Calcium Fluorescent Flash Evaluating Engine

CaFFEE a program for evaluating Ca dependent increase of fluoresence of Fluo-4 labeled cells over time.

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1. Introduction

1.1 Installing CaFFEE

CaFFEE is a 32bit program for Windows. It 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 operating systems. However I have **NOT** checked this.

There is no need for any registration or special rights for you to install and use the program. You just copy the CaFFEE.exe file from the CaFFEE.zip file onto your hard disk and it will run. Alternatively you can- of course- use the "extract" function to do this.

1.2 A few words of warning!

This program was custom made to solve the problems of measuring cellular fluorescent changes in our laboraty. The main issue was to capture relative changes of calcium chelated with Fluo-4 for hundreds of cells or subcellular structures per image field. It was programmed for using the formats we use, on our computer setup and with our aims in mind. Therefore, it is not as flexible as it could have been.

1.3 Experimental setup, for which CaFFEE works well:

For all our experiments in which we evaluate the Ca^{2+}-influx we use 96-well plates from Sarstedt or Falcon.

To analyze the results we use two fluorescent channels. The blue channel is used to visualize the nuclei stained with H-33342. The Ca^{2+}-influx is detected in the green channel by the use of Fluo-4AM. The present version of CaFFEE expects these two channels to be used, it is not possible to swap channels in the program.

For the evaluation of the plates we use the automated microscope system of Cellomics (Thermo Fischer), the ArrayScan VTI. Pictures are taken with a rate of approx. 1 per second. After ten seconds the ligand is pipetted in automatically and another 40 pictures are taken. Per assayed well a movie is exported in .avi format. Beware to set the export parameters to "Manual" and the brightness to "from 0% to 100%". The so created movies have a frame size of 512 x 512 pixels. Other sizes can be used, but be aware that larger images will be shown only partially in CaFFEE. Magnification can be 10x or 20x, both can be assayed without trouble using CaFFEE.

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

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

1.4 Potential uses:

Any fluorescent change may be recorded, e.g. using metal or pH indicators. Possibly, also mitochondrial fluorescence (e.g. membranepotential) or cell membraneptential can be quantified, but this has not been tested.

1.5 Overview of the three major features/objectives of the program

CaFFEE can be used to do different things. Depending on what you want to do with your movies, you can use just the corresponding part of the program. The different parts of CaFFEE are capable of:

I) Making an optimal movie showing the effects of your treatment in the clearest way possible (adaption of background, grayscale dynamics and saturation).

II) Visualizing the effects of the treatment in a single picture by showing the difference of the pictures before the treatment and those after the treatment.

III) Evaluating the quantative effects of the treatment for every single cell and the overall population, expressing this in numbers (curves) and exporting them to ex cel.

1.6 Running CaFFEE

For whatever feature you decided, first thing to do is load a movie.The menu of CaFFEE is extremely simple; there is only one item: "Open". So just load an .avi file you want to evaluate. You will see the film loaded and then CaFFEE will show you the green and the blue channel as two independent series of single frames. On the left the green channel - showing the Fluo-4AM signal corresponding to Ca^{2+}-influx. On the right the blue channel, showing the nuclei. As the starting images of the movie are shown, the neuritesare propably not very bright. As depicted in the example (left panel of Fig. 1.) cells and neurites give only a weak signal. However, this can be optimized, see Fig. 4. for an example of the possibilities of CaFFEE. The right panel of Fig. 1 shows another common phenomenon; bright dots that are much brighter than normal nuclei. These can be condensed dead nuclei (propably the case in Fig. 1) or organic matter from the medium. The program will give you the oppurtunity to eliminate these, see page 9 and next. After loading the movie and seeing the resulting single frames displayed you can now choose what you want to do. This handbook will cover the three possibilities of CaFFEE in the next parts.
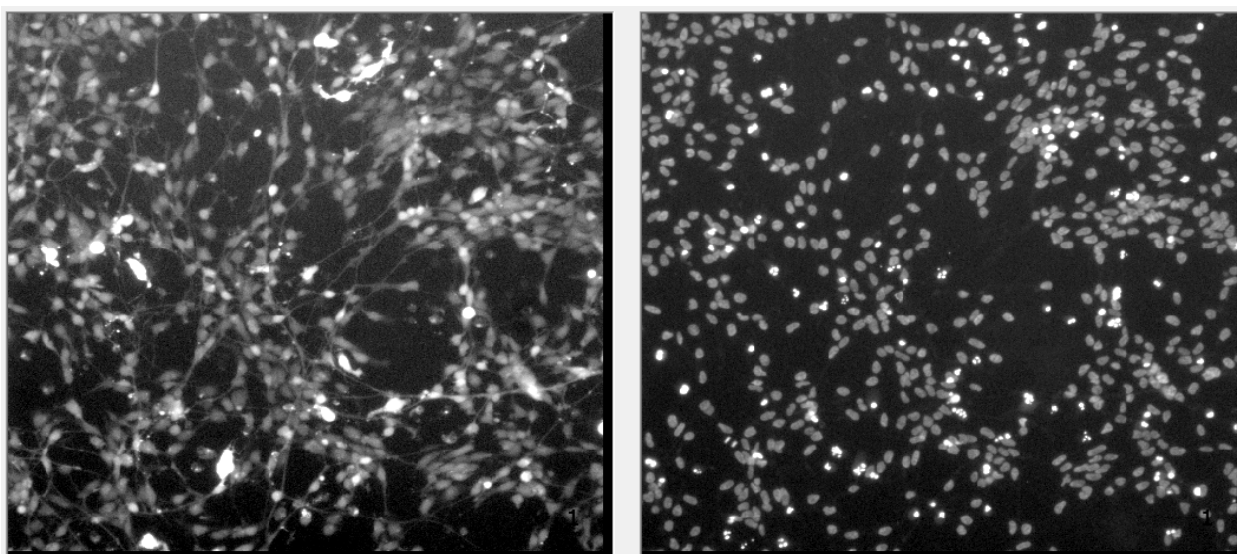

Fig. 1. The two color channels will be separated, showing the green (whole cell) channel on the left and the blue channel (nuclei) on the right. Both channels are shown in grayscale.
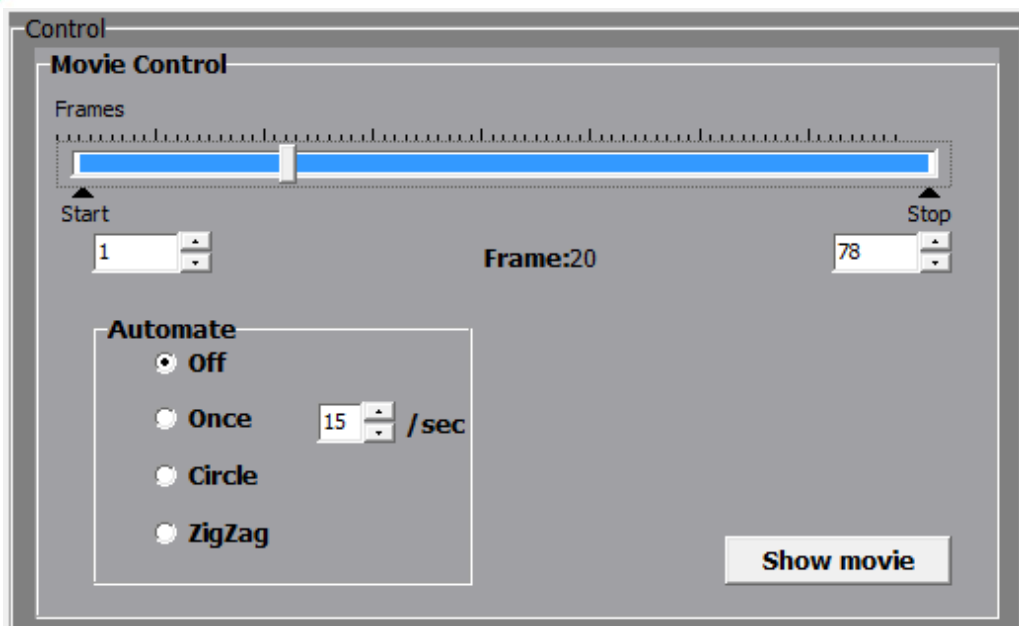
Fig. 2
General control
panel

2. Part I - Optimizing the movie

2.1 General control

On the top right hand side there is the panel for general control - see Fig. 2. With the slider on top you can move along your frames and the selected frame (with separated channels) will be shown on the left. It is also possible to select just a subset of frames for all further processing by setting "Start" and "Stop" correspondingly.

In the box labeled "Automate" you can select for the movie to run once/continiously while you play around with other settings. The zigzag options will have the movie run from start to end and then back. You can even select for the number of frames per second = the speed of the movie. The button marked "Show movie" will not only do exactly that but it will also give you the opportunity to save the movie or single frames of it. We will come back to it after I show you the further possibilities of enhancing your movie.

2.2 Enhancing the channels

You can enhace the channels using the parameters you can set in the panel underneath the pictures on the left - see Fig. 3.

All the setting are identical for both colors, so I will only describe the various possibilities just once.

2.2.1 "Black at:" and "Full at:"

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 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 (to the right) and the background for the corresponding channel will be forced to zero.
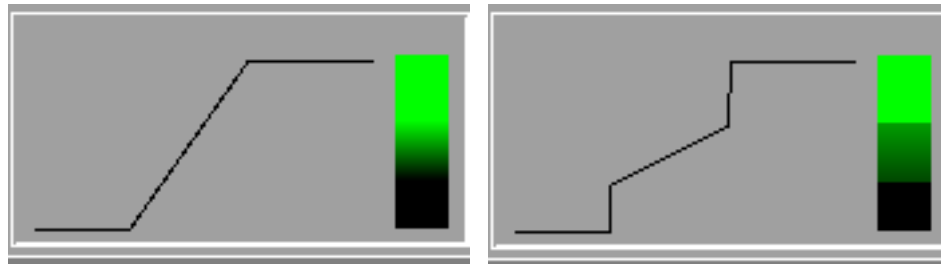
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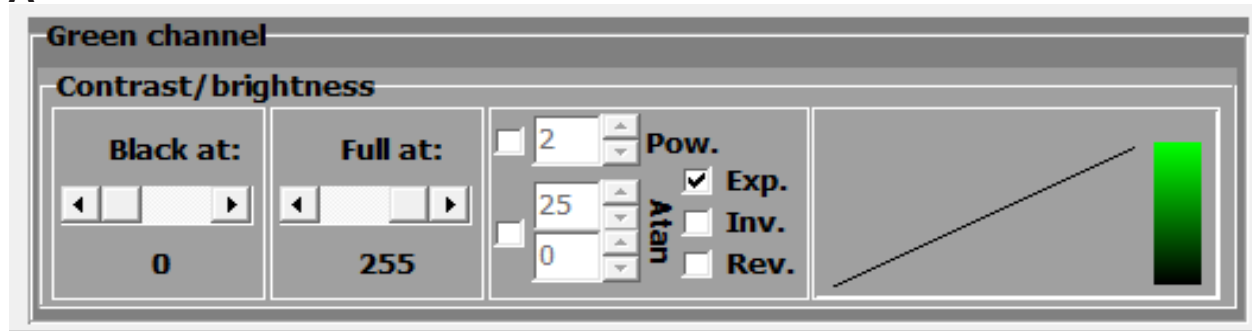
B

Fig. 3. the settings to enhace the green channel

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

The third part of the panel shown in Fig. 3A controls exactly how different shades of gray of the original picture are depicted on your screen. This process is controlled by the five checkboxes shown in Fig. 3. There are three basic shapes for the transformation of your gray colors. If both, Pow. and Atan. 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 graphical elements on the right hand side of the panel. Horizontally the original gray scale values are plotted, vertically the newly calculated values are shown.The distribution of the various grays is shown continuously in the represented shades of green in the shades-box (SB) on the far right.

As soon as the user changes the settings the graphs will change along. Let's have a look when we apply the "Black at:" and "Full at:" options.We put the first value to 60 and the second to 180. In the resulting graphs, see Fig. 3B, 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 SB on the right shows this as a black bar on the bottom, the greenish band in the middle and finally as the pure green bar at the top. The middle band contains all possible shades of green squeezed into the space between the black and pure green bars.

2.2.2 Expanding to full scale or not, using the "Exp." checkbox

In the right part of Fig. 3B., the results are shown for the same settings but with the Exp.-checkbox unchecked. In the SB the lowest values are again put to black and the highest to pure green. The big difference is in between; not all shades of green are squeezed in. Only those used in the original picture are used and they 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.
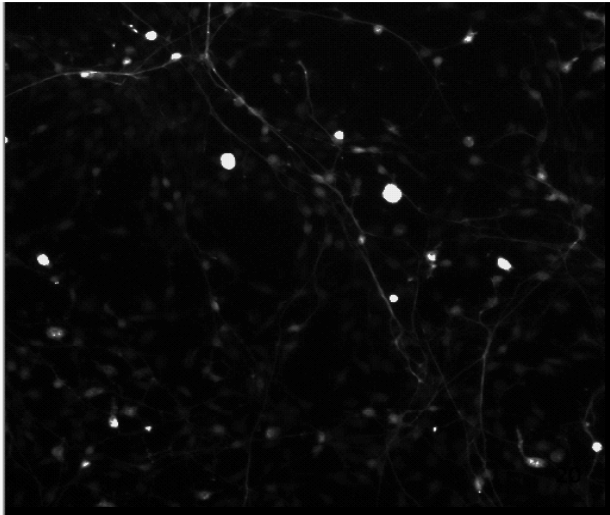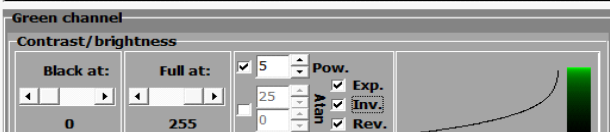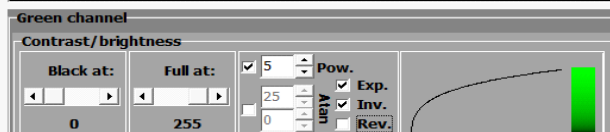
Fig. 4. The Power (Pow.) checkbox, using Inv. and Rev.

2.2.3 Power, Atan, Inverse and Reverse

The relation between the original image recorded value and display value does not have to be linear. Just click on "Pow." or "Atan" and you will see the difference.

Activating the "Pow" checkbox will switch to the power function. It will use a power function to caculate the relationship between original gray levels and those displayed in the picture. The simplest function is that using the power of 2; the quadratic function, but also higher orders are posible, the exact number is selectable between 2 and 6. Just try the different numbers and see how your picture changes. In Fig. 4 you can see the combination of a power function (in this case the fifth power is used) and the Reverse (Rev.) and Inverse (Inv.) checkboxes. In this example a rather dark picture was chosen, in which the

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signal is clearly above the background, albeit not very high.

The lines representing the transformation curves show their function the most clearly. The situation without Rev. and Inv. is below the picture in the lower left corner (Fif. 4C). This is obviously not the perfect way to look at this picture. If you look at the corresponding SB, you'll understand why. All dark green pixels are made even darker making differences between shades in this area even smaller. At high levels, the shades of green are "pulled apart" making differences larger. This is a setting very suitable to see differences in very bright pictures, but not so in our darkish picture. As you can see in Fig 4. there are two settings that give much better results. If you inverse the function, making it power to 0.2, the picture is much better, see the picture in Fig. 4B. In the SB you see why: differences between dark shades of gray are "blown up" making this part of the spectrum more pronounced. Personally, I think reversing the function (turning it around 180 degrees - Fig. 4D) makes it even better. The steepness of the curve is not quite as high as with the inverse function, making the transitions between black and gray more natural.

So, with these settings you can enhance either dark or bright pictures, but what about pictures that contain the interesting information in the middle part of the spectrum? Exactly, you guessed; use the "Atan" function see Fig. 5. This function can be changed by shifting the bend from left to right and by changing the slope. You play around with the two parameters and see how they influence your pictures.

 Fig. 5. The basic form of the Atan function.

Of course all these settings can be combined freely with "Black at", "White at" and "Exp.".

With the various settings and the combinations thereoff, you will see big differences between shades that your natural vision can not even distinguish. You can not only make everything visible but even clearly obvious.

If you are satisfied that all is visible that should be visible, now is the time to save the movie and/or single frames.

2.2.4 The "Show movie" function

So click on the "Show movie" button, as shown in Fig.2. A new window will open with the optimized picture in two colors. However, how these colors are shown in the final movie/frame is selectable. There are 12 combinations you can select from. Here is also the opportunity to see the whole sequence of the frames in the color scheme you selected. The speed you select is also the speed in which the final movie will be saved.

After you saved those parts you want, Part I of CaFFEE is finished so high time to close the movie-window and go on to the evaluation part.

Fig. 6. the Nuclei selection box and the Evaluation box

3. Prerequisites for Evaluation

To start with the next step: check the "On" checkbox in the middle of the "Nuclei Selection" box, see Fig. 6. As soon as you check this box several things will happen.
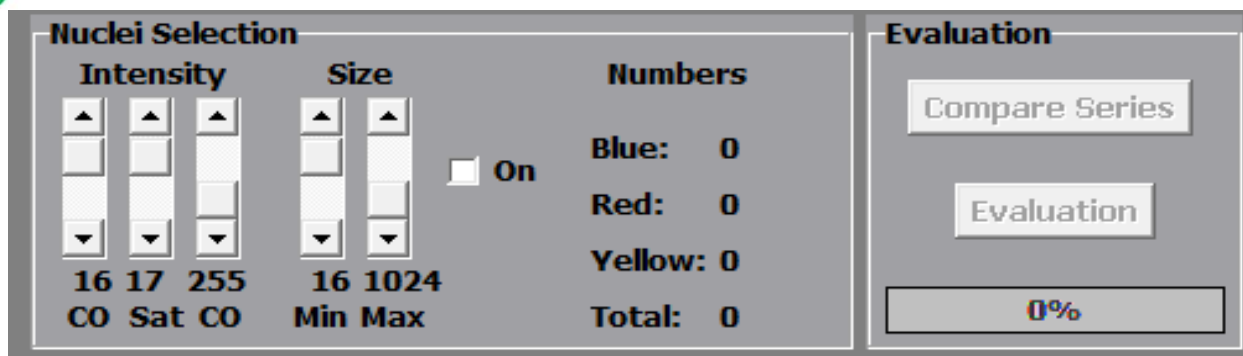
The most obvious is propably the fact that both pictures switch back to color, see Fig. 7. The left part - the Fluo4 picture - will keep the settings you selected in Part I but will show them in green. On top the nuclei are depicted. However they are no longer in their "natural" color. They are shown in pure 100% intensive blue. There are no shades of blue what's however. The only nuclei shown are those that are blue in the right most picture.

The right most picture - the nuclei picture - is showing the nuclei in three basic colors. Again there are no shades, this picture is showing what the program thinks are nuclei and what characteristics these have. These characteristics are determined by the settings that are also in the "Nuclei Selection" box, again see Fig. 6. There are two major parameters you can use to select nuclei: their intensity and their size.

3.1 The intensity.

There are three sliders to set different aspects to select nuclei. The left most tells the program what intensity a pixel should have minimally to belong to a nucleus. It is a cut-off value (CO), all below this threshold will no longer be considered. These pixels will not be
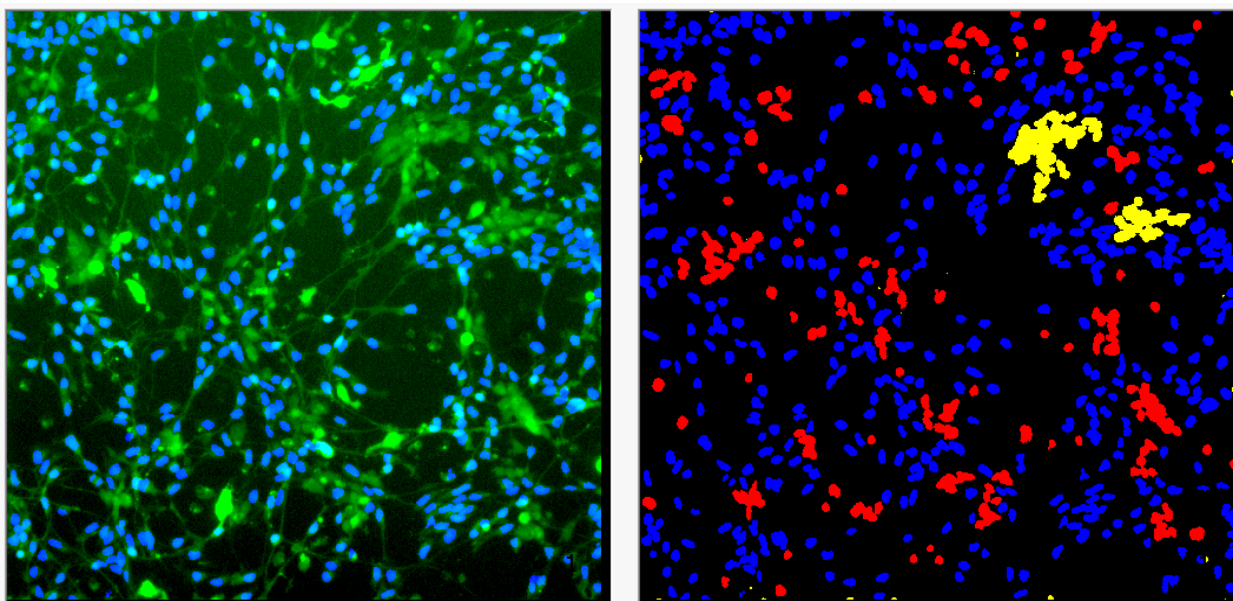

Fig. 7. The view after switching "On" the "Nuclei Selection"

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part of any nucleus and therefore invisible in both pictures.

All pixels that have intensities equal or higher than the value of the second slider are considered to belong to a nucleus. Their values will all be set to 255 in the blue channel, making them as blue as they can be: saturated (Sat). Sometimes you will notice small intensly colored blue specks in your culture. These can be kicked out of your evaluation by setting the third slider, another CO, to a value below the intensity of these specks. This setting will result in showing them in red and no longer in blue. All structures shown in red in the nuclei picture will no longer be shown in the "Fluo-4" picture.

If these specks are really small, obviously smaller than real nuclei, you can also get rid of them by setting the second parameter:

3.2 The correct size.

I think the two sliders will give you no trouble; with the first you loose everything that is too small, with the second everything that is too big. These objects, of the wrong size, are shown in yellow, see the right part of Fig. 7.

The exact numbers of the various objects are listed next to the sliders. These numbers are for the actually selected frame. If you still have "Circle" or "Zigzag" clicked you will see how these numbers change along with the colors in the pictures.

Now, you have defined what are the real and relevant nuclei that should be evaluated. The controls to do so were activated as soon as you clicked the "On" checkbox. Both are in the "Evaluation" box also shown in Fig. 6.

Just click on the top button, labelled "Compare Series". Now we reached:

4. Part II - Evaluating the effects graphically

As soon as you click on the button a new window will open. Again it contains the space for two images next to each other and a lot of radiobuttons and checkboxes. But let's start at the very right hand top part, there is a checkbox like in Fig. 8.

When the window opens this will be unchecked = off. This gives you the change to set all the different parameters without the program updating all pictures continuously, it can be annoying. However, when fine tuning your settings, there is nothing like directly seeing what is happening. Well, whatever you personally like; here is the way to select how the program should behave.
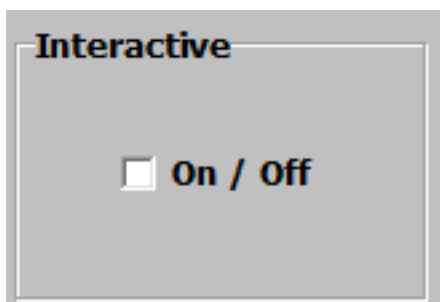

Fig. 8. The central control switch of the graphical evaluation window

Fig. 9. The Reference Frame(s) Panel

4.1 Working principle of the "Graphical Evaluation"

It tries to help you in your quest for: a simple graphic that shows the difference between before and after stimulation in a clear and quantitative way.

So what do you need for it?

4.1.1 Reference frames

You first define one or a few frames of your movie as control frames. You can do this in the "Reference Frame(s)" panel - see Fig. 9. Control frames are frames that show your cells in the inactive state. If you think frame 5 is showing the perfect control then you put a "5" in the "First frame" line and again in the line marked "Last frame". The image will show you frame No. 5 (if you switched "Interactive" to "On"). The image shown is not identical to frame No. 5 as there is some background correction done - see further below.

In most cases you will have a number of frames before stimulation and you assume that the average of these frames will represent the truth much more than any single frame can ever do. So let us assume 10 frames were taken before stimulation occured. Frame 3 to 7 show stable, minimal activity. Load these five frames into "Reference Frame(s)". Just put a "3" into "First Frame" and a "7" into "Last Frame". Depending on the settings in "Signal over background" the selected frames will be averaged and shown.

4.1.1.1 Background correction
4.1.1.1.1 "Fixed absolute"

Now back to the background correction. How this is done exactly, can be set in the box labelled "Signal over background", see Fig. 9. There are three options for background correction and all three have a further parameter you can set. The first option is called "Fixed absolute" and is the most simple option. The value you give is the cutoff for every picture you selected. So in our example; only the signals above the cutoff of our pictures 3 to 7 will be averaged. It is an easy and fast way but it is very dependent on the brightness of your pictures. In a series over highly exposed pictures the background is, of course, much higher than in a darker series.

4.1.1.1.2 "Fixed over Average"

The other two options have less problems with this kind of variation. Both "Fixed over Average" and "Percent over Average" will first calculate the mean intensity of the whole picture. If you select to use the "Fixed over Average" method, all values below this Average with a fixed number added will be dismissed.
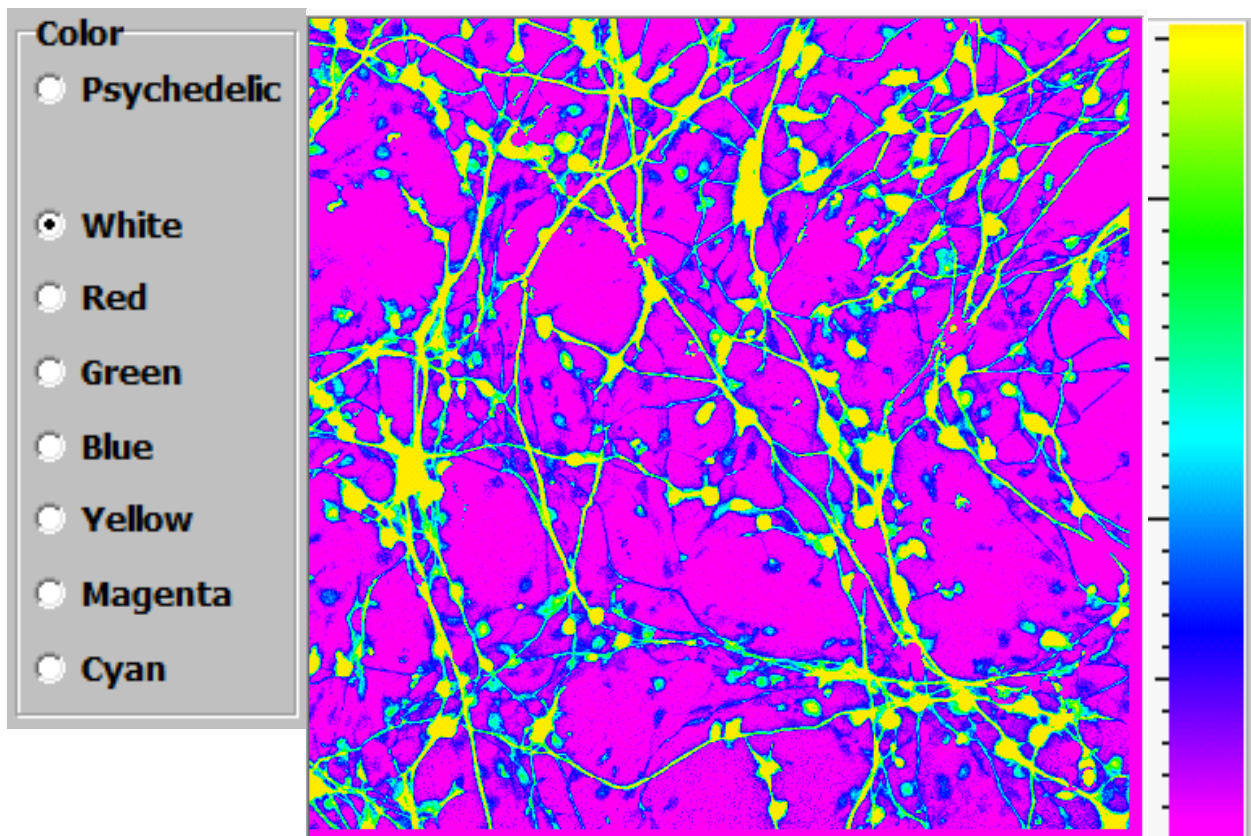
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Fig. 10. Color selector and an example of the "Psychedelic" option.

4.1.1.1.3 "Percent over Average"

The last option "Percent over Average" will take a percentage over the average as a cutoff. With both of the latter options it is also possible to enter negative numbers, so you can choose not only 110% (10%) of the average but also 90% (-10%) in "Percent over Average".

If you have a background problem, you play with these settings. Do not forget to try the combination with or without the "Expand" checkbox checked. Comparable to the "Exp." checkbox in Fig. 3.

Now you have defined "Before Stimulation" and you just have to tell the program with what to compare.

4.1.2 "Difference to reference"

As you already guessed, the second box labelled "Difference to reference" is exactly what you'll need. It looks very similar to the "Reference Frame(s)" panel, so most of the options will look familiar. Again, you can select one or more frames and the background correction to use. Again, it makes more sense to take a series of frames as input. In this case the program will **not** take the average of the pictures. A compilation of all the frames is made in which all signals are kind of added up. Finally, the averaged reference frames are substracted, once for every frame you selected in the "Difference to reference" box. This now leaves you with the difference you were looking for. To be quite honest, this gives very bright pictures indeed. There will rarely be a reason to use the "Expand" button, since all picture will have at least some pixels at the 255 level.

The result is shown directly as the second image. Be aware that you see the **activity of the neurites**, not the neurites themselves!
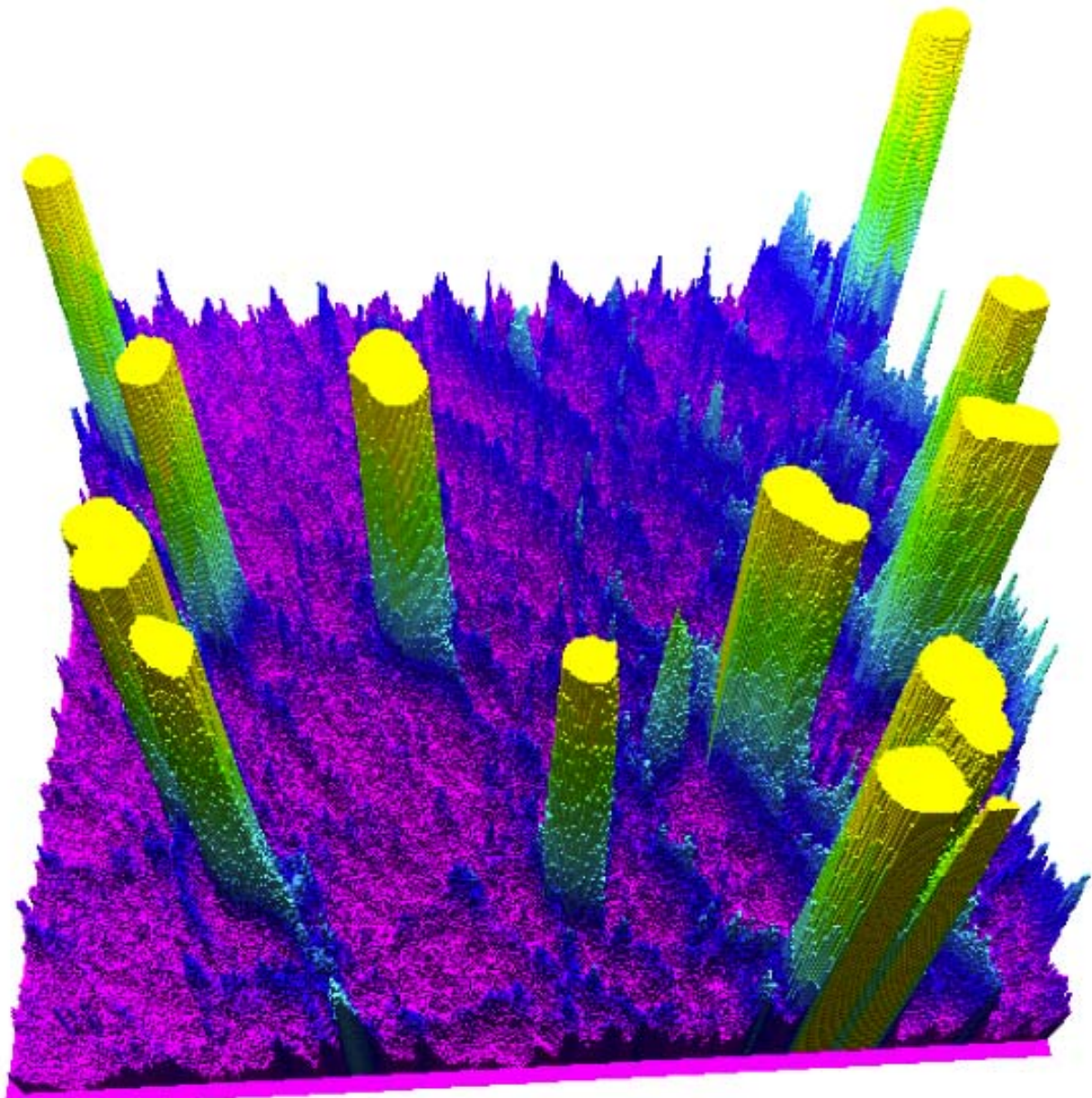
Fig. 11.An example of a 3D picture rendered by the Pov-Ray program. The bright cell bodies result in steep yellow table-mountains towering above the purple low lands that are crossed by blue neurite mountain ranges.

4.1.3 The Nuclei checkbox

Sometimes the bright cell body can make problems. They can be so active that the signal coming from the neurites can be reduced to almost insignificancy. Differences between neurite activities can be almost nothing at all. That is why there is a checkbox labelled "Nuclei". If checked it will black out the nuclei and calculate with just the rest of the picture. The size of this cut-out can be determined by setting the number within the box behind the the checkbox.

This setting can be counterintuitive the smaller the number, the more is cut out. For those that are interested in the workings, I will explain how it is calculated, all others just skip the next paragraph :)

4.1.3.1 The watershed method

The method used is the Watershed method. It calculates with a semi-3D model of your picture. All nuclei that were found are considered to be pits in the picture. If you have

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problems imagining this; look at Fig. 11. Just think that you are under the picture and you are looking up and seeing the bottoms of these pits. Their depths are according to their brightness. So the maximum depth of a nucleus is 255. Now switch to a position above your picture and look down. Imagine the pits are filled with a black liquid. If everything is filled up to 200, the deepest parts (= the brightest parts) of the nucleus will become invisible. Filling further - to only 50 deep - will make much more of the nucleus disappear. At even higher levels (lower number!) sometimes two pits will run together and in the end (depth=0) the whole picture will be drowned in the black liquid; resulting in a completely black picture.

So now we are all back on track and look at some options by what color the activity should be represented. Of course, this is selectable; a number of colors are available, see Fig. 10. A special case is "Psychedelic", when you choose this option the image of the activity will be shown in false color. The values of the activity will be shown in colors ranging from purple (low activity) via blue and green to bright yellow (high activity), see Fig 10. on the right.

4.1.4 Saving the results
4.1.4.1 Saving the picture

At the very right hand bottom you will find the opportunity to save the picture you created in jpeg format. The default name will contain the numbers of the frames you selected for easy reference.

Of course there is also a way to close the graphical window completely. It comes in two flavours: "Quit" (close this window) and "Exit" (quit after saving).

4.1.4.2 Pov-Ray (Persistance of Vision Raytracer)

The last button (second from the top) in this part is labelled "PovRay". When clicked this will export the image in a format that will allow the rendering of the false color image as a 3D object. The Pov-Ray progam is a ray tracer and is freeware, please refer to: "http://www.povray.org/" for more information. Fig. 11 shows an example of a picture generated with Pov-Ray using the output of CaFFEE as source.

5. Part III - Single cell evaluation

Part III will describe how to get information about the activity of single cells. Start the process by clicking the "Evaluation" button, see Fig. 6.

5.1 "Overview"

After clicking on the Evaluation-button, a number of extra panels will become visible, showing you a lot of extra info. For those who like numbers, most information is shown in spreadsheet style on the panel called Overview, see Fig. 12A. It will show you the number of the individual reacting cell, the start level (base), at what frame it reacts to the stimulus (EC50), the highest point it reaches (top) and the difference between top and base (delta). The two last entries give you the relative increase (Delta divided by Base) and the relative top (Top/Base). The latter two measurements are quite common in some fluorescence research areas. If you click on any of the top row cells of this table all values will be sorted

A

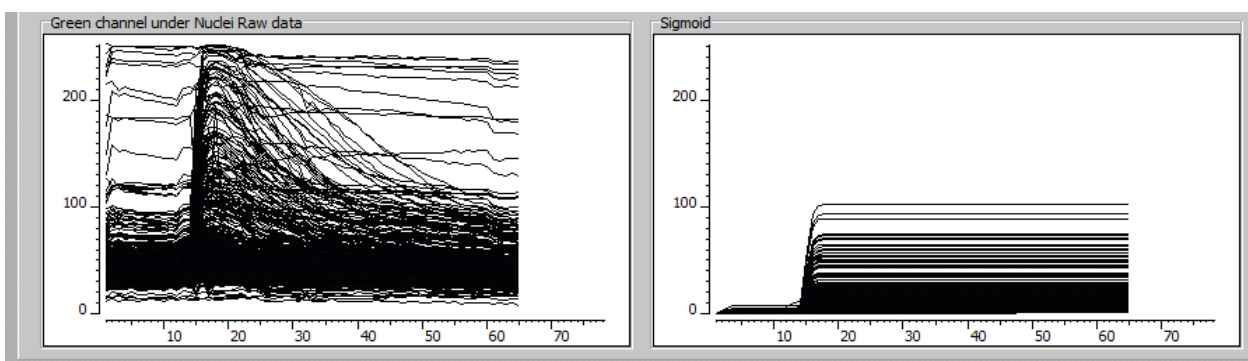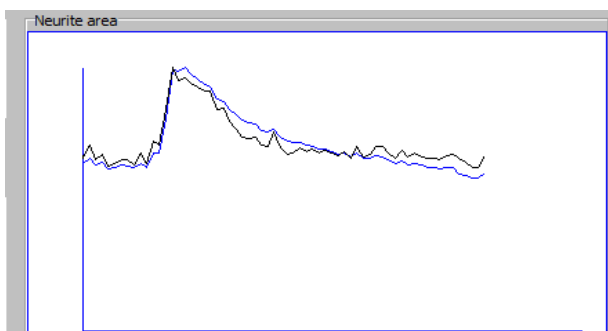| #	Base	EC50	Top	Delta	Delta/F0	F1/F0
478	72	14	138	66	0,92	1,92
479	42	14	44	2	0,05	1,05
480	47	14	50	3	0,06	1,06
481	46	14	61	15	0,33	1,33

B

C

Fig. 12. The new panels containing the information of the individual cells. Depicted is the situation directly after the calculations of the evaluation have completed.

according to the one you clicked.

5.2 "Green channel under Nuclei Raw data" vs "Sigmoid"

Let's now have a look at the graphs in the middle (Fig. 12B). The first panel "Green channel under Nuclei Raw data", shows the raw data of every single cell. Horizontally the frames are shown, vertically the intensity of the various cells. As you can see in this example there are cells that do nothing; they show an almost straight horizontal line. However, there are also cells that react to the stimulus, in frame 14/15 you see the increase for a lot of the cells.

In the second panel all the the curves are approximated by a sigmoid curve. All the graphs are corrected for the start fluorescence of the individual cell and now all start at 0. In this way all cells can be compared to each other and the begin and end values are nicely averaged.
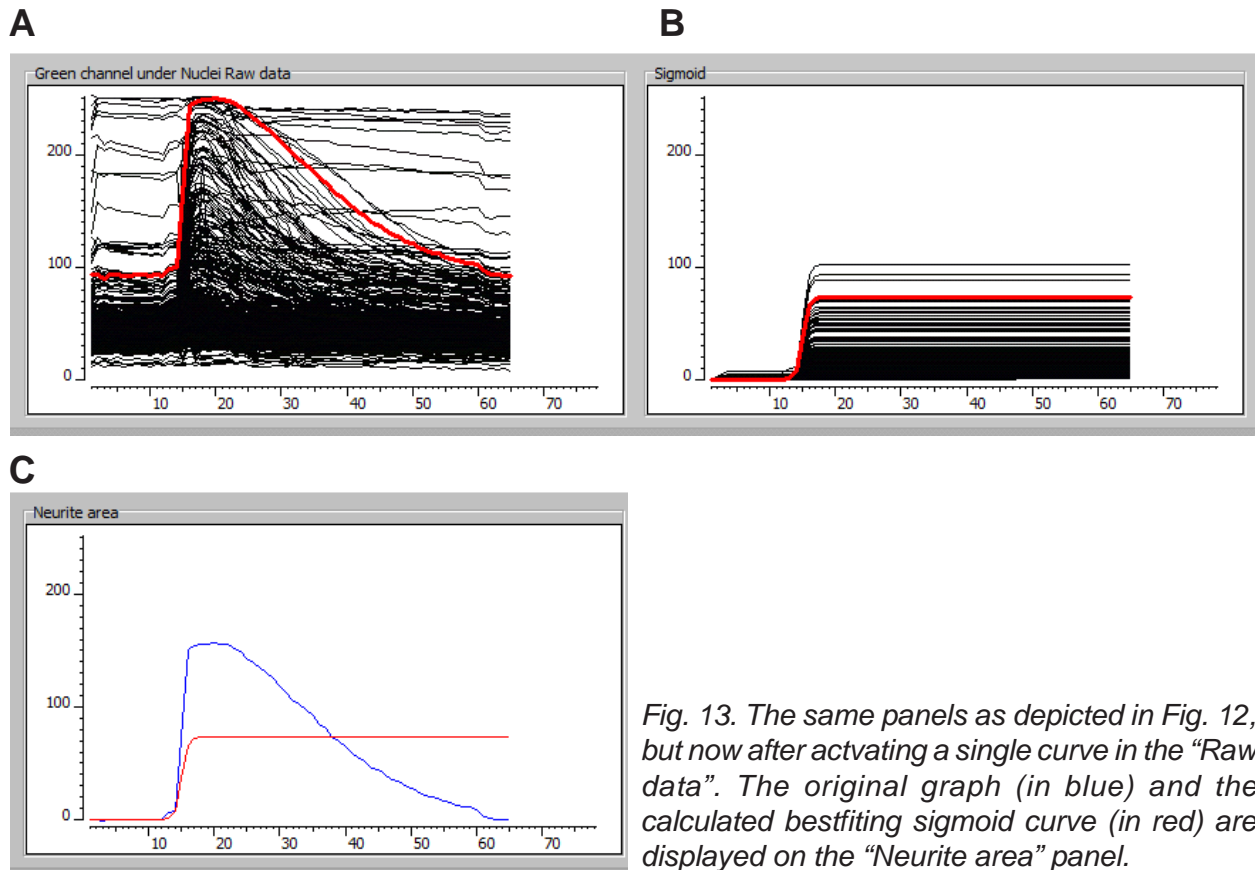
A

B

C

Fig. 13. The same panels as depicted in Fig. 12, but now after actvating a single curve in the "Raw data". The original graph (in blue) and the calculated bestfiting sigmoid curve (in red) are displayed on the "Neurite area" panel.

5.3 Total green vs Cellular green

The last panel (Fig. 12 C) shows the behaviour of everything green in the movie (the black graph) vs. the behaviour of all collective cells (in blue). As you can see for this example the behaviour of the cells is resposible for the behaviour of all green signals in the movie. This means that there are no major other causes for the increase of the green signal. Just as it should be.

5.4 Data that does not fit over the whole time period.

Still there are some problems with this evaluation of the behaviour of the cells.

When we compare the raw data with the smoothened sigmoid curves and just look at the deltas, one thing is obvious: this is **NOT** the ideal way to describe the behaviour. There is just no sigmoid curve that shows a delta that comes even close to those of the really high "raw data" curves. To show this fact even more clearly move your mouse over the "Raw Data" panel and stop while the point of the mouse arrow touches one of the steep curves like that in Fig 12B. You can see what will happen in Fig. 13A.

The touched graph will turn red and the corresponding sigmoid curve also (Fig. 13B). As a matter of fact you might notice that the overview now shows the data for the highlighted graph and that in both pictures there appears a small cross indicating the responsible cell. This effect works in all ways, CaFFEE will always try to show all data that belongs to-gether. Moreover the neurite area panel now shows both curves to show how good the fit is - see Fig. 13C. The beginning looks quite OK. The point of elevation is detected accu-rately. But then the curve calculates all the values of the next frames, averages them and takes this value as top value. Much too low.
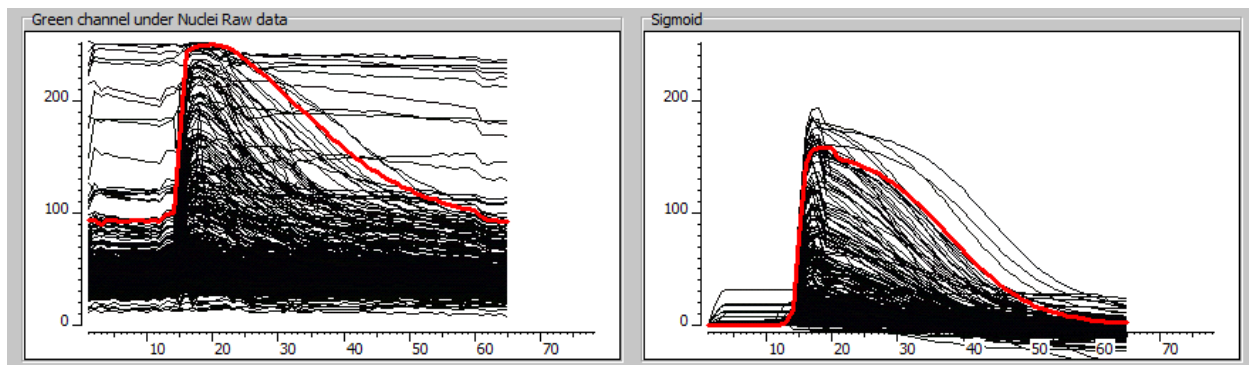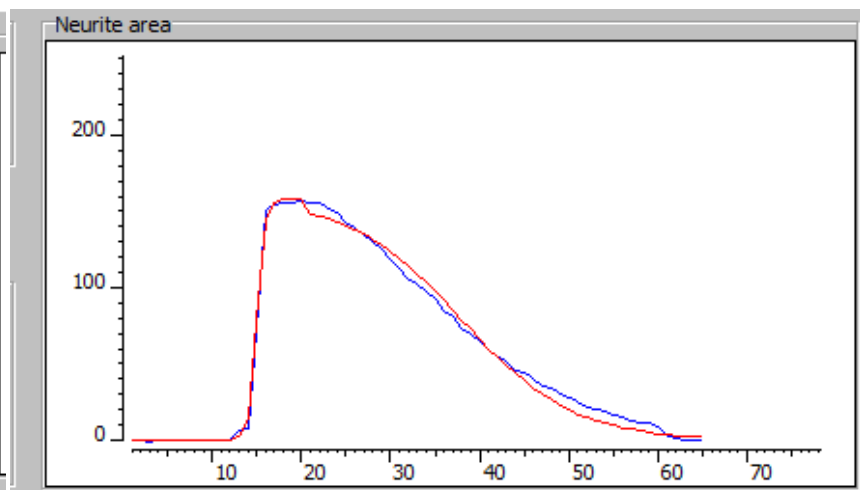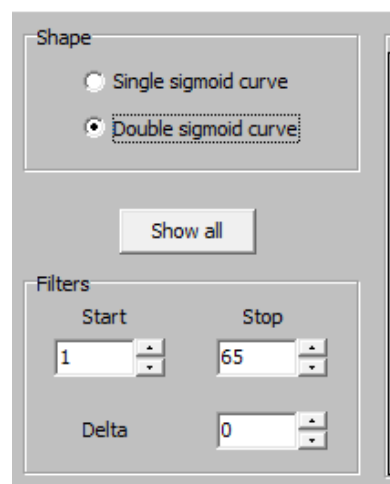
Fig. 14. selecting the double sigmoid curve

The behaviour the sigmoid curve describes best is that generated by a cell that is activated by a stimulus and stays in the activated state for a long(er) time. In some experiments you will really find this kind of behaviour. However, if you wait longer and longer, you will notice that the signal of the cells drops slowly back to the level it started with. This plays hell with the math that calculates the curves. The last part of the curve - there where it drops lower and lower will decrease the value of the **calculated** top. So, in theory, there are two approaches to deal with this situation.

First, cut the movie just after the first frame with a high Ca^{2+} signal and analyze just this first part. Which somehow does not sound right to me.

Secondly: use another math to describe the situation. I like this option more than the previous.

5.5 Evaluation of non-monotonic behaviour with "Double sigmoid curve" functions

In Fig. 14 we see this approach for the same data set we used before. However, this time we selected the "Double sigmoid curve" option from the panel right of the "Sigmoid" panel (Fig. 14B). We selected the same curve as before by placing the mouse arrow on top of it. This time the collection of sigmoid curves looks very different. As can be seen in Fig. 14C the fit of the sigmoid curve with the raw data points is now very good indeed. The values in the "Overview" panel will now also display better values. It will also display info about the

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speed of how quick the curve goes down: the EC50dwn.

5.6 Some more controls

As you can see there are several more options on the panel depicted in Fig. 14 B. Next to the choice you have about the shape of the fitting curve, you can visualize all the numbers of the cells as used in the "Overview" panel. Just click on the button marked "Show all" and the numbers of all cells will be displayed in the second picture. In principle the counting is from the left to the right and from bottom to top.

5.6.1 Filters

The part called "Filters" allows you to select which curves you want to see - only those with a delta above a certain threshold, or what part of the curves. The latter is selectable by setting the first and last frame.

5.7 Connection with Excel

So we have almost reached the end of this handbook, there are only two buttons left: the first on the very right of the window is marked "Excel". When clicked all data; the raw data, the calculated sigmoid data and all information about the original movie itself will be exported to an Excel file. Needless to say that this Microsoft program should be installed to use this feature.

5.7.1 Whole folders

If you want to analyze a whole set of Avi-files the final button labeled "Folder in Excel" is the very thing to use. It will export all the above mentioned data of every file found, into one Excel workbook. Every movie will get its own sheet. As an extra a summary sheet will be created with all important data of all the different movies in a single table.

The summary sheet will show you the behavior of the green signal over time for every file. This is a nice way to view the reaction as a whole. More important are tables where the averaged behavior of the cells are shown. The values of the Delta, Delta/F0 and F1/F0 for every frame / time point is shown.

As in a lot of our experiment cell mixtures are used, in which just a small percentage will react to any given stimulus, the response can look rather small. This is the reason CaFFEE offers you the opportunity to enter a minium Delta. You can select to evaluate just those cells that react (measured by their Delta) above a certain increase in fluorescence.

To say it simple just those cells that react at all.

As a matter of fact this kind of data is also included on every single sheet for the different files. The program has some extra columns showing those cells that were used to calculate the averages for the tables on the summary sheet.