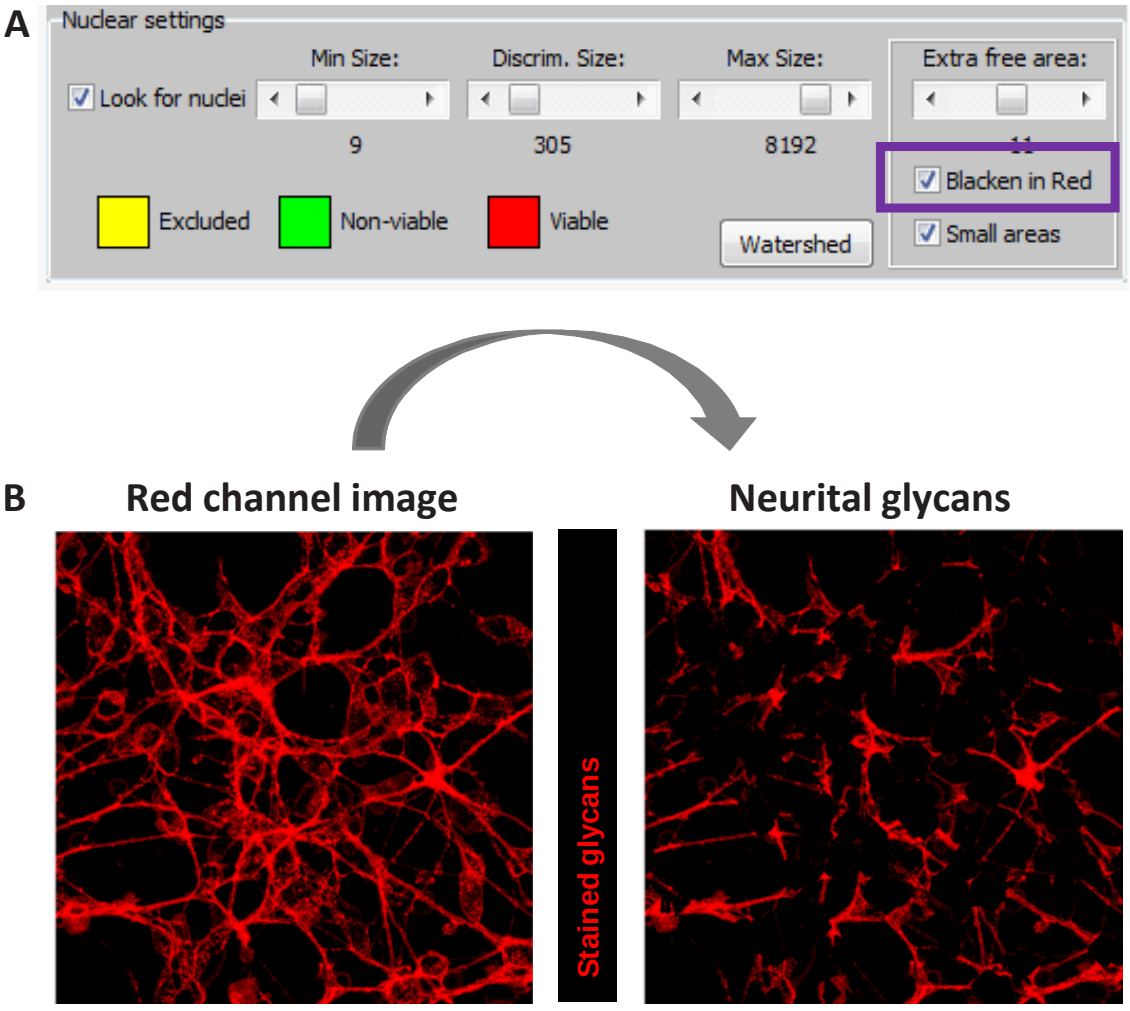


Fig. 7 *Defining neurital glycans.*

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.

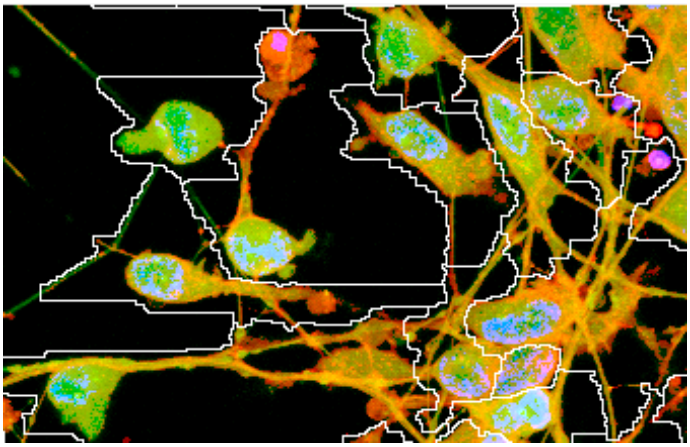


Fig. 8 *The result of watershed, showing the cellular areas.*

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	61/(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.*

The **Total number of green pixels** is the number of pixels that are green (calcein staining) in the original photo. This means, they have to have an intensity value higher than the "Black at" value for the green channel. Just remember the total of pixels for a 512 x 512 picture is 262144 pixels, so a value like that in Fig. 9 means that approx. 42.6% of the pixels are green. Albeit most will have only a very low green intensity.

The value under the heading **Green pixels in neurite area** gives you the number of green pixels in the picture as shown in Suiker. This means: those pixels that have a value above background and are not located in the nuclear area.

To quickly evaluate the health of your cells the field called "**Neurite area per cell**" shows the average number of pixels that make up the neural area of one cell. It is: [**Green pixels in neurite area**]/[**Viable Nuclei**].

The next few values are about the incorporated sugars - the red channel. The entry marked "**Red pixels in shown picture**" tells you how many red pixels there are in the picture shown by Suiker above the background level.

You probably can already guess the next field; **Red pixels that are also green** stands for the number of pixels that are red but also are green. As calcein (green) shows the total neurite area (remember the nuclear area is blacked out) this translates into; count those parts of the neurites that show an incorporation of sugar = colocalization.

The next entry; "**Red pixels in neurites per cell**" is the number of sugar positive pixels per cell.

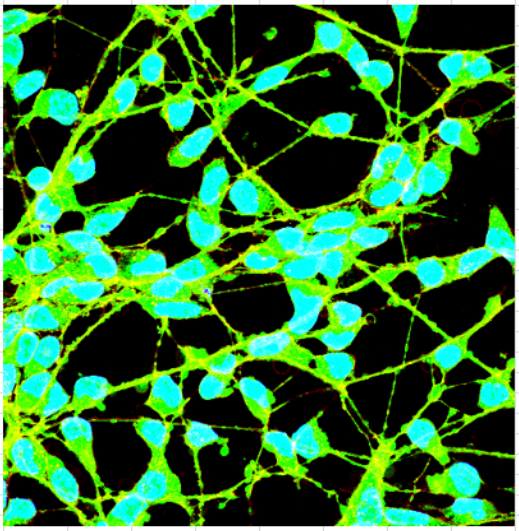
So far, all values represented the number of positive pixels. However, the intensity of the signal is also important. In some cases the percentage of positive pixels can be equal but the signal can be twice as high, telling you that the area of the sugar-covered part of the neurites is the same, but in one experiment the amount of sugar/area is larger. That is the reason why there is "**Total red itensity per cell**". This value is calculated by taking the red channel value of every pixel in the original photo that is above the threshold and adding these all up. This sum is then divided by the number of cells on the photo to get the average.

In the same way **"Red intensity in neurites per cell"** is calculated, albeit that only those pixels are used that also show a green value. So the nuclear areas are taken out and the values represent only the neurite area.

6. Export to Excel

The very last button of Suiker is the "All to Excel" button. If you have Excel installed clicking on it will export all files that are activated (see page 11) in your selected folder. Suiker will do this according to your actual settings. Suiker will create one big workbook with a different sheet for every file exported. The name of the files will correspond to the names of your images opened and analyzed in Suiker. All settings will be included, as well as the results and the corresponding composite picture (Fig. 10). An extra sheet called Summary will be included for a rapid overview of all sheets (Fig. 11).

Folder:	Q:\Petra\inhibition of sialylation\180322_d5_mitostressors_n3\confocal_180322_d5_mitostressors_n3\180322											
File:	ctr2_4.czi.png											
Low CO	15		Inverted	set								
High CO	255		Low CO	25								
Expand	set		High CO	105								
Squared	not set		Expand	not set								
Third	set		Squared	not set								
Inverted	set		Third	not set								
Low CO	98		Inverted	not set								
High CO	255		Min Size	9								
Expand	set		Max Size	8192								
Squared	not set		Ring	11								
Third	not set											
	Viable Nuclei	Percentage of viable cells	Total number of green pixels	Green pixels in neurite area	Neurite area per cell	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		
	92	82.1429	122496	28753	313	125138	28015	305	82447	21825		



ctr2_4czi | ctr2_3czi | ctr2_2czi | ctr2_1czi | ctr1_4czi | ctr1_3czi | ctr1_2c

Fig. 10 *Results Excel sheets.*

Folder: Q:\Petra\inhibition of sialylation\180322_d5_mitostressors_n3\confocal_180322_d5_mitostressors_n3\180322_d5_mitostressors_n3\slide 1Merges\

File name	Viable Nu	Percnt of	Tot Grn pi	Grn in Ne	Neur area	Red pix	Red AND	Neur Red	Red Int/ce	Neur Red Int/cell
1a_1.czi.png	78	92.85714	81324	17275	221	43622	10393	133	15965	4073
1a_2.czi.png	96	84.21053	102270	20577	214	54308	13014	136	15769	4106
1a_3.czi.png	56	70	91385	22973	410	48729	14048	251	26213	8516
1a_4.czi.png	56	82.35294	69866	12989	232	36736	7889	141	18519	4576
1b_1.czi.png	73	83.90805	96251	12242	168	46144	7319	100	17272	2978
1b_2.czi.png	79	79	81281	12621	160	48043	8305	105	16264	3224
1b_3.czi.png	96	79.33884	109825	16397	171	61108	10734	112	18325	3604
1b_4.czi.png	88	76.52174	131874	22934	261	72213	14305	163	23419	5197
2a_1.czi.png	103	94.49541	113577	25556	248	90201	21892	213	33911	9363
2a_2.czi.png	59	83.09859	91316	25129	426	79674	21581	366	52746	16823
2a_3.czi.png	79	85.86957	107777	22128	280	82396	18510	234	38809	9984
2a_4.czi.png	65	84.41558	102141	24737	381	82839	21440	330	47729	14871
2b_1.czi.png	104	89.65517	131692	26616	256	116708	24676	237	51524	13448
2b_2.czi.png	112	87.5	150938	32213	288	136811	29025	259	55153	13995
2b_3.czi.png	68	86.07595	92378	23372	344	78343	20356	299	44341	13345
2b_4.czi.png	65	82.27848	111645	28533	439	97391	25036	385	61721	17489
3a_1.czi.png	69	88.46154	102200	25046	363	89294	21650	314	53422	15388
3a_2.czi.png	68	88.31169	86266	19024	280	78415	16426	242	44237	11095
3a_3.czi.png	94	91.26214	106683	25420	270	103455	23409	249	46333	13564
3a_4.czi.png	81	92.04545	109849	22550	278	98585	20477	253	52029	12988
3b_1.czi.png	61	78.20513	97054	22985	377	88438	20758	340	63361	17942
3b_2.czi.png	45	75	90518	27681	615	89830	25650	570	92167	31364
3b_3.czi.png	38	84.44444	72196	21956	578	74619	20267	533	85585	29053
3b_4.czi.png	65	89.0411	91344	22023	339	91566	20395	314	63335	17835
ctr1_1.czi.png	78	78	110607	25735	330	110165	24711	317	83099	22505
ctr1_2.czi.png	66	75	103546	29156	442	99907	27903	423	91657	31794
ctr1_3.czi.png	63	82.89474	100123	29252	464	103811	28060	445	94819	32143
ctr1_4.czi.png	82	86.31579	112152	33285	406	118183	31603	385	76043	23541
ctr2_1.czi.png	78	86.66667	110587	22694	291	115697	21837	280	86697	20752
ctr2_2.czi.png	70	79.54545	114215	26461	378	123889	25740	368	110055	28426
ctr2_3.czi.png	61	69.31818	111572	33527	550	55147	32449	532	50435	39876
ctr2_4.czi.png	92	82.14286	122496	28753	313	125138	28015	305	82447	21825

... | 2b_1czi | 2a_4czi | 2a_3czi | 2a_2czi | 2a_1czi | 1b_4czi | 1b_3czi | 1b_2czi | 1b_1czi | 1a_4czi | 1a_3czi | 1a_2czi | 1a_1czi | Summary

Fig. 11 *Summary sheet.*