Thomas Rutten Projects

In EasyClus© & in EC LIVE

EasyClus© & EC LIVE software for (autonomous) flow cytometric data analysis/ v1.31

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1. Introduction

EasyClus LIVE is software for the full autonomous data analyses of flowcytometric (CytoSense) files directly after the instrumental analysis. All kind of procedures are performed by EasyClus LIVE from the checking of the instrument up to the detailed cluster analysis and database matching. Results are also transferred to a website enabling the near real time (appr. < 3 minutes) reportage of the status of the sampled (natural) water and instrument.
The idea behind LIVE analyses by a CytoSense and EasyClus is the ability to high frequent algae monitoring LIVE in a river, lake or on a ship, giving information about the phytoplankton dynamics and status, which can fluctuate within short time. An idea is that it also might give information about chemical pollution of the water by (sudden) changes of the phytoplankton communities.

1.1 What's NEW in EasyClus LIVE v1.31

New (1.31) options which have been implemented:

1) NEW: Cyanobacteria towards other particles module by Orange/Red ratio and new plot options
2) NEW: International standard groups pico-nano-micro-crypto-cyano groups determination by different methods including easy saving and plot options
3) NEW: Specific only SWS (prot1) triggering data analysis module to collect turbidity-extinction information from waters
4) NEW: Options to change the website from 15, 9, 6, 4 figures representations
5) NEW: Immediate export to .txt of cyano-pico-nano-etc. groups (counts, FWS, FL Red, median, mean length and more)

Previous (1.30) (new) options which have been implemented:

1) More clustering methods are possible now.
2) Specific cluster as well as single cells counts
3) Alarm email possible (but sometimes locally blocked)

2. Installation/ upgrading of EasyClus LIVE

EasyClus LIVE is installed automatically after the installation of EasyClus.

2.1 Installation

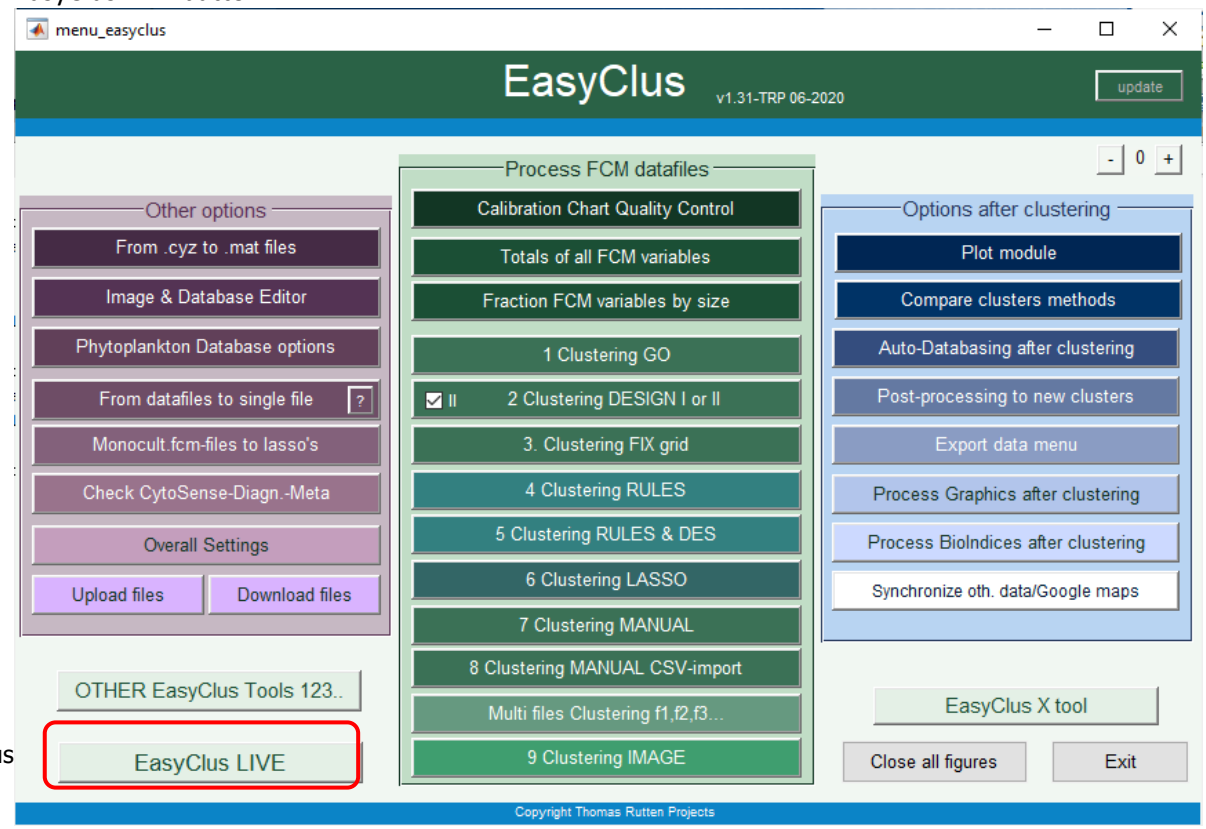
In the online mode it is recommended to install EasyClus LIVE in such a way that it automatically starts after starting the computer, to avoid manual starting after a computer shut off, which happens often to install upgrades of all kinds of software. Autostarting is better in the online mode, because the processing continues automatically after restarting the computer.

<u>startup AUTO installation procedure</u>

1) We will make a new startup shortcut, so we have an usual EasyClus shortcut and here we create a second EasyClus LIVE shortcut. To do so, copy shortcut of usual EasyClus in/on your desktop.
2) Make a copy of your whole latest EasyClusxx\ including all folder \cytoxx\ and put this second version next to the existing one. It will create a folder something like \EasyClusxx-copy\. Change 'EasyClusxx-copy' in e.g. 'EasyClusxxLive'
3) Look in this new EasyClusxx-LIVE\cytovxx\ startup.p in the cyto-\ directory is the first file that is used by Matlab-EasyClus. Rename this file in startup-old.p. Look (in the same folder) for the startuplive_plugplay.p file and rename this to startup.p. Now this file will be used as the first file.
4) Change path in the new EasyClus shortcut, right mouse button-properties in 'begin in' to path of new copied EasyClus ..:\EasyClusxxLIVE\cytoxx. You can also change the icon to the icon of LIVE which is put in the live folder.
5) For autostarting LIVE, put (a copy of) this EasyClus shortcut in the (windows) startup directory usual in : \program data\microsoft\windows\menu start\programs\startup).
   Windows 8 and lower: easy copy and paste to startup directory found in programs
   Windows 10: App: System/TaskManager/StartUp OR use
   Open link : Windows flag + R-> *type* shell:startup - copy short cut here

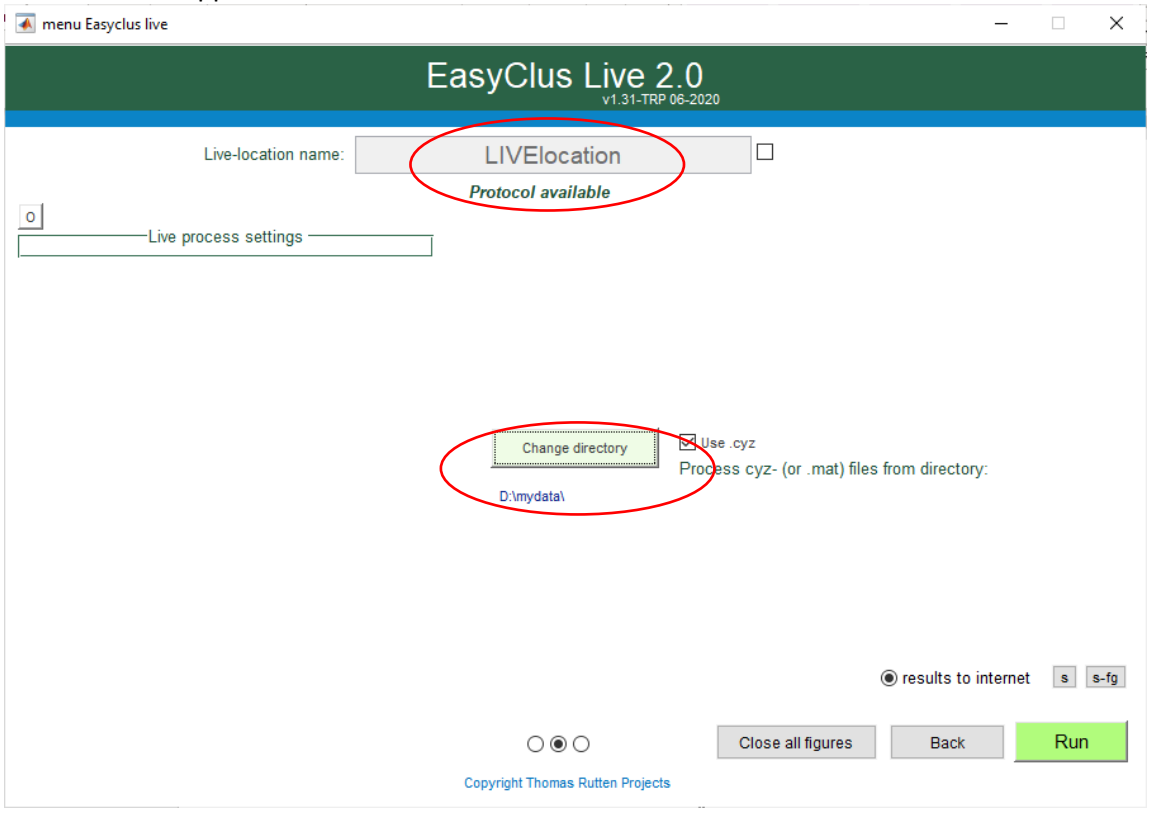
3. The application Easyclus LIVE

After starting, the Easyclus menu appears on the screen. EasyClus LIVE is started by using the EasyClus LIVE button.



Start button EasyClus

The EasyClus LIVE menu appears:



- Choose a file from the folder button to select where the (CytoSense) cyz-files are saved to and thus from where they have to be imported for processing, here c:\mydata\. Difference is now that you need to import a file from this folder first.
- Give a specific name for this 'LIVElocation' project. This name will be used as a tag and for saving settings
- Check if 'results to internet' should be activated or not. If yes, results will be forwarded to a server or an internet website.
- Press the 'Run' button and EasyClus LIVE will be started running samples and running beads and processing them according to default LIVE settings.

3.1 Some simple rules before starting the LIVE application

All default data process operations are already put in the LIVE software, just follow above instructions and everything will be done autonomously for you.
There are however some basic rules:

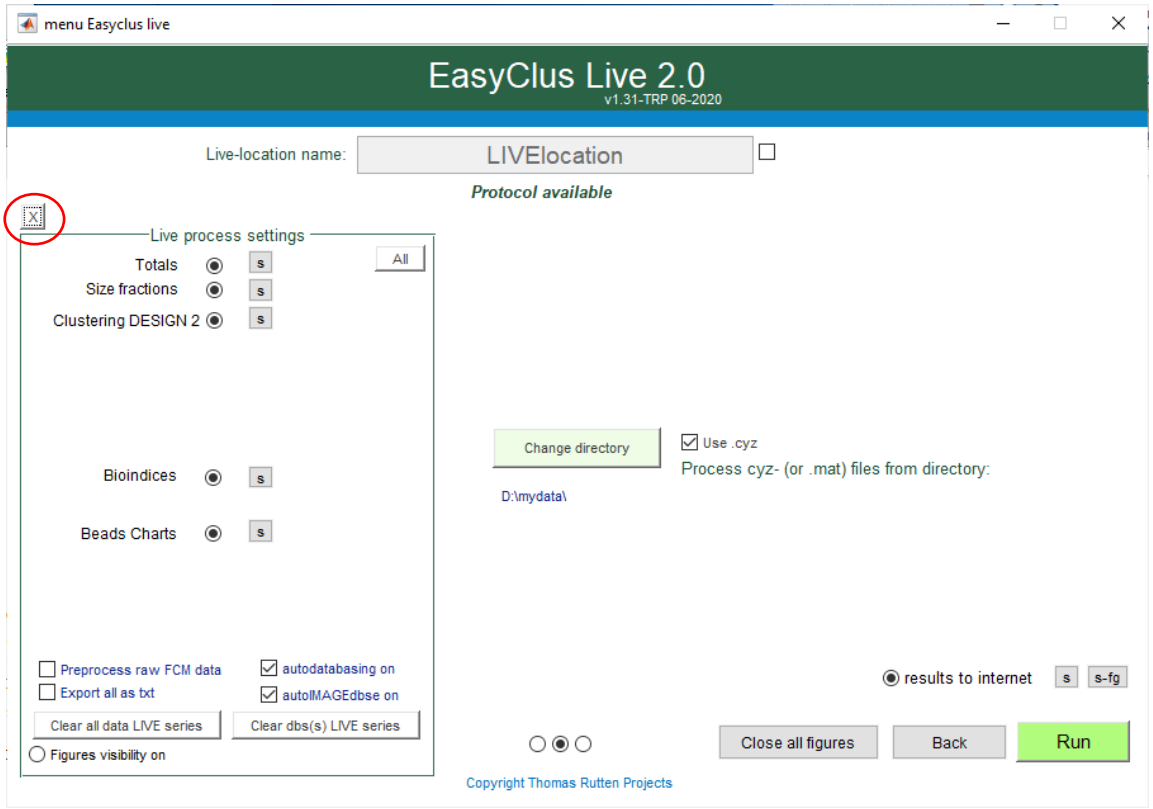
- All files in the given directory will be processed, also the files, which are only used for testing. Remove files before, if they are not of interest to be processed.
- Beads files are recognized by using the word 'bead' in the filename.
- 'Turbidity-extinction' cyz-files (only SWS triggering) are recognized by using the word 'prot1' in the filename.
- Be sure that the settings of the instrument and beads reference charts are rather similar to enable the mutual comparison of long term datasets. So, keep for example the maximum peak heights of a specific bead cluster around the same value during a longer period.
- Be sure that the total particle rate is not too high (<6000-10000 /s) to prevent coincidence of particles (algae as well as other particles).
- If you have HS+LS detectors, be sure that the LS detector is about 100x less sensitive i.e. at 5000 mV HS, LS should give about 50 mV maximum height.
- Be sure to store enough phytoplankton particles (>10000) for instance using smart triggering (SWS as well as FL Red).
- Results and figures can be transferred to a website. If using website reportage (e.g. to www.fytoplankton.nl / www.phytoplanktonlive.com), the live-location name is used as a website location name. TRP needs to activate your website name on the mentioned website. Be sure to keep exactly the same name for future website access.
  You can also choose to make/use your own website.

4. Settings

4.1 Default Settings

The default settings are found by ticking the checkbox ‘o’ on.

The settings that are default activated are:



Only the visualized bullets and checkboxes are used. Other available options

Totals: The sum of all individual particles of each FCM variable. Each sum variable is stored in a file to a certain maximum files to prevent very big storage capacity. Afterwards these files can be merged together. Only some totals are also continuously stored without interruption (LT-files). These files are stored as .mat files and forwarded to the website for downloading.

Size fractions: The sum of all individual particles of each FCM variable subdivided in size fractions between (D.L.- 5-10-20-50-100-250-500-1000-Max.Length Instrument). These files are stored as .mat files and forwarded to the website for downloading.
Checkbox: use subselection set to on means that the above results will be performed on a subselection of the data (selection set).

Clustering DESIGN II : Clustering according to the DESIGN II clustering method using three default scatterplot combinations – 55-3-950-97%-700.

Bioindices: Shannon Wiener – Simpson –Rutten index – Maximum found nr of clusters – Euclidean distance based bioindices (next year) are calculated on basis of one of the applied clustering methods. These indices give information about the biodiversity – dominance or other algae characteristics.

Traffic lights: Several checks of the instrument are put into so called traffic lights, which is green (ok), orange (critical) or red (not ok). Long term information is also given (Temperature, Pressure and more). This feature is set on.

Beads Shewart Charts: Beads are used to check the instrument in between sample analysis. The automatic analysis of beads and the making of so called Shewart calibration charts is set on. Beads are automatically determined according to the DESIGN clustering method and beads recognition criteria. The smallest beads are chosen if more than one type of beads is used.

Autodatabasing on checkbox: is set on for the unique clusters found in the DESIGN cluster method. Only unique clusters are put in the database automatically.

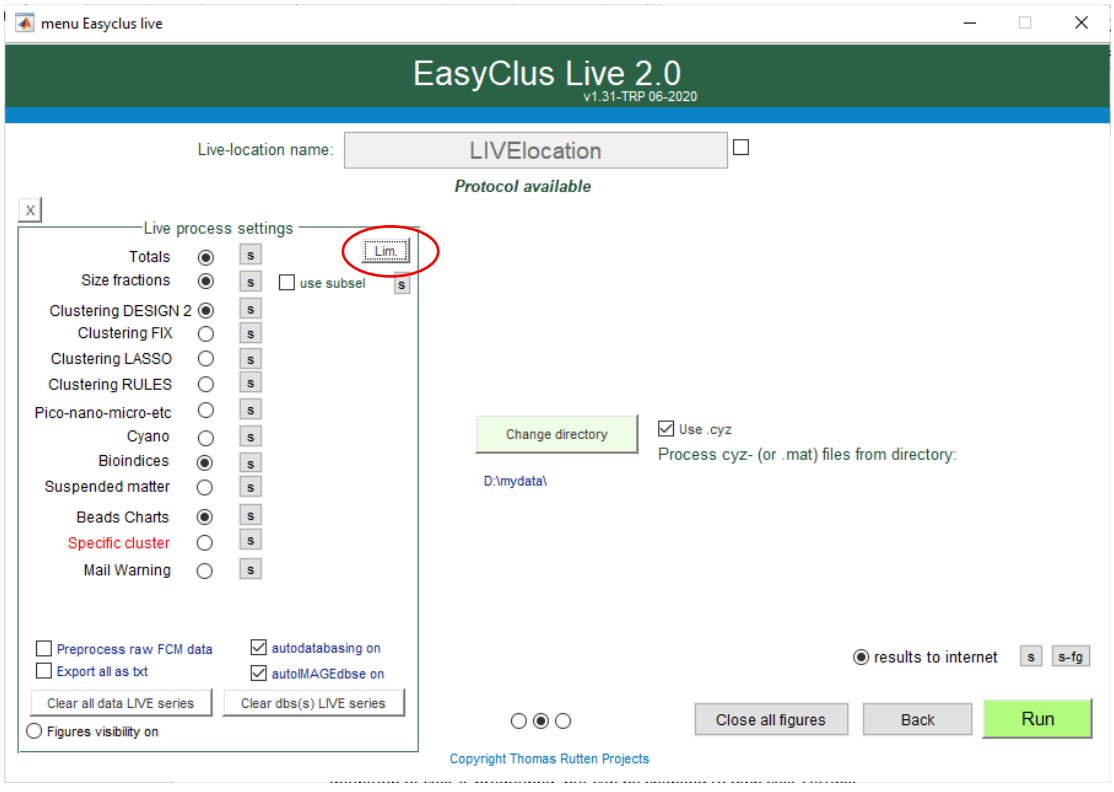
AutoIMAGEdbse on checkbox: is set on. Images in FCM files are clustered and recognized using the DESIGN method and stored in a separate database*.

Results to internet is set on: Results will be sent to a server and website. During the first operation of your location, an ftp-(data transfer) dialog menu will appear asking the properties of the server. For fytoplankton.nl users, only ftp.fytoplankton.nl should be filled in, the other windows will be autofilled.

* IMPORTANT : When using autoimagedbs 'on', the phytodatabase.mat will increase after each analysis with images and particle information. Since the CytoSense-Sub produces many images, this will create a very big file after many samples, which might slow down everything from saving-running and especially transferring files to the internet. There should be a filter to prevent unlimited adding of particles to the phytodatabase.mat. How and what criteria to apply is still a question for the author. Better NOT to put this option 'on' to prevent slowing down the process.

NOT performed in the default mode

More options are available in LIVE but not performed as a default. They become visible with the 'All button'



Clustering FIX: Clustering according to the FIX clustering method dividing all FCM data in fixed spaced uninterrupted cells, so each possible event will fit in one of the predefined cells. The definition of cells is predefined, but can be changed to own cells settings.

Clustering LASSO: Supervised clustering by lasso's. Not performed in the default mode.

Clustering RULES: Supervised clustering by classification rules. Not performed in the default mode.

Pico-nano-micro-crypto-cyano : clustering of groups according to the classification of an international standard

Cyano : Clustering of the cyano group towards all other particles by using the ratio of the orange vs red fluorescence

Suspended matter : counts-FWS-SWS over (adjustable) size fractions of only SWS triggering samples (prot1)

Specific Cluster: This option enables the user to produce graphs of specific clusters or species, which are recognized by one (or more) of the clustering methods.

Mail warning : Set up of a mail warning if something exceeds a specific value. This option is sometimes blocked by local network safety actions

Preprocessing raw FCM data checkbox is set off.
Only for one specific customer the preprocessing is set on for:

- HS-LS profiling (profiling of flattened saturated peaks) (only one customer default on)
- Only use HS data for further processing (only one customer default on)

On request, specific preprocessing operations can be set on as a default.

The (settings) button are giving access to the (applied) settings menu in order to change them for instance. It is strongly recommended to run a sample in the default mode at first, before changing the settings. By running, the default settings are activated which is in most cases close to the desired settings, and better to change them from here than to try to find your own settings.

Preset of settings

For some customers there is the option to have pre-setted settings. These are activated by using the 'right' location name (in the live location name edit box), which is recognized by the software automatically.

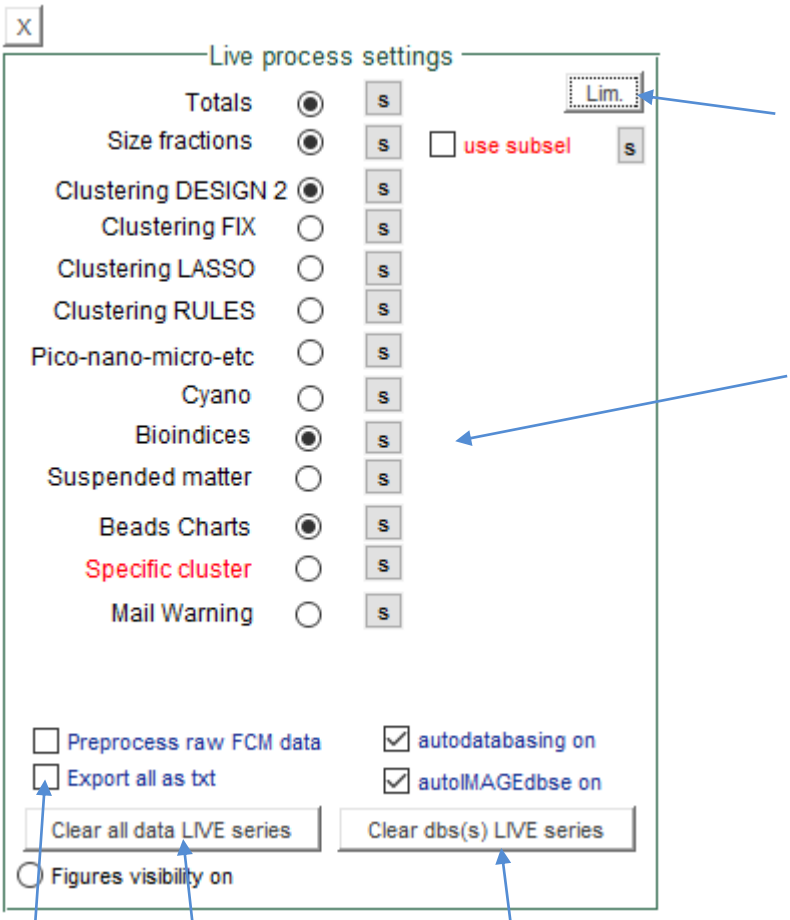
A second option of the preset of settings is the use of earlier settings by you, which are automatically imported from the EasyClusLIVE folder while using the same live-location name as before. This live-location name works as a 'tag' and the location name is used a kind of signature and imports files with this signature. Earlier settings can also be copied to a new location name (see chapter 6).

4.2 Change specific settings?

Stop running LIVE processing. There are two options the quick menu (4.3) or the more detailed settings menu (4.4).

4.3 Quick menu

Tick the 'Fast settings on/off' checkbox, to activate/deactivate specific operations



Activate operations by ticking this on

Clear all data at the actual location

Clear the database(s) at the actual location, not the other data

Export all data as .txt files too

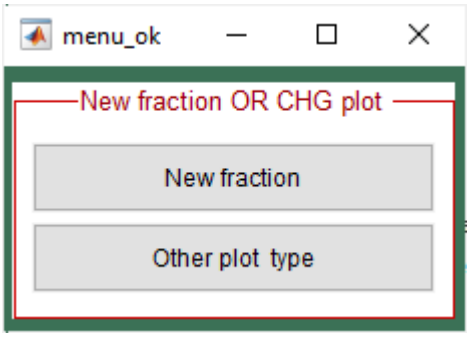
It is better to run a sample first and then change settings, to be sure that all settings are prepared and set!

4.3.1 Totals

Totals is not implemented yet here.

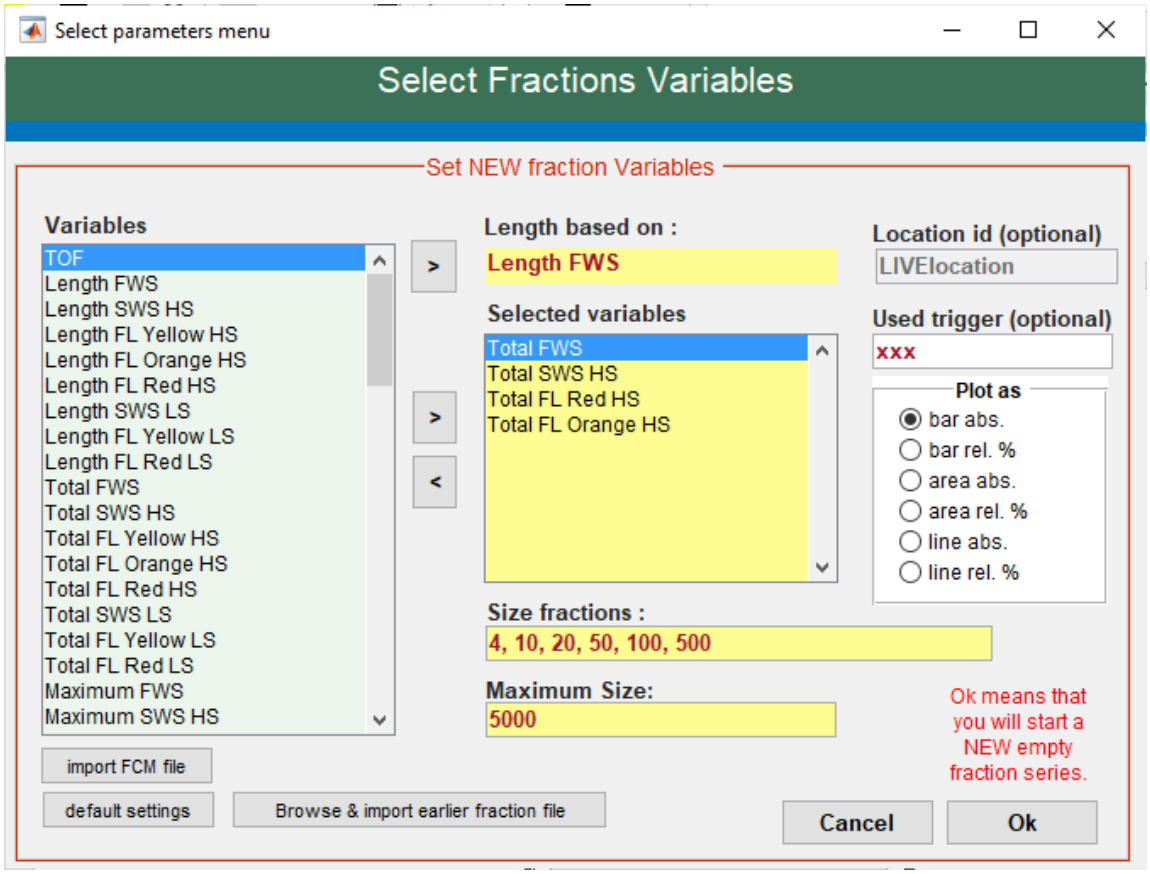
4.3.2 Fractions

Following menu appears:



You can change the fractions (and start new) settings itself or only change the graphs type which are made.

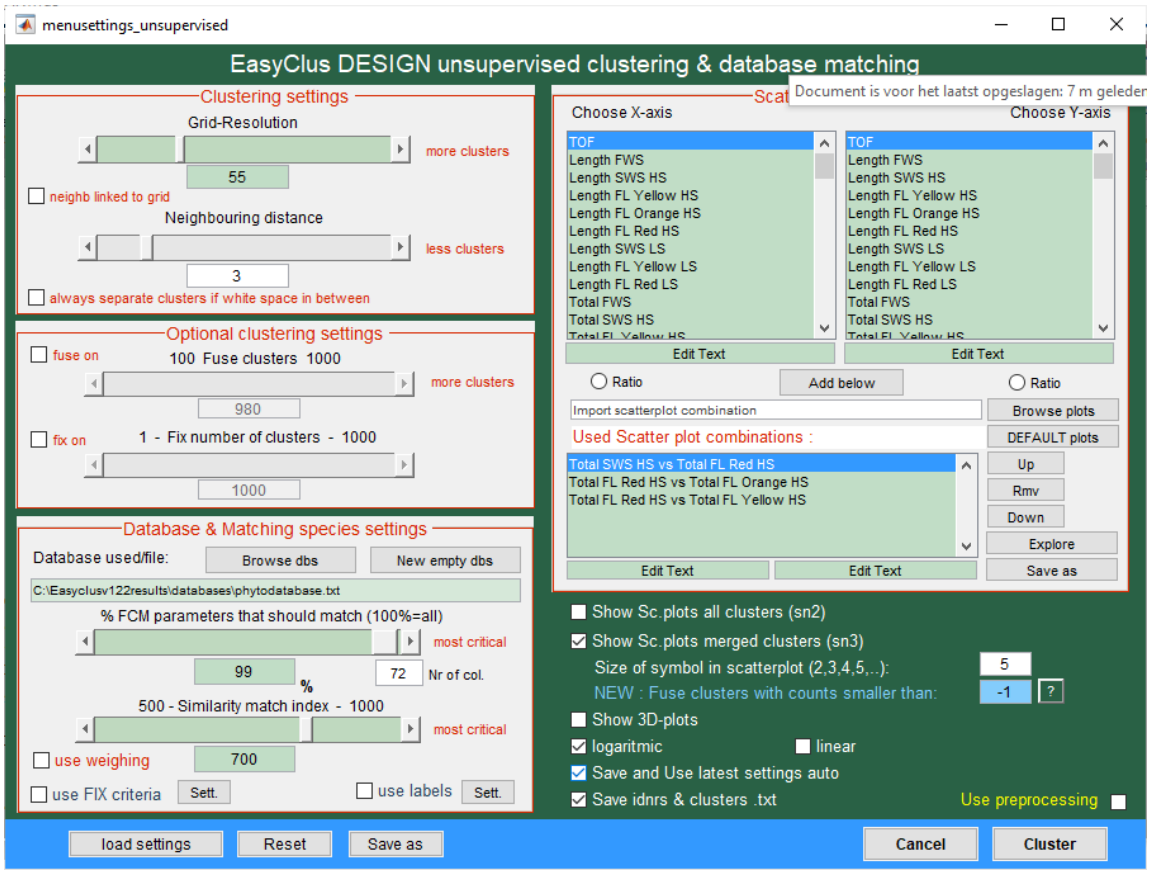
New fraction : Here you can change everything which is in the menu :
(only 'Used trigger' doesn't change anything, it is just used as 'text'. Changing triggering is done in the CytoUSB-software.



Other plot type :

4.3.2 Clustering Design 2

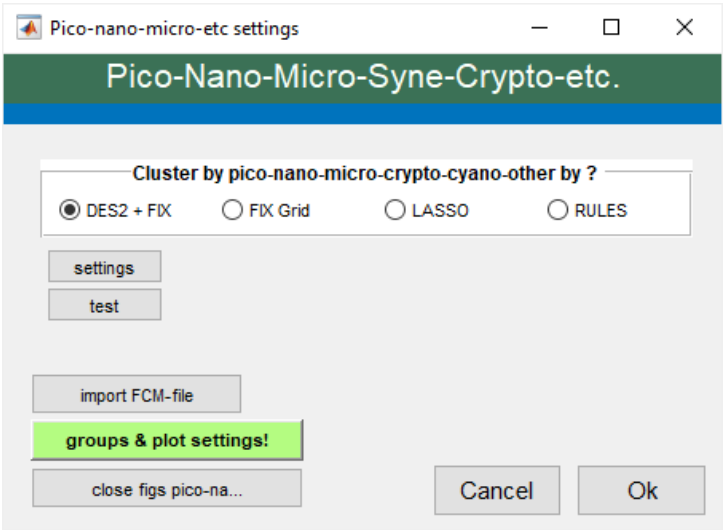
The design 2 settings menu which also appears in the usua;l EasyClus LIVE appears and can be changed here.



The same kind of menus will appear for the other menu settings of other clustering methods and this is not shown here.

4.3.4 Pico-nano-micro-crypto-cyano groups

This (new) method determines the international standardized groups of pico-nano-micro plankton by size class and typical fluorescent properties (high orange=crypto) specific orange to



red ration (=cyano).

Choose which (clustering) method should be used to determine the specific groups :

Pico : < 4-5 micrometer
Nano : 4 – 20 um
Micro : > 20 um

Crypto : Particles (FL Orange/FL Red) > x2 value

Cyano : Particles (FL Orange/FL Red) > x1 value AND particles (FL Orange/FL Red) <= x2 value

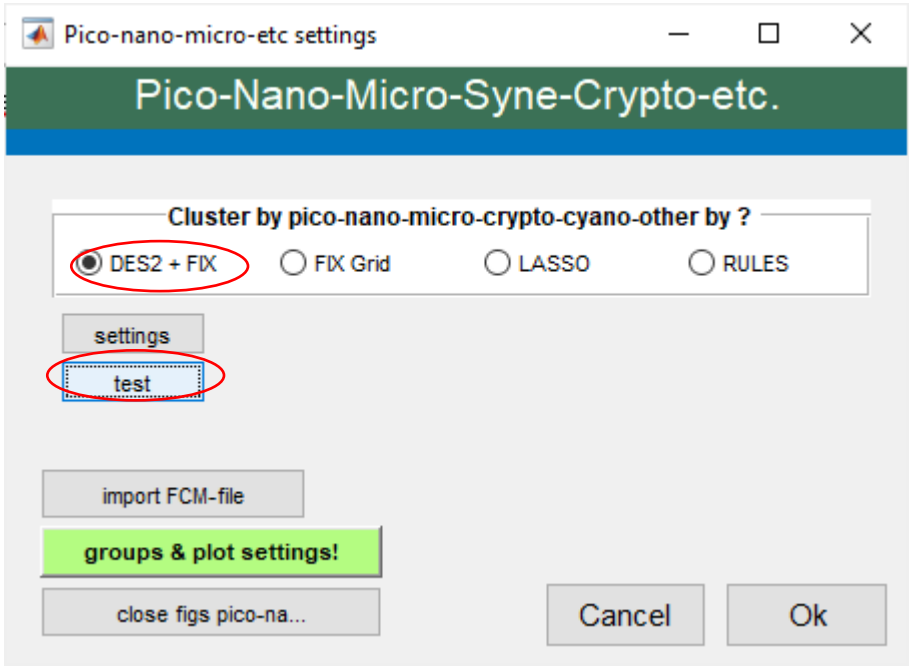
A. DESIGN 2 + FIX : The sample is unsupervised clustered and the clusters averages – medians are assigned to the specific groups. All individual particles within this cluster are assigned to this matching group. This methods prevents that a cluster is split into two pieces due to one of the criteria. Each unique criteria combination defines a specific group, which can be labeled to one of the international groups 'pico','nano' etc. The matching of the clusters and the naming of the groups is done by using FIX and LABELing checkboxes in the settings menu.

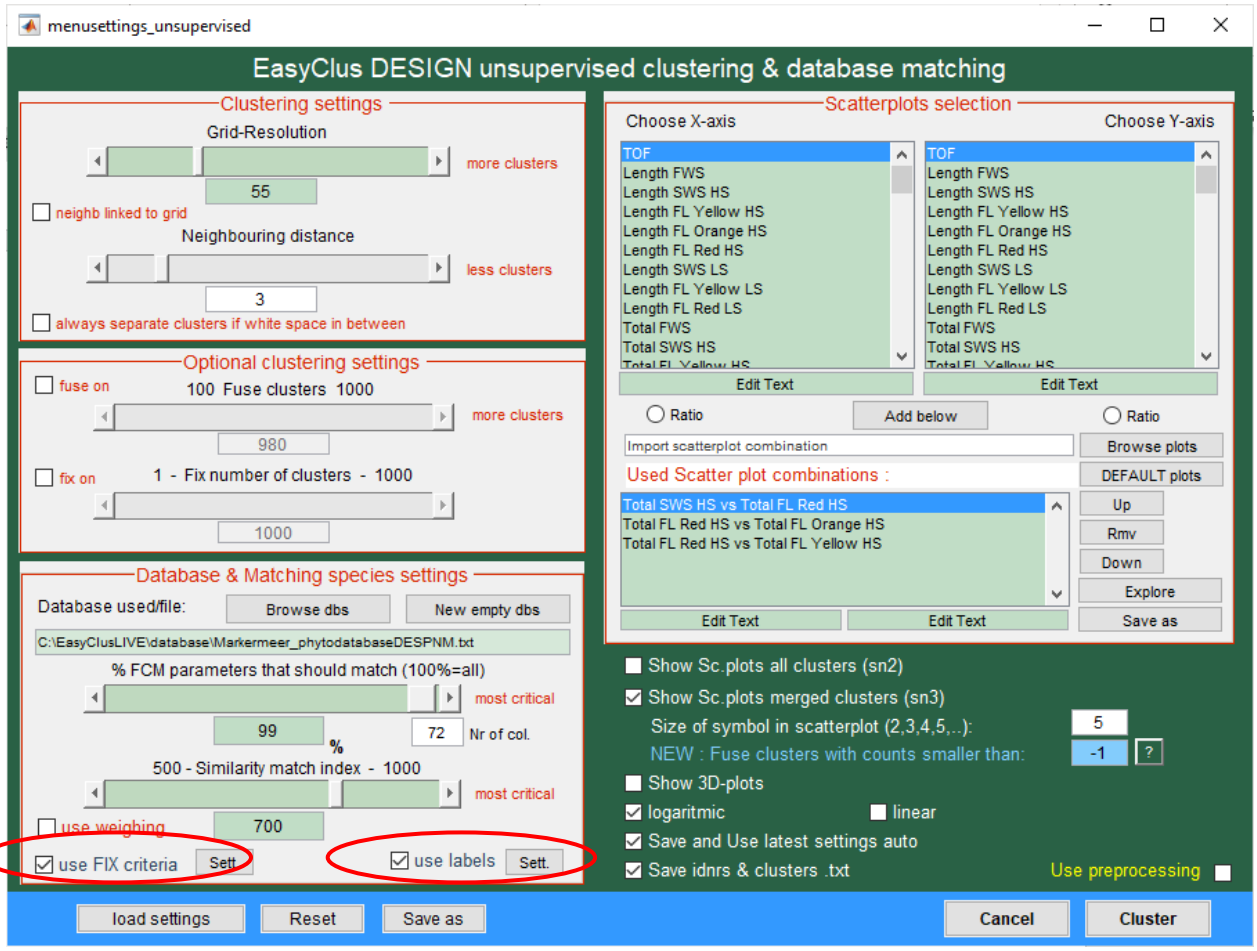
B. FIX Grid : This clustering method uses direct criteria for each individual particle, to assign it (directly) into one of the groups. Each unique criteria combination defines a specific group, which can be labeled to one of the international groups 'pico','nano' etc. The difference with the previous method is that previous method assigns a whole cluster, and this method assigns each individual particle. In this method a 'visual' cluster in a scatterplot might be split into two or more groups.

C. LASSO : The grouping is done on basis of previously defined (supervised) training sets containing the information for the assignment of the different groups. This information is put in a database.mat or as selectionsets (lasso's)

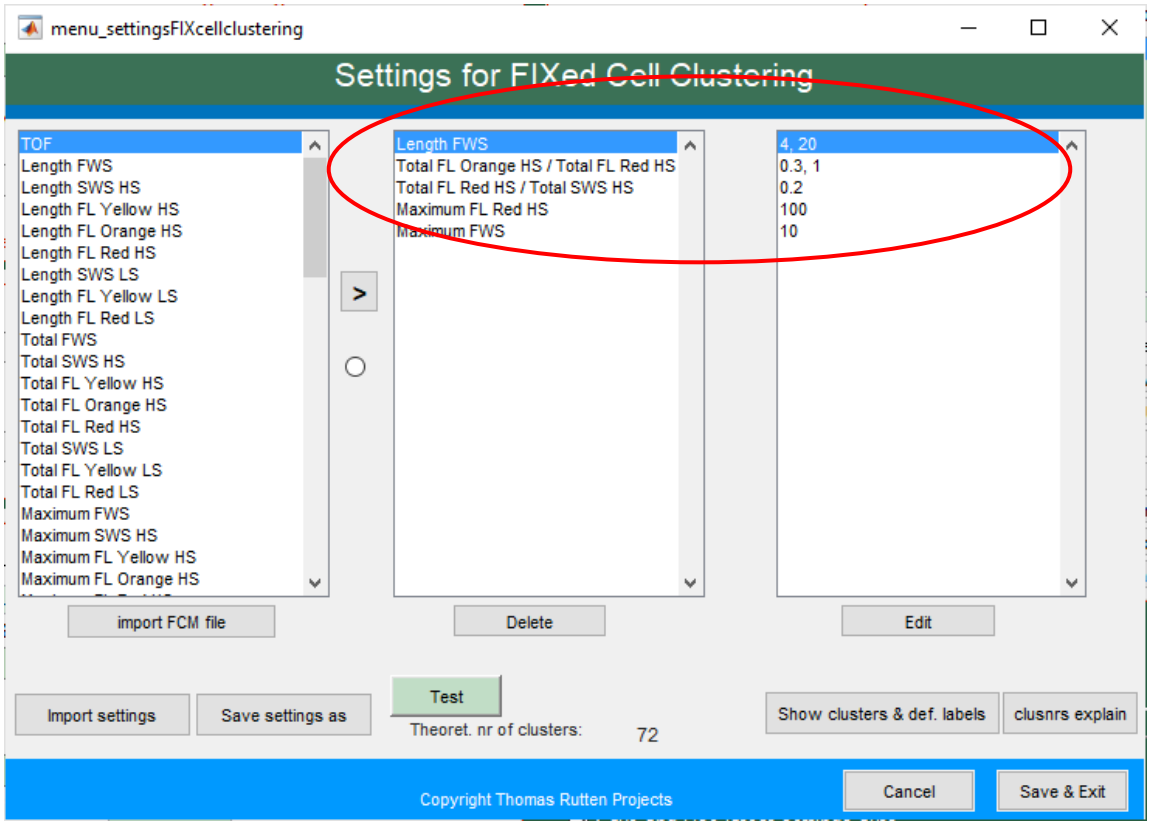
D. RULES : The grouping is done on basis of previously defined (supervised) training sets containing the information for the assignment of the different groups. This information is put in a database.mat.

A. DESIGN 2 + FIX :

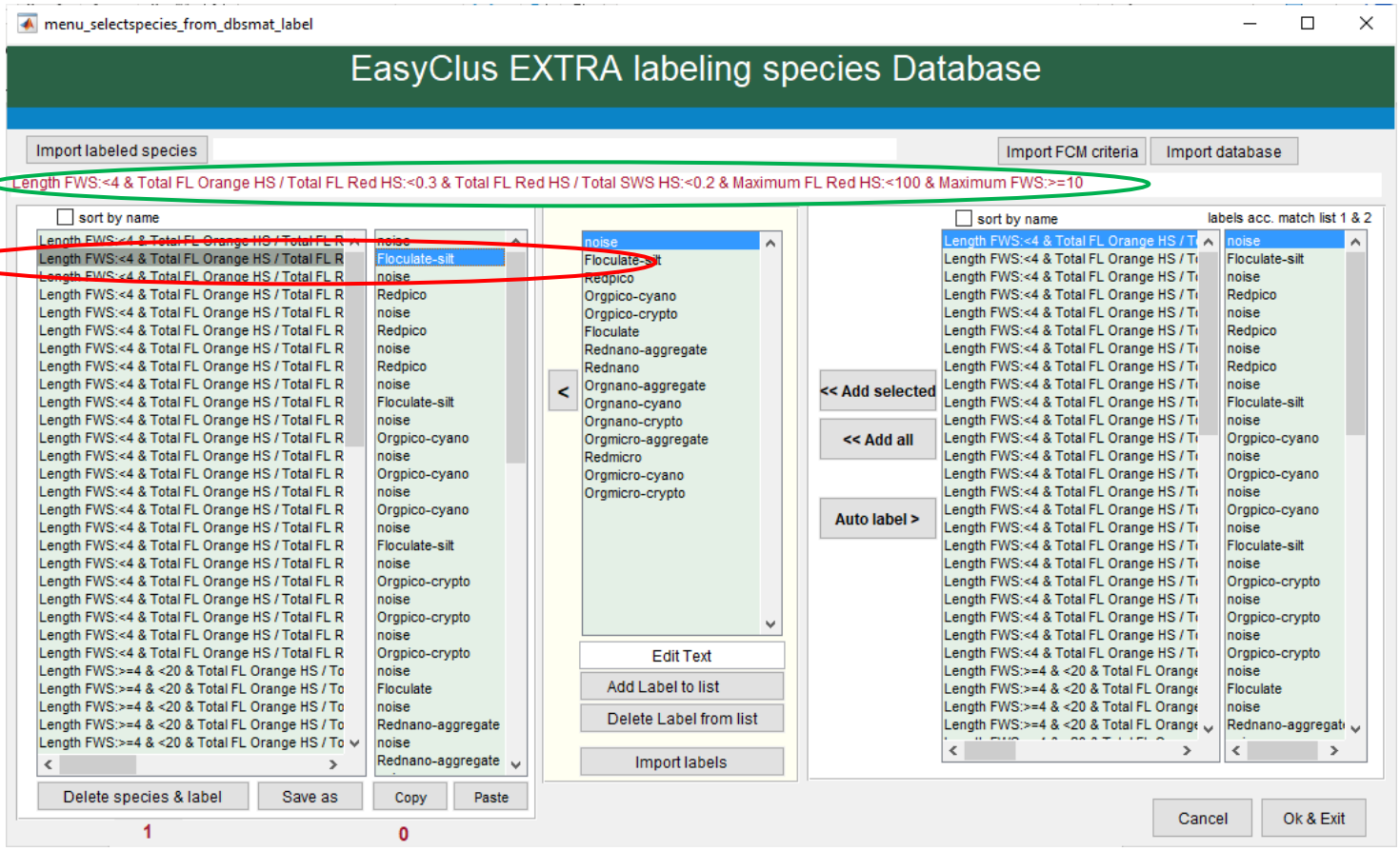




Settings up the cluster assigned criteria by clicking the use FIX criteria checkbox

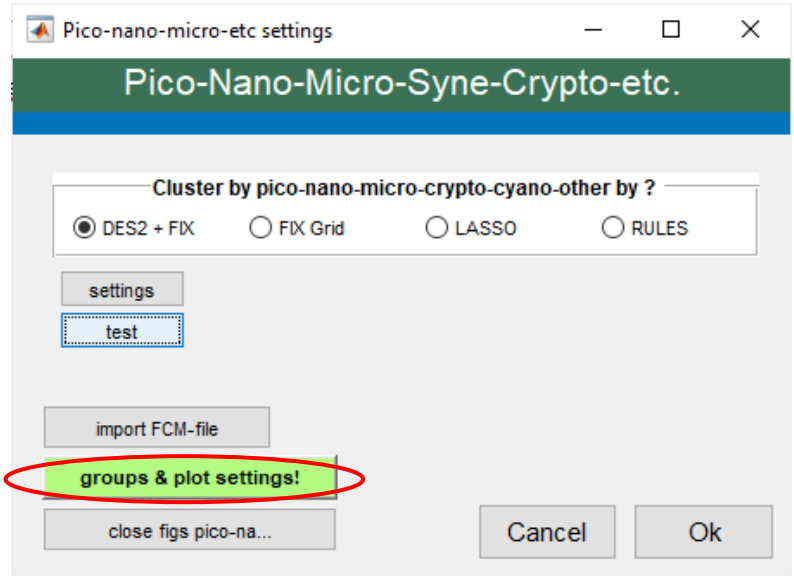


And the assignment of 'FIX criteria names' to new LABELS

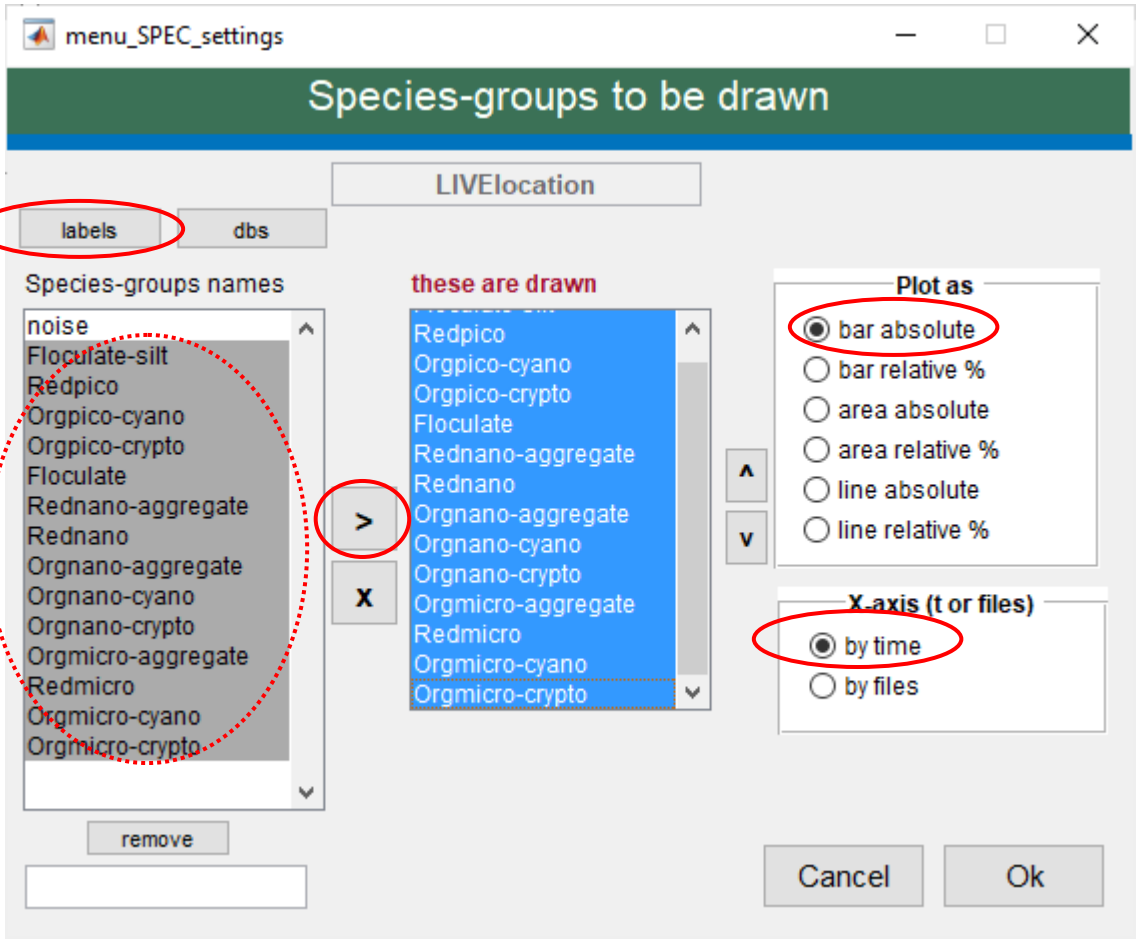


The 'long criterium name' will be changed to the short 'Floculate-silt' LABEL name given by the user.

After that we need to define what to put into the graphs

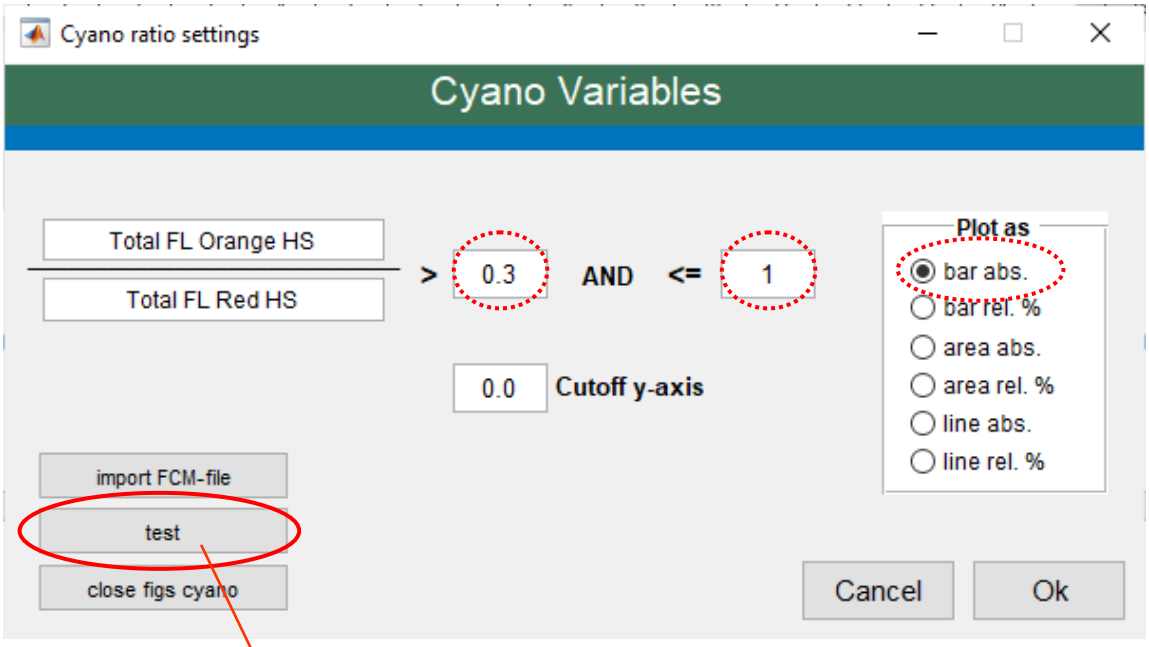


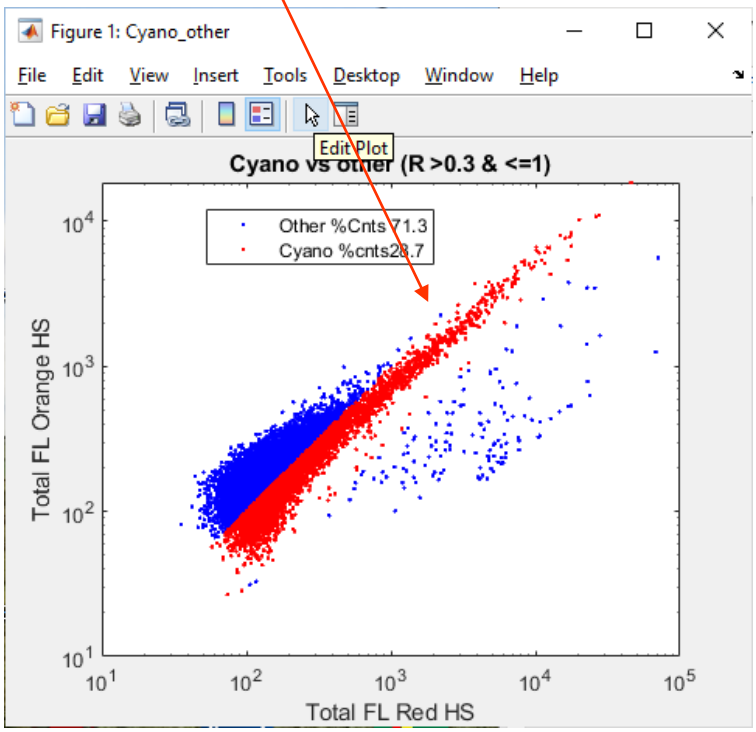
We select (from the earlier defined labels) which labels are put in a graph (e.g. 'all', here 'all except 'noise'). And we define to use a graph (stacked) bar, with absolute values as a function of sample time. The file can also be saved as a. txt file.



4.3.5 Cyano

After pressing the setting button : Adjust the right ratio values for the assignment of the Cyano group. The counts, all Totals (FWS, SWS, FL Red etc.) median and mean length are automatically determined and saved in a separate file. One or more graphs will be drawn according to the right figure (here absolute bars are chosen). Default figures of counts/ml, Total FWS/ml and FL Red/ml are drawn. The file can also be saved as a. txt file.



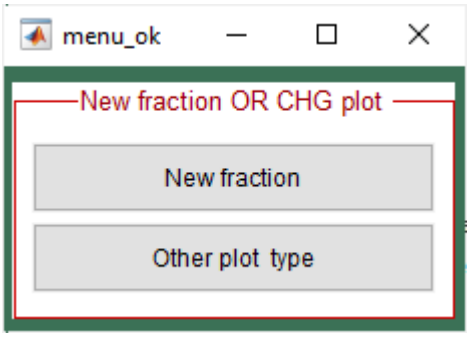


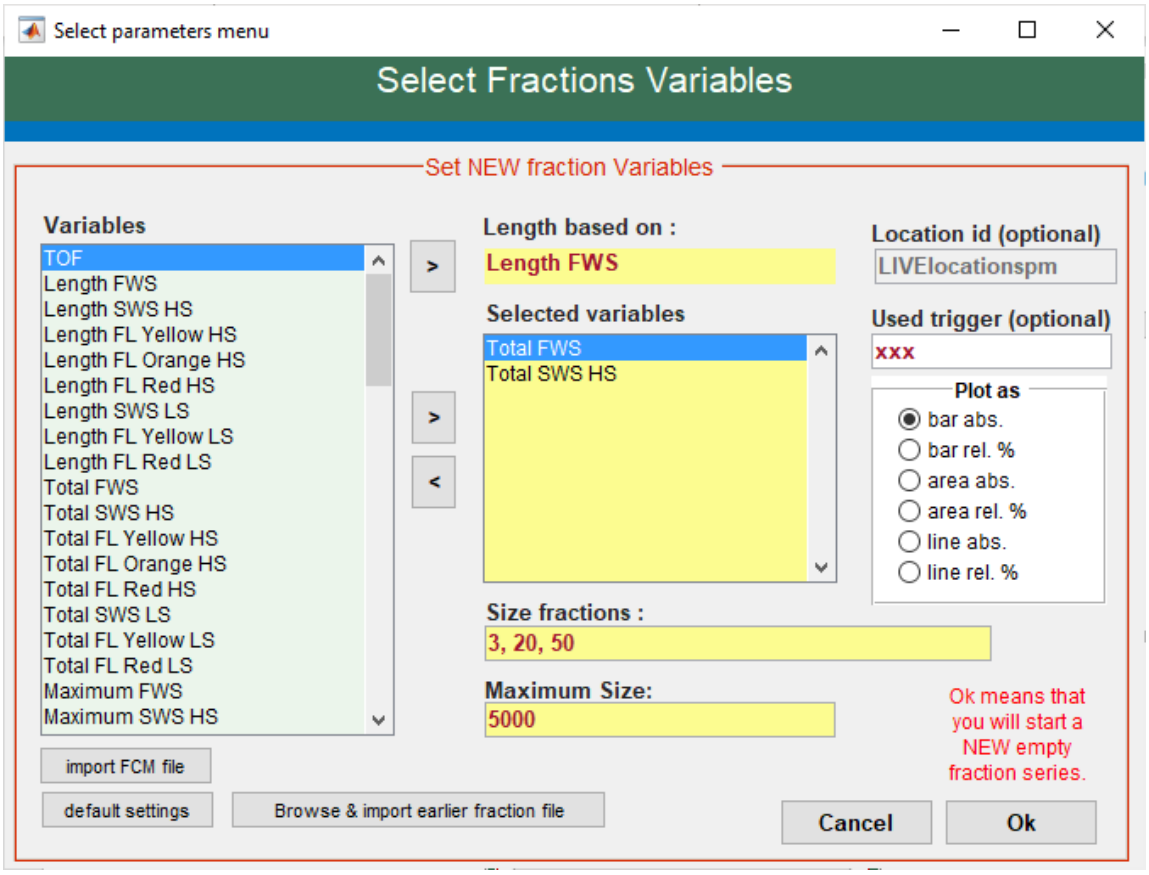
4.3.6 Bioindices

There is no settings given here yet.

4.3.7 Suspended matter

Works the same as fractions but here only for suspended matter .cyz files ('prot1' in filename), which are only SWS triggered samples (so all particles are measured, not only algae).





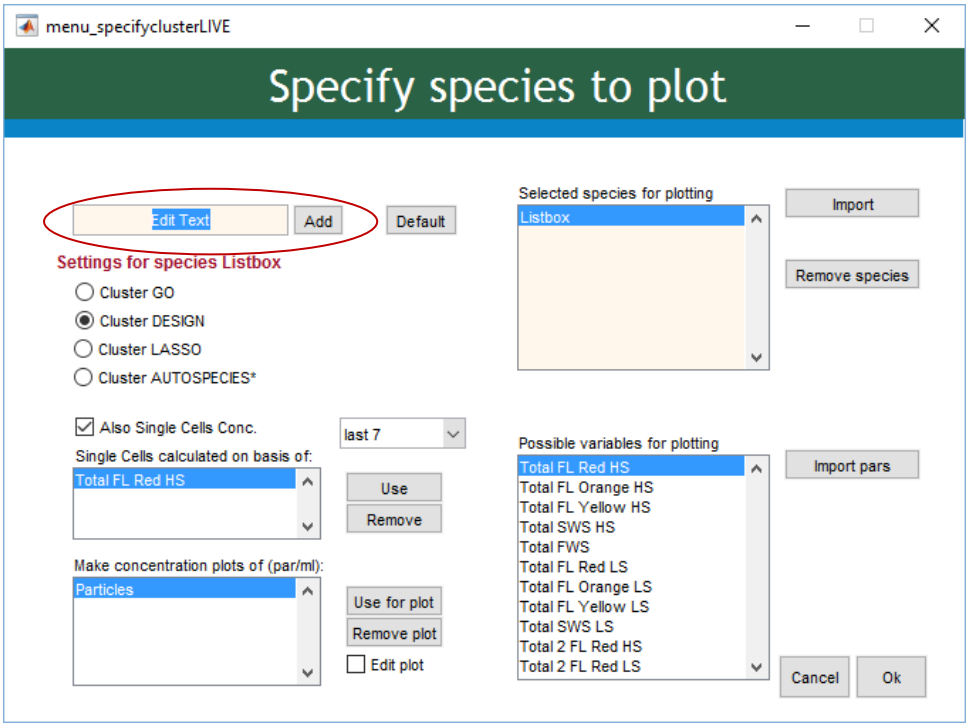
Counts/ml , Total FWS/ml and Total SWS/ml are default variables which are stored.

4.3.8 Beads charts

There is no settings given here yet.

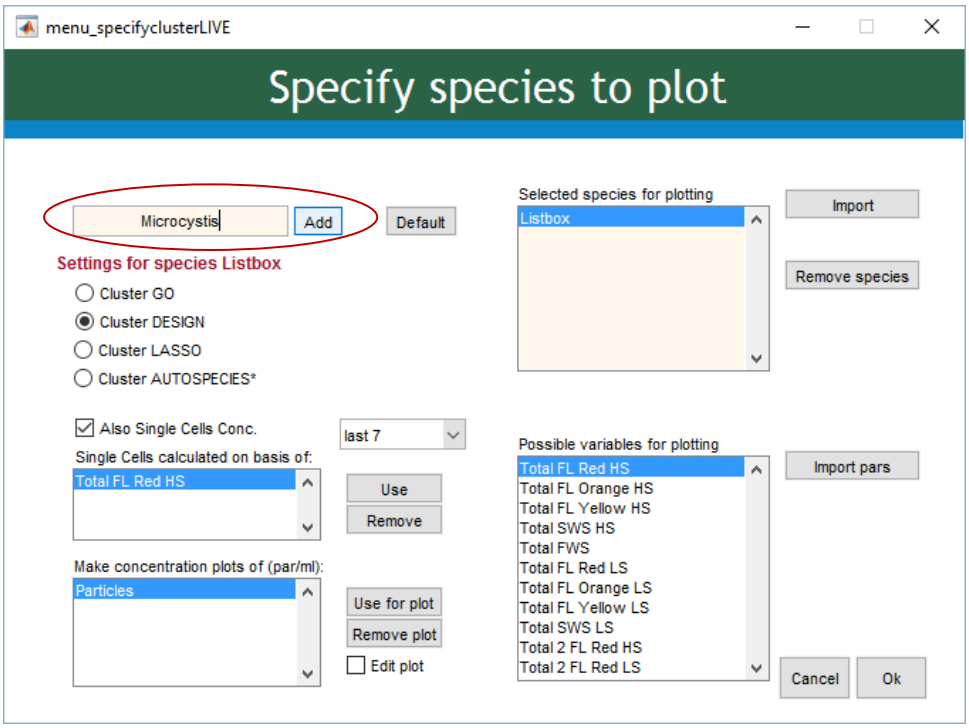
4.3.9 Specific cluster

A specific cluster (in combination with one or more clustering methods) can be visualized. Do not use this option with pico-nano-micro groups, because they use their own visualization method.

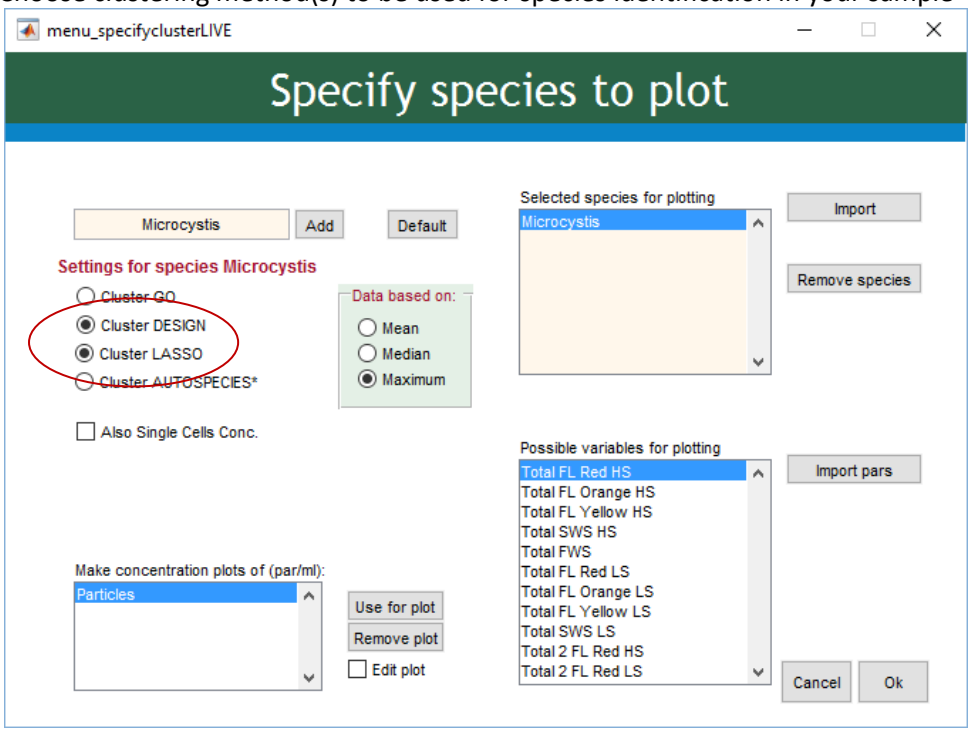


Activate which species to extract out of clusterdata:

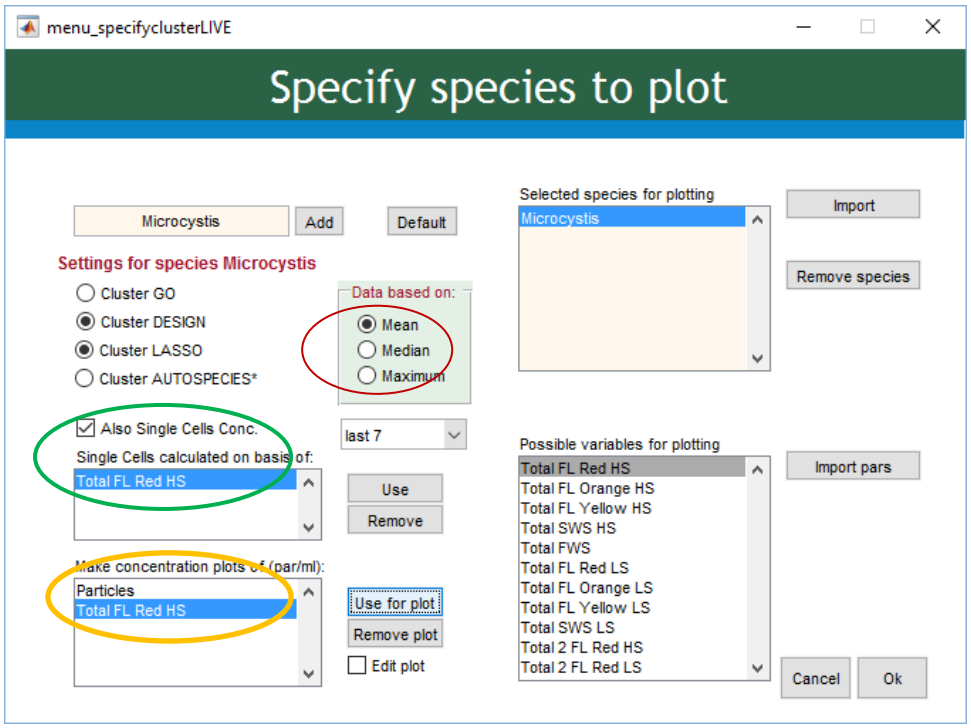
Type species name to make graphs of (here e.g. Microcystis) and press the 'Add-button'. This name should be exactly similar to the name used in the clustering methods (database, lasso or other), because this name will be used to filter the clusters out of the data results. In case 'single' cells are calculated be sure that there is a separate Microcystis single cluster & Microcystis colony cluster.



Choose clustering method(s) to be used for species identification in your sample



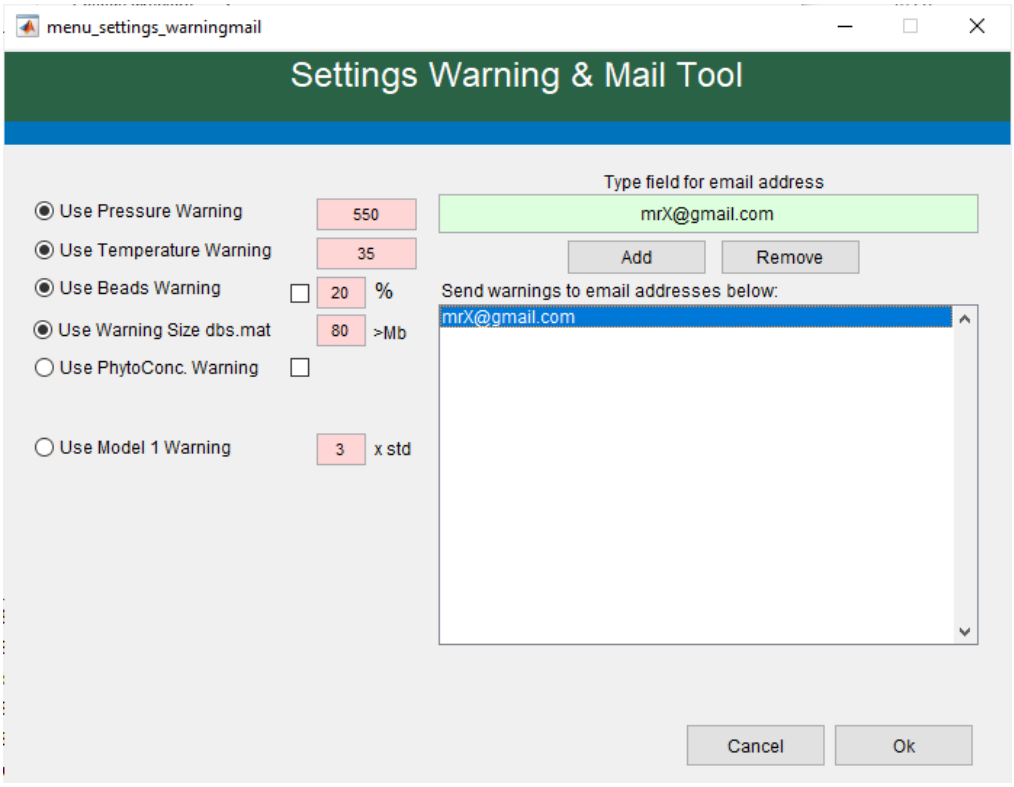
Put the checkbox ‘Single Cell’ concentration ‘on’ if single cells estimation – recalculation are of interest. These single cell calculation are based on the total sum of all found clusters with the species name in it, divided by the average of one single cell of the same species. The variables in the green circle are used for this recalculation.



Graphs in the orange circle are drawn by EasyClus LIVE. The red circle is used to draw a surplus graph based on the mean or median or maximum of data of all applied clustering methods and variables. The option 'last 7' means that the last 7 found (known) values of single cells will be used to calculate the average value of one single cell. This 'last 7' option can be changed to other values (last 1,2,3..,48) or a fixed value.

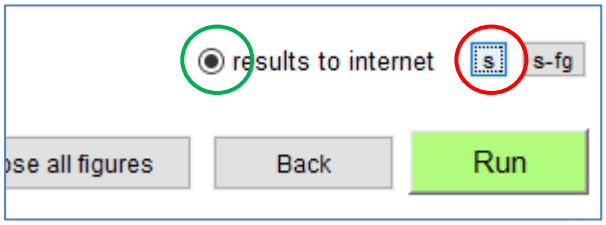
4.3.10 Mail warning

To set up specific warnings and send to given email addresses. The sending of emails by EasyClus LIVE needs internet and no blockage by any local security.

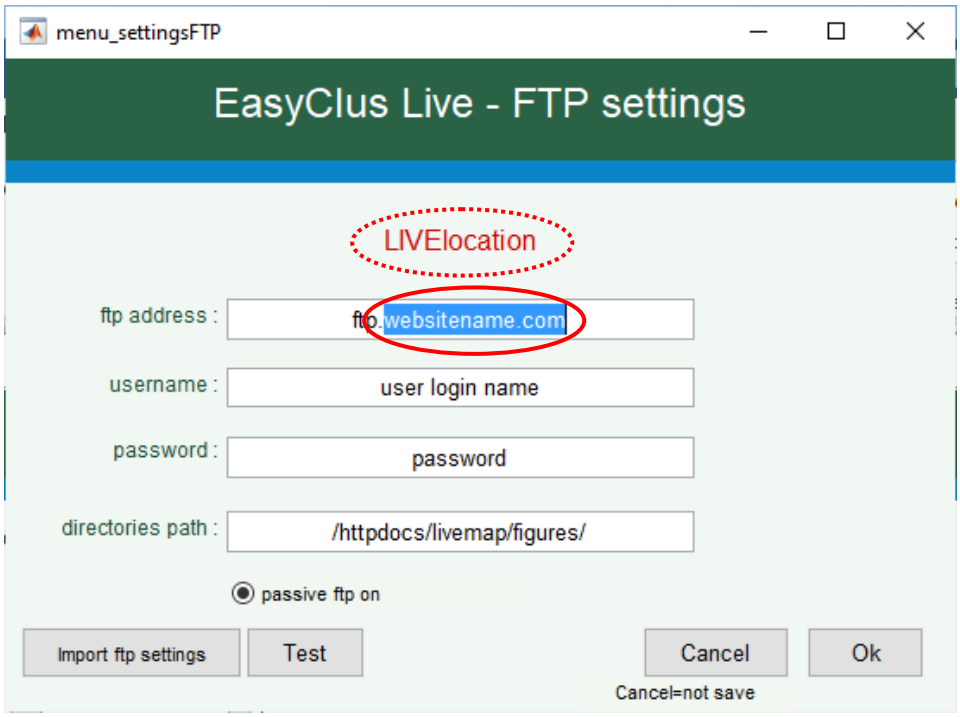


4.3.11 Internet FTP

Set the right FTP settings by clicking the settings (red circle) button

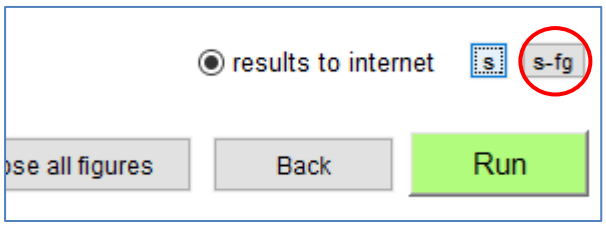


LIVElocation is the website server location, where all results are uploaded to. Only Thomas Rutten Projects can make a new location name for you (for free) on the phytoplanktonLIVE.com (fytoplankton.nl) website. ONLY change 'ftp.websitename.com' in 'ftp.fytoplankton.nl', the other info is automatically filled in. Press 'test' to test if you are able to upload something to this location.



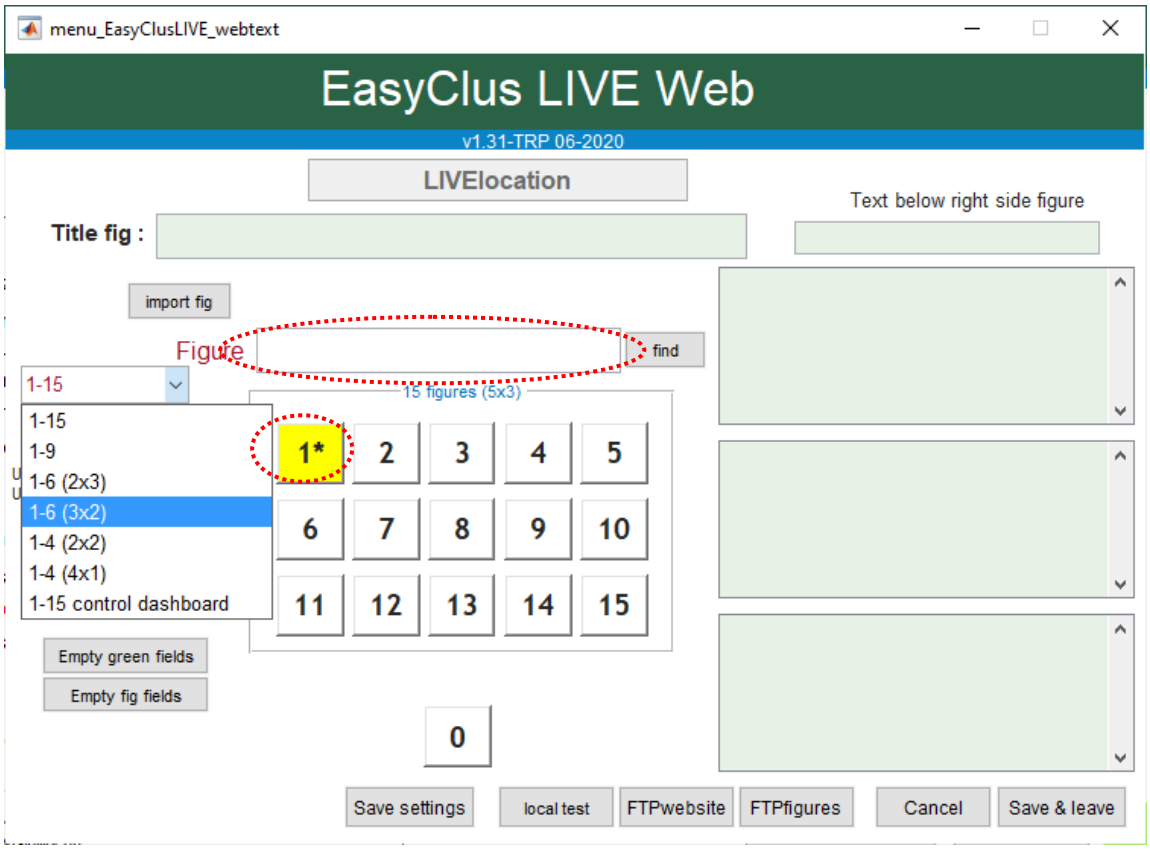
4.3.12 Website & Internet FTP

New is the option to change the figures and number of figures as well as text on the website.



Press the s-fig (settings figures) button. The following menu appears:

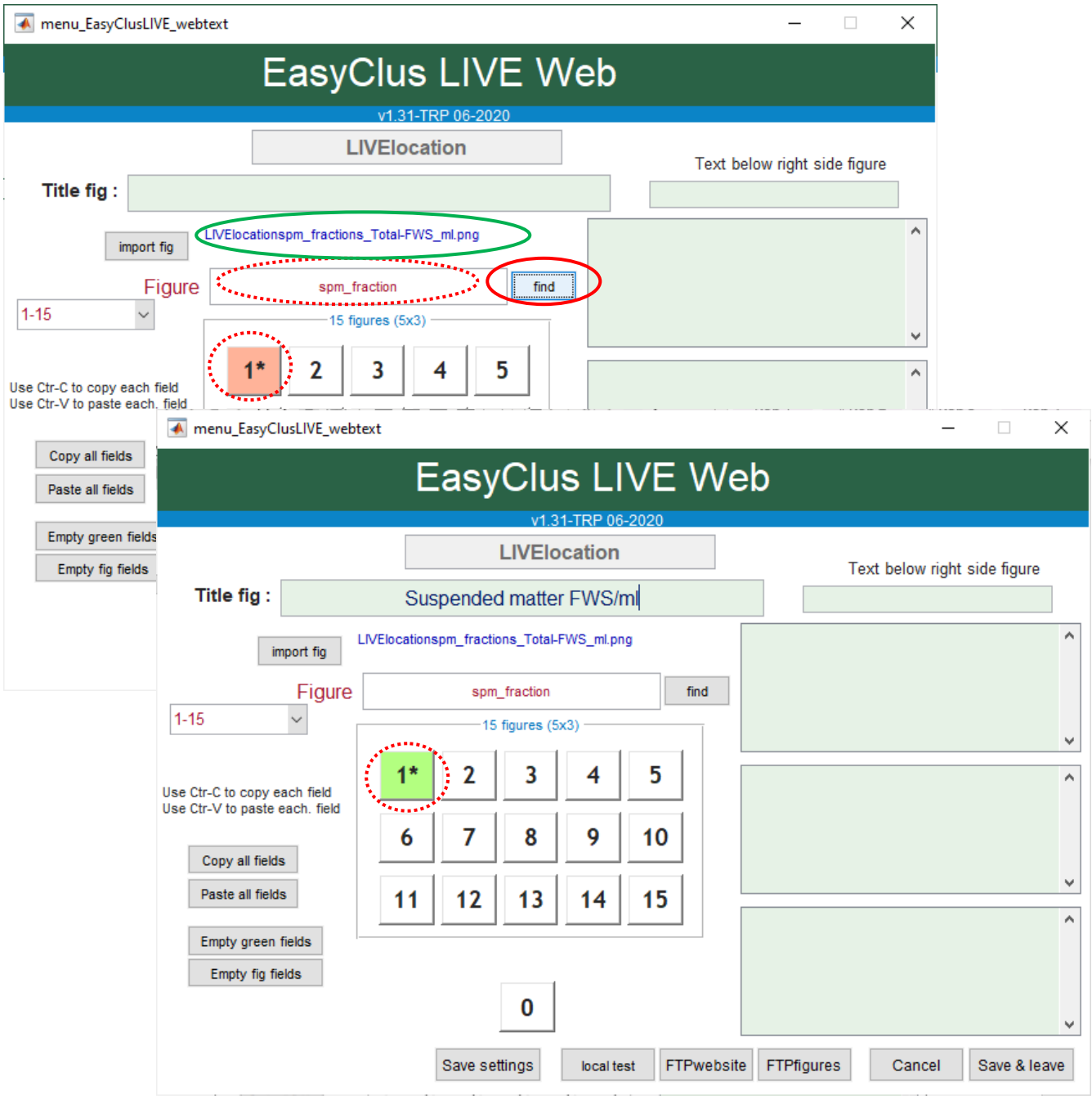
Here you can select the number of figures and the figures, which should appear on your



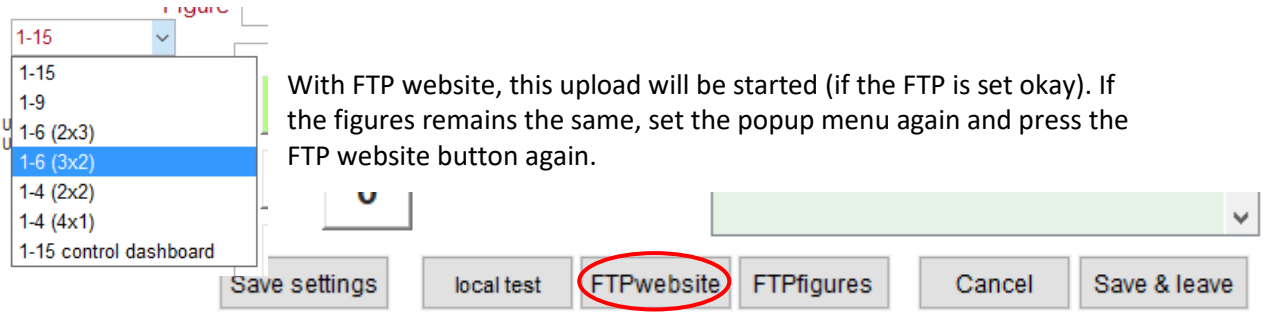
website as well as the title and other textboxes around this figure. You can fill it in with the text you want.

In 'Figure' a tag is written (by default) which is coupled to the figure you want to have at position 1. If you have run LIVE with this location, there are already some figures stored in EasyClusLIVE\figures. If a figure fits with your 'tag' it (find), this figure will be put above the tag and will be put to the internet.

The color of 1. (here yellow) becomes green if there is a figure found and if there is at least one textbox filled.



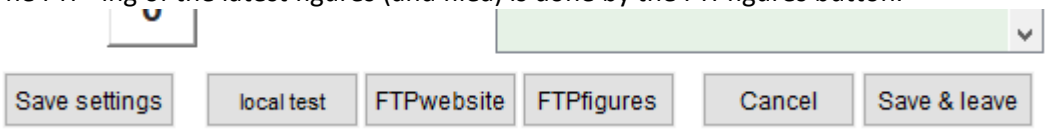
The popup-menu is used to change the number of figures, which should be uploaded to the website.



With FTP website, this upload will be started (if the FTP is set okay). If the figures remains the same, set the popup menu again and press the FTP website button again.

A local test can be done by using the 'local test' button. Unfortunelately, the text is not displayed in the local mode due to unknown reasons.

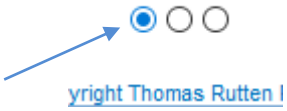
The FTP'-ing of the latest figures (and filea) is done by the FTPfigures button.



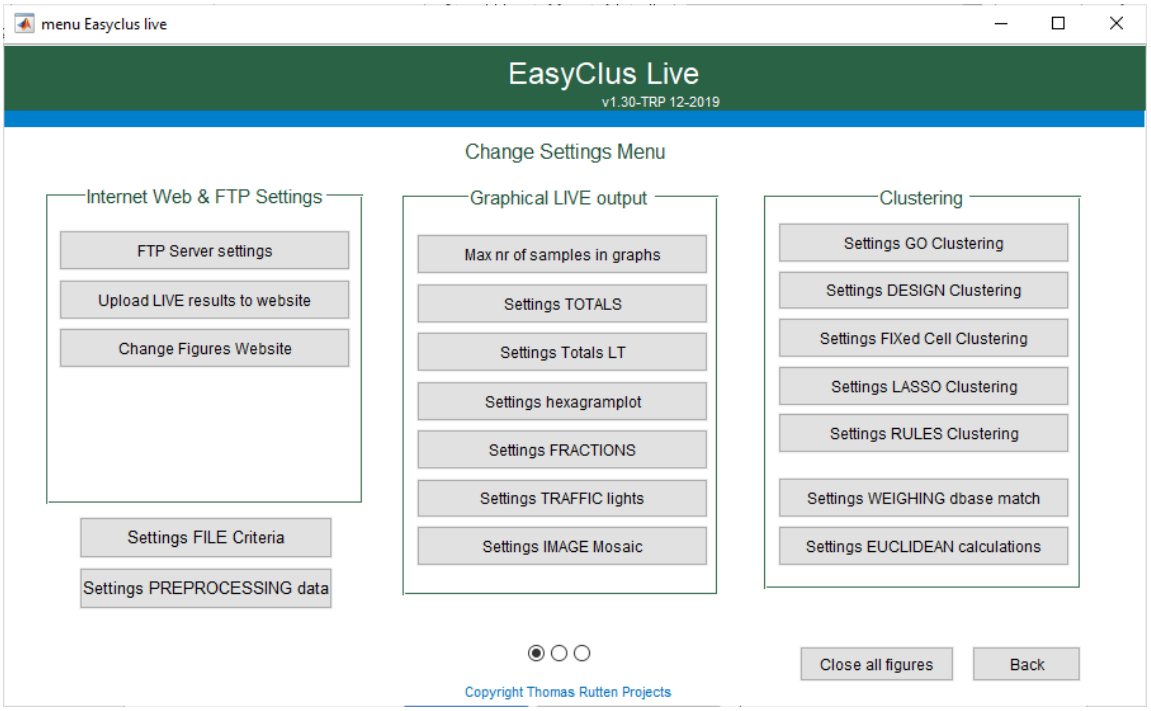
If the settings of the figures and text are okay, don't forget to save them!

4.4 Detailed Settings menu

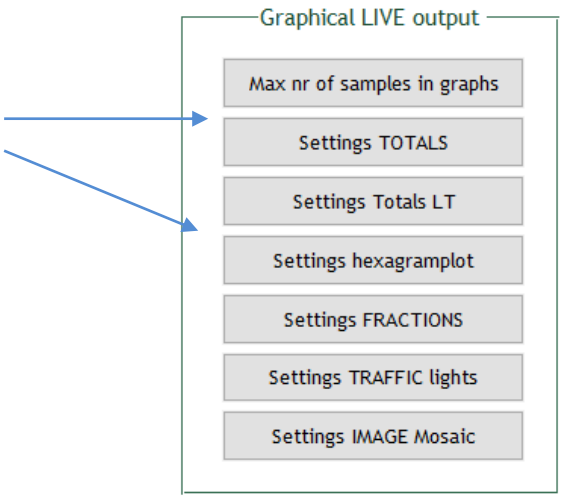
In the middle below: Tick the left bullet to get access to all kinds of LIVE settings.



Detailed Settings Menu



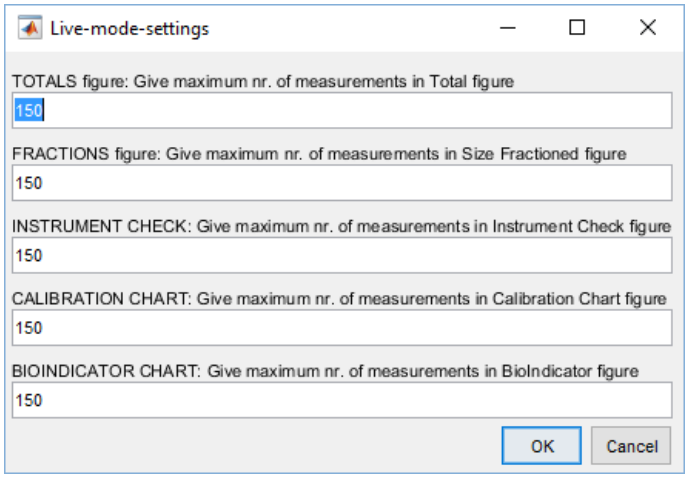
4.4.1 Settings Central part - Graphical LIVE output



4.4.1.1 Maximum number of samples in graph

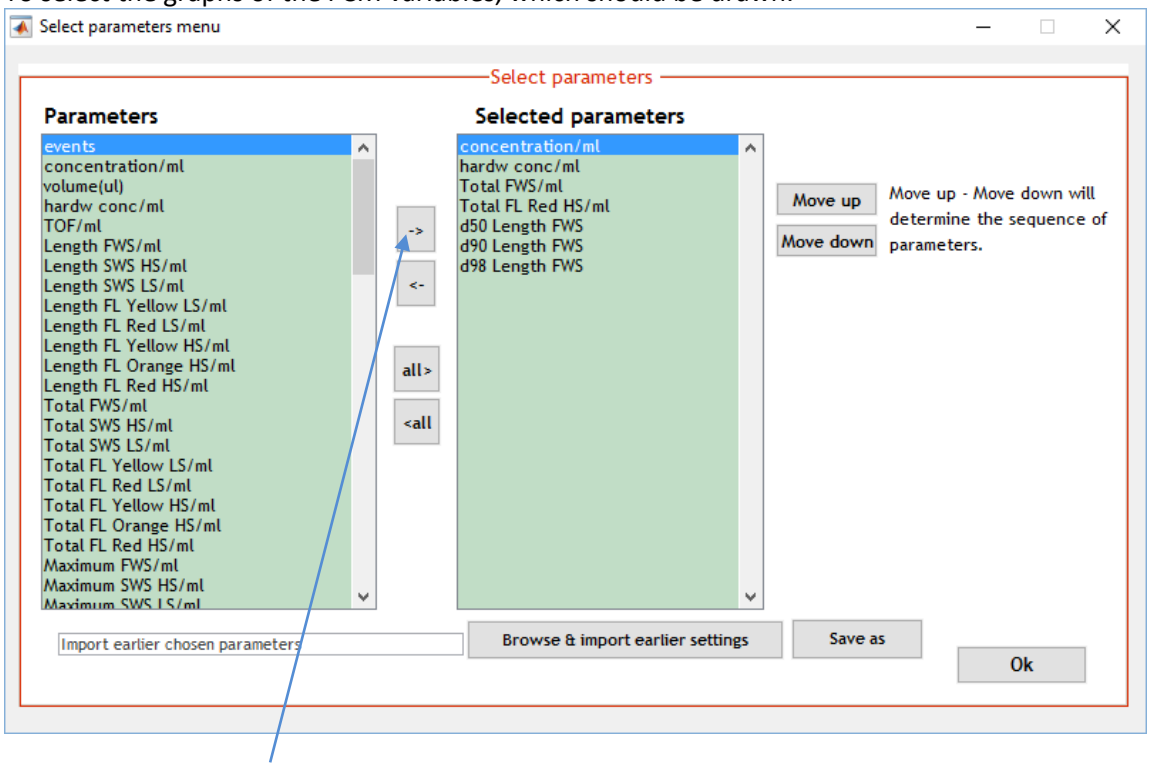
To change the number of samples/files, which will be drawn in the (LIVE) graphs. The latest given number of samples will be drawn for each operation (Total, Fractioning etc.). The saving of the files is also based on this number according to the rule that a new file is started after 4 times the given number of graphs (in this example 150 files are drawn, after 600 files the save file is starting with a new file). This saving procedure is done to prevent the building of huge datafiles.

One exception is made for the LT-total files (Long Term Totals). They will always be drawn and saved completely, without subdividing operations.



4.4.1.2 Settings Totals

To select the graphs of the FCM variables, which should be drawn.



Just choose one or more FCM variables at the left side, select by the arrow button, and the graphs that will be made are presented in the 'selected parameters' window.

4.4.1.3 Settings Totals LT

Identical to the previous menu, only the selection of parameters is limited to those which are saved as Long Term data only. This limitation of parameters is used to limit the size of saving.

4.4.1.4 Settings polygon plot

The polygonplot could also be called, penta, hepta or octagramplots. These plots gives you the values of the selected FCM variables at each edge relative to the values of the last 24 samples. The green surface is the 25 samples average of all FCM variables, the blue line the individual sample values towards the averages.

This settings button gives the opportunity to select the FCM variables, which should be used in the hexagram plots.

4.4.1.5 Settings Fractions

Not activated yet.

In the near future, you can choose if you want to use another length parameter, other fraction sizes or other defaults parameters. Every change here will activate a new file.

4.4.1.6 Settings traffic lights

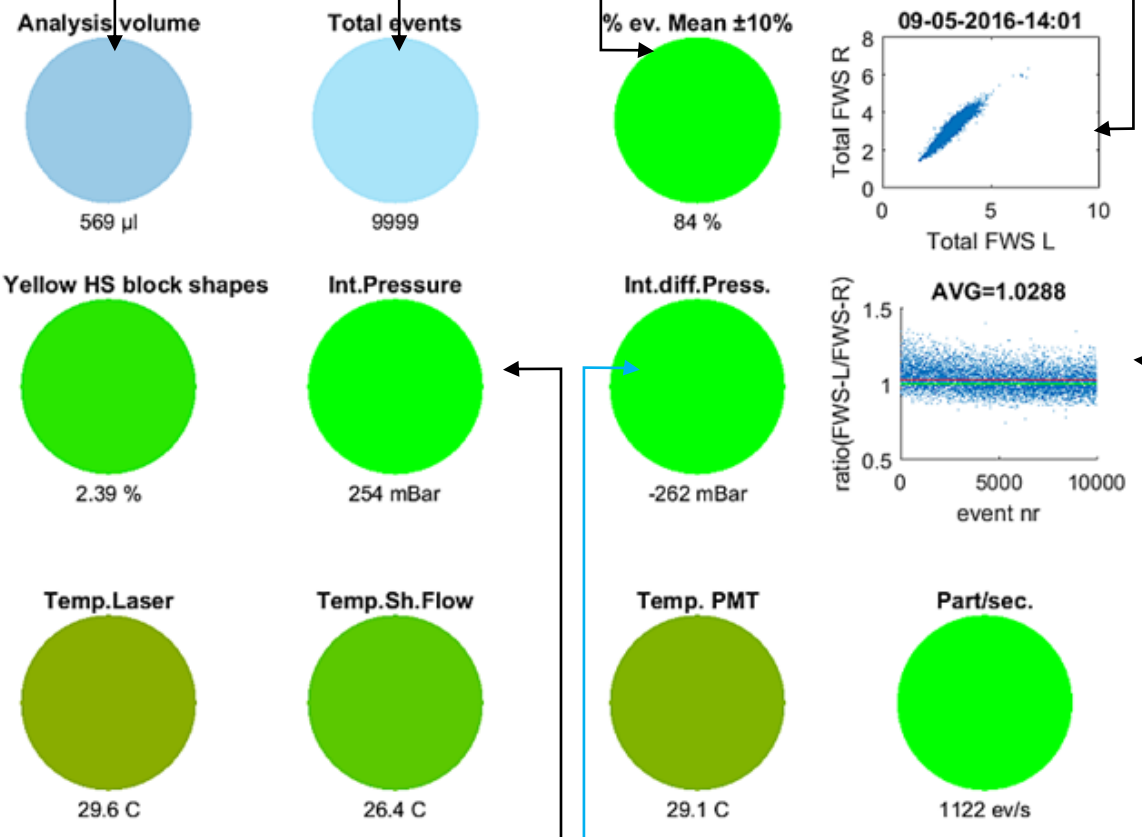
The traffic lights are used as a control dashboard of a good functioning instrument and sample analysis. The traffic lights are explained:

Analysis volume : All data in the FCM file is taken from the given volume of sample in microliters (10^{-6} l) and is lower than the FCM pumped volume (not given here).

% ev. Mean 10%: The percentage of particles between .9 and 1.1 of the ratio Total FWS R / Total FWS L. If it is 84%, it means that 84% of all particles is between .9*ratio and 1.1*ratio. If it is less than 30%, 70% of all particles is outside this bandwidth, which might be an indication of fluctuations of the core for example caused by air in the fluid path.

Total events: The number of particles (= events) that has been stored according to the trigger settings.

Plot 1: Scatter plot Total FWS L vs. Total FWS R. Each particle gives Forward scatter on the left and right curvature detector. The value of the total signal area left and right is shown in this scatter plot. It should be a linear relation. Title is the date and time of the measured sample.

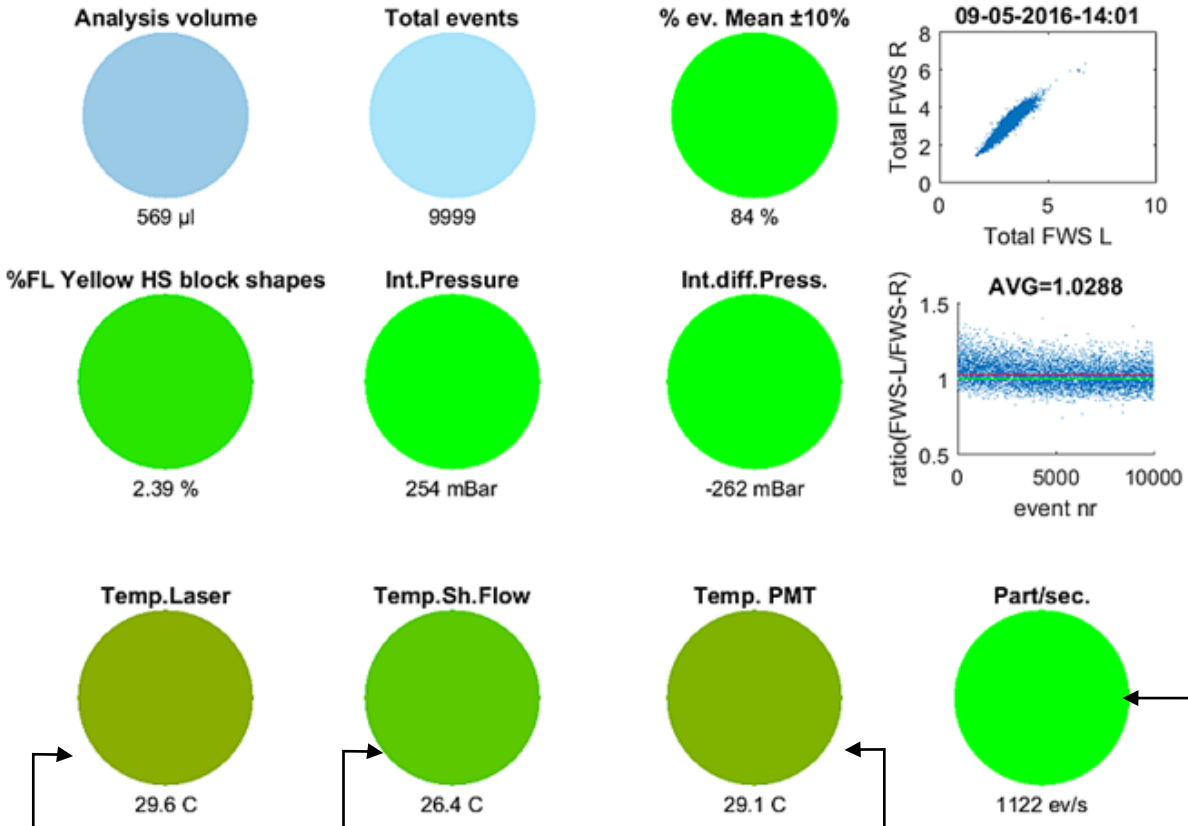


% Par block shapes: The highest found percentage of block shaped signals in the given parameter. Sometimes caused by very small signals just above threshold, sometimes by other not well known reasons.

Plot 2: Scatter plot event nr vs. ratio (FWS-L/FWS-R). The ration value of FWS-L/FWS-R. 1=perfect, but some difference is not a problem because it is very sensitive and some particles are irregular shaped. The AVG=1.0288 in the title is the average ration of all particles.

Int. Pressure: Internal Pressure of the fluidics in mBar. If it becomes higher than 900 mBar, it indicates that the internal pressure is increasing towards the outside pressure, e.g. by blockage of the fluidics path (filter, kuvette channel).

Int. diff. Pressure: Internal Differential Pressure of the fluidics inlet in mBar. If it becomes higher than -900 mBar, it indicates that the outside pressure is increasing towards the inside pressure, e.g. by blockage of the fluidics path (inlet tube).



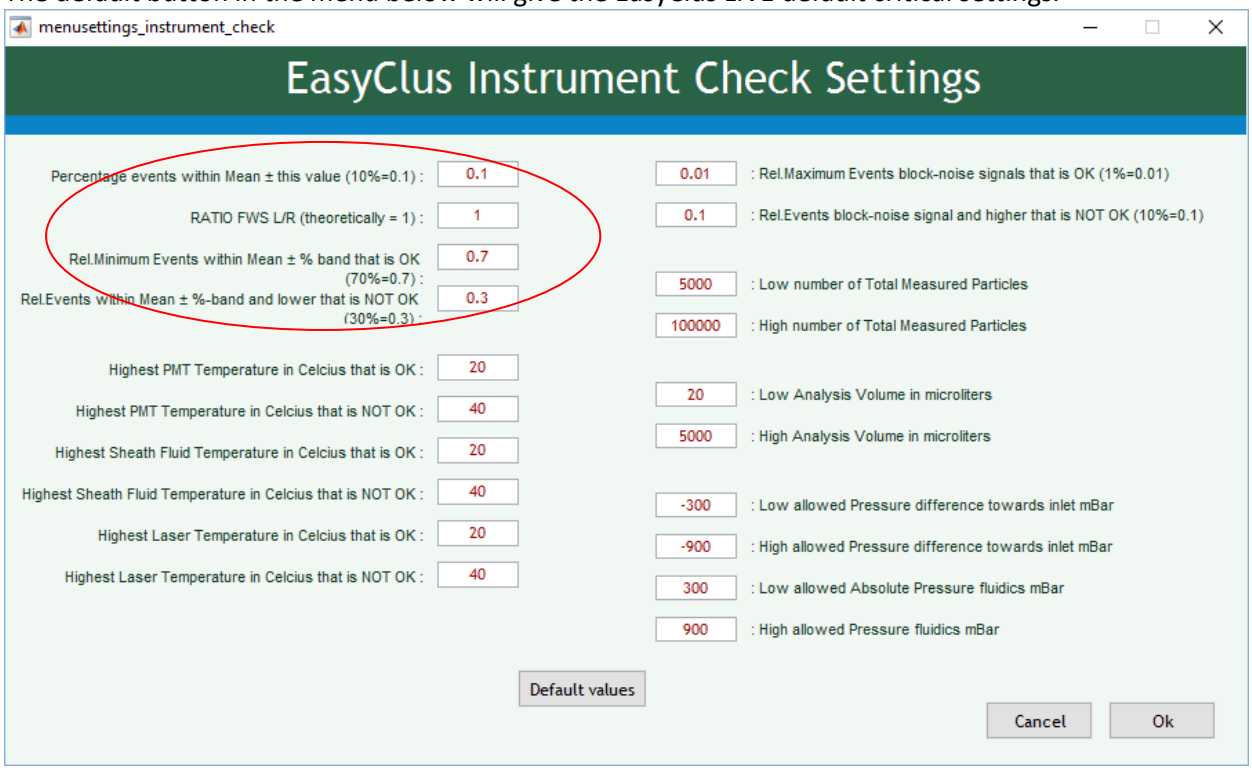
Temp. Laser: Temperature Laser is the temperature in Celsius in the laser to check if it is not too high. Temperatures higher than around 36-39 °C are high.

Temp. Sh. Flow: Temperature of the Sheath Flow (internal cleaned sample stream which takes the sample to the laser) in Celsius. Temperatures higher than around 35 °C are high.

Temp. PMT: Temperature inside the PMT detection house in Celsius. PMT's are temperature sensitive (more noise with increasing temperature). Temperatures should not fluctuate too much. Higher than around 35 °C is not recommended.

Part/sec.: Particles per second that crosses the laser beam. Each particle from approximately .2 µm and larger, which crosses the laser beam, will give signal in the detectors. If the particles are too close after each other, they will be interpreted as one signal. More than one particle in the laser beam at the same time is called coincidence and should be avoided. The chance of coincidence increases from particles rates of 5000/sec. and higher. Decrease the sample rate to decrease the particle rate.

To change instrumental and analysis critical settings. A settings menu will appear, which can be changed individually. These settings will used as critical settings. Close to critical gives an orange traffic light, critical or beyond a red light and a green light if it is ok. The long term instrumental and analysis light graphs are drawn to give an overview of long term functioning of the instrument and analysis.
The default button in the menu below will give the EasyClus LIVE default critical settings.



4.4.1.6.1 Curvature detection as alignment tool

The red circled area contains criteria for the curvature detection, which is used as an alignment check of your system. The curvature forward light scatter contains a left and right detection and is very sensitive for changes of the sample core position and air bubbles in the flow cell. A perfect alignment is a system with a core exactly in the middle of the flow cell and the curvature detection exactly positioned towards the middle of the core. This will give similar signal detection on the left and right FWS curvature detector, their ratio is 1 (second window in the encircled area). Most of the particles will give curvature ratios around this value, let's say between 90% and 110% of 1 (10% = 0.1 in first window in red). The instrument is alignment is well if 70% of all particles is detected within this ratio of 0.9 and 1 (1 ±10%). It is not okay if this ratio is lower than 30% of all particles (third and fourth window in red).
Air bubbles in the flow cell will immediately push the sample core to the left or the right and thus will affect the ratio to critical values. Pinch valve hitting and flushing can be a method to get rid of air in the system.

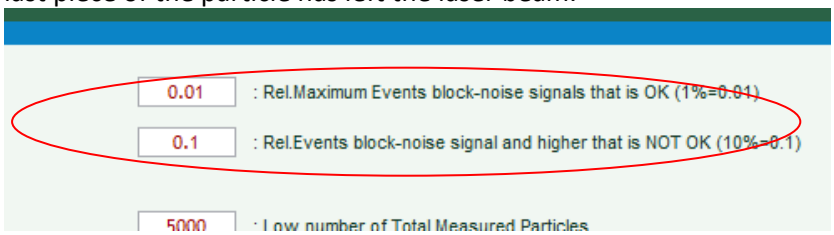
4.4.1.6.2 Temperatures

There are temperature sensors in the CytoSense, in the PMT detector house, in the sheath fluid and the laser temperature. The highest acceptable and unacceptable temperature values are given.

4.4.1.6.3 Block noise signals

Block noise signals are block formed signals, almost rectangle signals, that can occur due to unknown reasons (sometimes bad triggering settings, electronic base line correction). In principal, block formed signals are not okay. Each particle which passes a laser beam, should

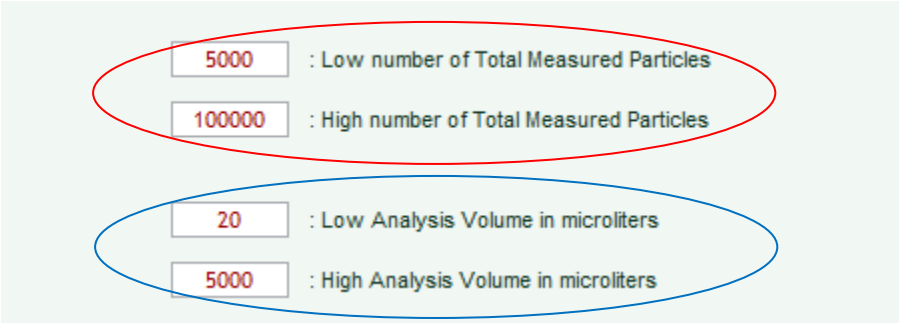
give an increasing value until it is completely in the laser beam and decreases to zero after the last piece of the particle has left the laser beam.



The number of particles which is blocked formed should not be higher than 1% of all particles (lower is better of course) . Higher than 10% is really too much. Values 1% is the first value (0.01), value 10% is the second one (0.1).

4.4.1.6.4 Measured particles and Analysis volume

The total number of particles (in red circle below) and the effective analysis volume (in the blue circle). The number of particles measured is dependent of what's in the sample of course, but also on the applied instrument triggering settings and applied sample rate. A lower trigger is more sensitive to smaller particles and will lead to more measured particles. The use of (red) fluorescence triggering will only store fluorescent particles. Criteria can be filled in here to define what's suitable for you.



The analysis volume is the effective analysis volume. This is different from the pumped analysis volume, because the effective volume is lower than the pumped volume. Why? The instrument has dead time, time needed for calculations or other processing and during this dead time, the instrument is blind to incoming particles. This dead time is also dependent of the memory block size.

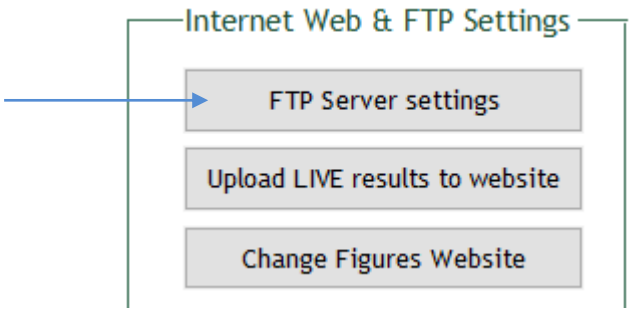
Be aware that you have used enough sample as a representative for the whole sample. Too low applied volumes (<50 microliter) are not recommended.

4.4.1.6.5 Pressure

The pressure in the fluidics is measured by sensors. One at the inlet suction part (differential) , the other inside the fluidic system (absolute pressure). High pressure occurs if there is a blockage in the fluidics part, e.g. by blocked filters (full of particles) or by a big particle inside the flow cell. The system should be stopped immediately at a high pressure, because it can lead to leakage of fluids. Low pressure can also be a problem, because it can point to 'open to air fluidics', fluidics that are in contact with open air caused by leakage too. Therefore the pressures are monitored too, long term as well to have an overview of the historical pressure behavior. Critical values can be changed by the operator in the menu.

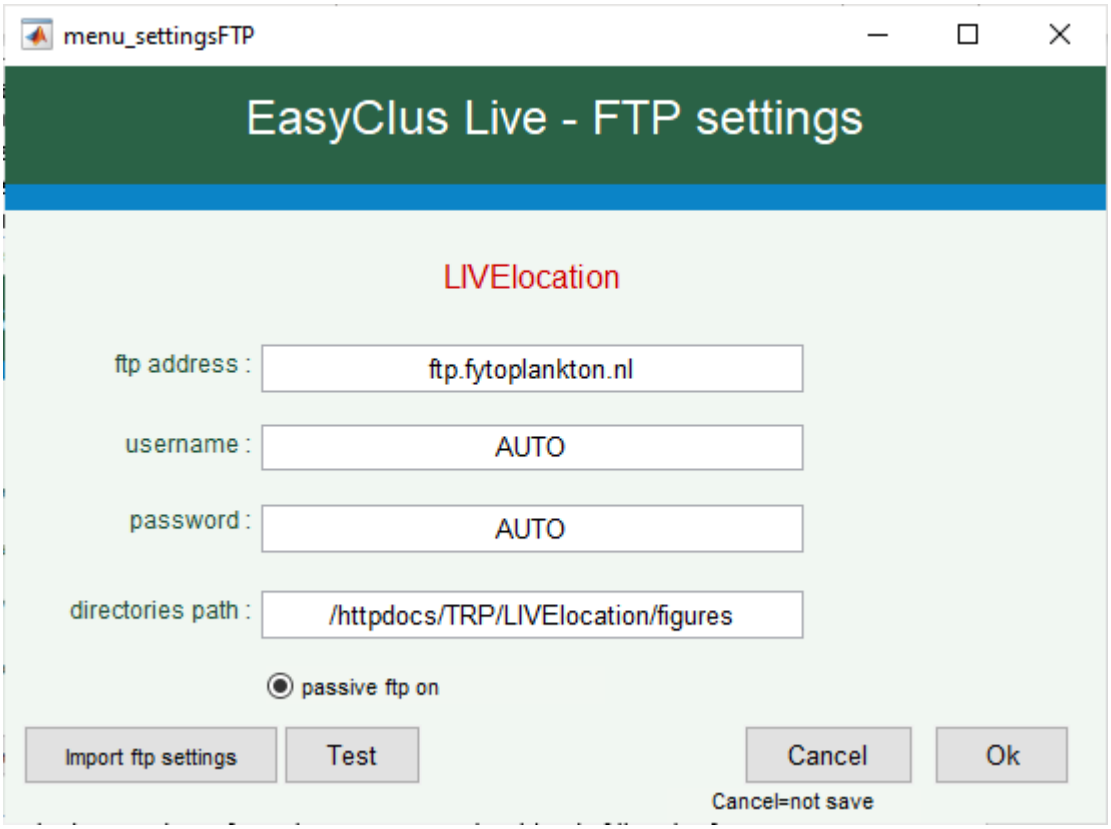
4.4.2 Settings LEFT part – Internet Web & FTP Settings

These settings have to do with everything related to the sending of files figures and data to a server by ftp'ing or to an internet website.



4.4.2.1 FTP Server settings

Graphs, data and text can be transferred to a sever after each analysis (i.e. within 3 minutes after your sample analysis). The protocol used for sending data is the FTP protocol. The ftp server address, username, password and 'put in' directory of the server, should be given on beforehand by using this menu.



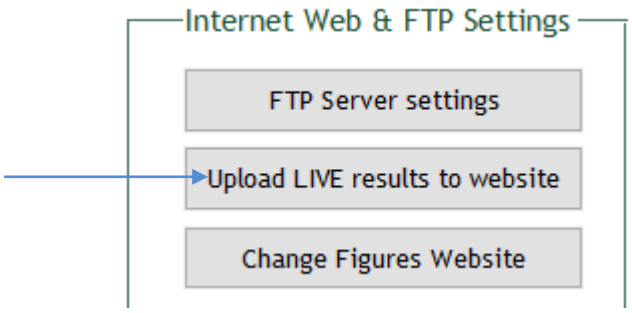
Customers using TRP's fytoplankton website for online reporting, should only fill in the first window (ftp.fytoplankton.nl), the other windows are AUTOfilled. The directory path is made customer & location/project name dependent, because these are used in the directory path of the server. Be aware that the location name is server directory specific, meaning that changing it will put the files to another directory (fytoplankton.nl users).
In case of fytoplankton.nl users, your results are transferred to:
httpdocs\yourEasyClusname\livename\ (yourEasyClusname=AUTO done, Livename=name given by you in LIVE Run).

The website (s)html files are put on the fytoplankton server by TRP including your specific location directory (ies). The shtml-files should be in the /livename directory; all data & figures are sent to the livename/figures directory.
Images are transferred automatically to httpdocs\gallery\albums\yourEasyClusname livename.

For all other options: ftp.domain.xx
The server paths are given by yourself as well as the username and password. In case data & figures are sent to path1\path2\. The images are sent to path1\path2\images\
The test-button is used to check the connection and the transfer process. A file will be transferred to the given directory. Be aware that the server directory already exist. EasyClus will not activate this directory (only images directories are made automatically).

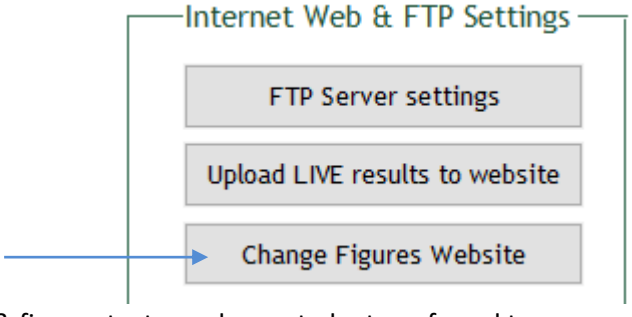
Results to internet (FTP) problems?
It is very likely that the firewall is blocking Matlab. FTP cannot take place with this firewall. To 'unlock' the firewall or to give Matlab permission to ftp do go to the Windows configuration panel - security settings – Windows Firewall – Give permission to program to pass windows firewall – programs list to give permission - put checkbox on of running Matlab.

4.4.2.2 Upload LIVE results to website



Uploading to the website takes place automatically after data processing (if activated by the bullet box 'Results to internet' in the main menu of EasyClus LIVE. It might however be useful to upload results again, for instance when the internet connection has been bad. This button enables to re-upload the latest results on your computer, which are stored in the EasyClus LIVE directory dealing with the location showed in the main menu of EasyClus LIVE.

4.4.2.3 Changes Figures Website

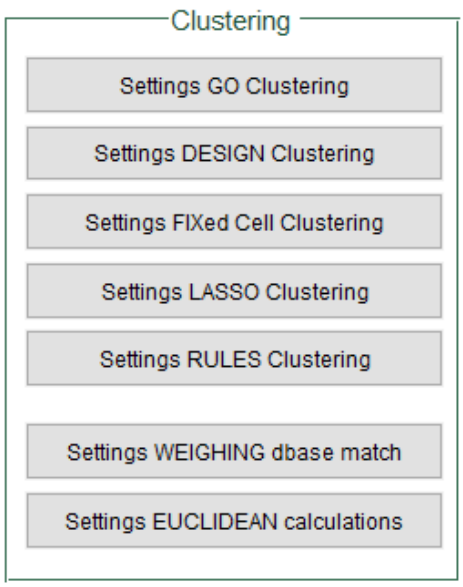


Default figures & figures text are chosen to be transferred to a server. Changes can be done here e.g. other figures which should be put on the website or other text. This is explained above in 4.3.12.

Changes of the figures of the dashboard control is not recommended.

4.4.3 Settings RIGHT part – Clustering settings (and Euclidean settings)

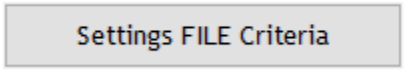
Applied cluster settings in the LIVE method are changed by the right settings 'Clustering menu'.



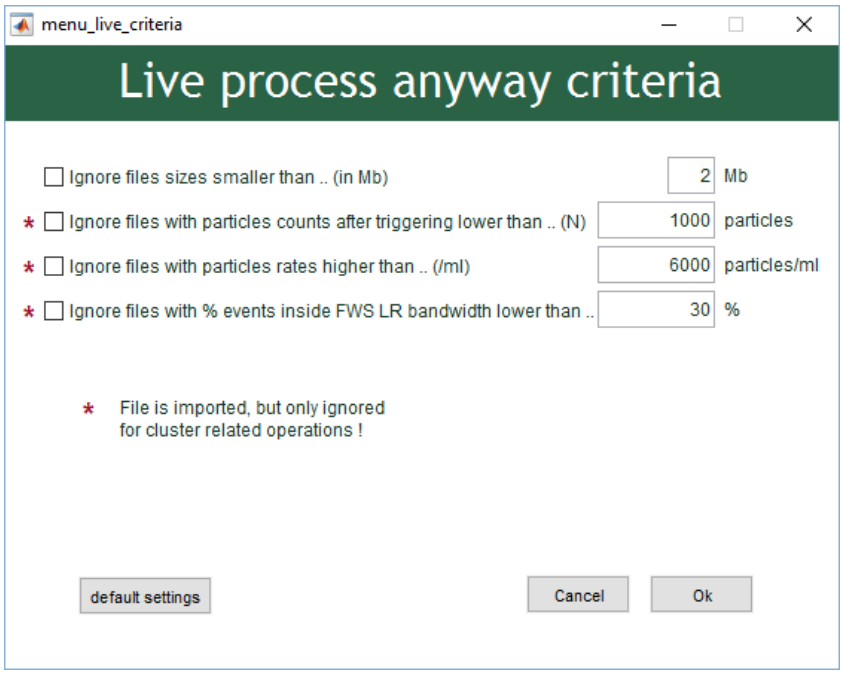
Each cluster method is identical to the usual EasyClus clustering method and not explained in detail here. Their settings will be specifically changed only for the actual location.
Only the LASSO selectionssets/lasso's method is different compared to the usual LASSO method. In LIVE, al used selection sets or lasso's, should be put in x:\EasyClusLIVE\lasso and do not have to be selected separately in the lasso settings menu.
All other settings are identical, but location specific, to the usual EasyClus settings and don't need to be explained again here.

4.4.4 Settings LEFT part –FILE criteria

Sometimes you 're not interested to further process files because they have low counts or too high particle rates (coincidence problems) or maybe wrong data by an air bubble. This less reliable data (to your opinion) can disturb the long term data monitoring. With the option

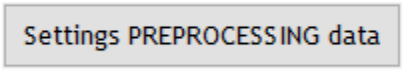


A menu will appear to activate certain file ignore operations as well as their condition.



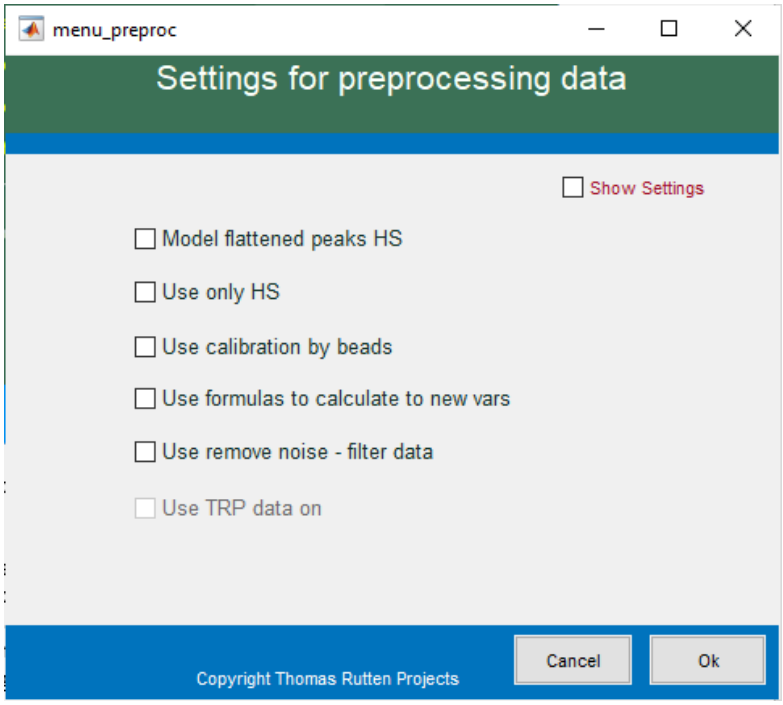
4.4.5 Settings LEFT part – Preprocessing

The preprocessing of raw FCM data is applied before further data processing will be acquired.



Preprocessing can be very useful to fine tune your data, get rid of redundant data and to calibrate your data before further operations.
There are four options for preprocessing the data.

- Profiling flattened saturated HS signals on basis of LS signals
- Use of only HS signals and ignore LS values
- Correct FCM data towards assigned values of reference beads and the closest reference beads results
- Calculate new variables (e.g. chlorophyll) by using own formulas for each particle



Each preprocessing operation has its specific settings. A lot is explained in the EasyClus manual. In short:

4.4.5.1 Model flattened peaks HS

Use settings to give the maximum height of your HS detectors (default=5000 mV). The software uses these maximum values to recognize the maximum HS signal. The HS-LS twin detector combination uses the LS signal to model the flattened HS peak. The HS is modeled/estimated on basis of an without LS algorithm.

4.4.5.2 Use only HS

Information in LS is usually redundant information especially when the modeling flattened peaks option is also used. In that case, the LS information is used in the HS signal too. The question is what to do with the LS information? Here you have the option not to use it anymore, although for modeling it is used of course.

4.4.5.3 Use calibration by beads

The beads measurements in between samples are used as a reference for recalibrating the raw data. This means that if the average beads cluster has decreased in a certain variable to 90%, all data of that variable will be corrected with 100/90.

Using reference beads for calibrating (raw)data
- EasyClus LIVE: first time start at a location (there is no reference value). Look in EasyClusLIVE/defaults/ for a settings_calibdatavsbeads.mat file (but there isn't at first time use), after that a second chance look in c:\cytoxx\defaults\ for the same file (but there isn't at first time use). Last option is that the (unknown) reference values will be processed during the building of the Shewart chart of beads (at that location). Every run the average values of all measured beads samples up to 7 times will be used. After 7 times, these averages are expected

> ***Background using beads as a reference:***
> In general: Beads clusters form stable reference values which are put in a so called Shewart calibration chart to monitor the performance of the variance of the instrument. Beads results will fluctuate around a certain average value in each FCM parameter. If beads are below this average, all results measured in this period will be increased to 100%, if they are higher than the average, all results will be decreased to 100%. These averaged values are defined by the user OR defined by the software. In the software case, no earlier settings are available and the most recent beads results will be used. The average values are the known values until so far. If seven beads samples are measured the software will automatically generate the average values for your system and save the settings results. Other locations will use these values too, to keep the mutual comparison of results easier. It might even possible to use the settings of another CytoSense to keep the instrument around the same absolute levels.

to be the settings_datavsbeads.values (as well as settings_datavsbeads.colheaders & settings_datavsbeads.shewartpathfilename). This is saved as filetag_settings_calibdatavsbeads.mat. If there is no settings_calibdatavsbeads.mat in EasyClusLIVE/defaults/ this file is expected as a reference for other locations and therefore also saved as settings_calibdatavsbeads.mat.
- EasyClus LIVE: 2nd, 3rd time start at a location 2 or 3 (There is a reference now). The reference in EasyClusLIVE/defaults/settings_calibdatavsbeads.mat is used. Only the settings_datavsbeads.shewartpathfname is changed into this new location2 (or 3) filename.

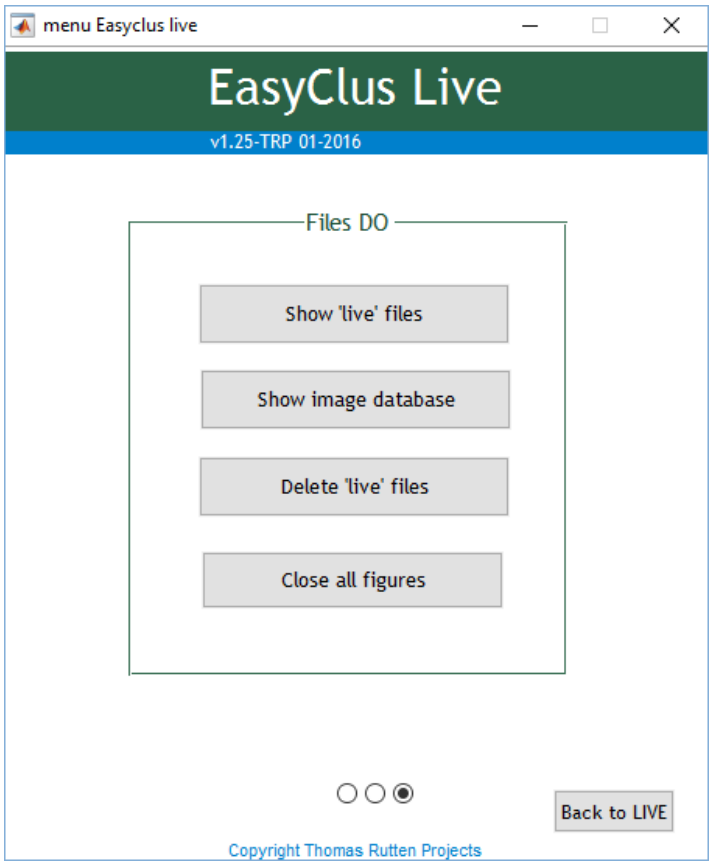
Shewart beads charts are location specific saved, because a user might want to use other settings beads (Be aware that the right reference values are used then). If location 2 is using the same beads and settings, the right file EasyClusLIVE/defaults/filetag_settings_calibdatavsbeads.mat is made and ready for further use. If there are other settings_calibdatavsbeads.mat e.g settings_calibdatavsbeads2.mat, the user is offered automatically a popup menu and asked to choose one of the options during the starting of the new location settings.
- EasyClus LIVE: If settings for location_new are copied from an existing location, the stored reference values are copied, the new right shewartbeadspathfname is changed here.

4.4.5.4 Use formulas to calculate …

In the settings you are able to define formulas to calculate specific variables e.g. ug chlorophyll. These formulas are added to the raw data and stored automatically.

5. EasyClus LIVE Files DO menu

The right bullet leads to the files DO menu to view, delete, change specific files.



The features are not specifically explained, because they explain themselves and tells you what to do during handling.

Show ‘live’ files	: To visualize one of the LIVE datafiles
Show Image database	: To visualize or change information and images in the Image database.
Delete ‘live’ files	: Files or samples are deleted in the LIVE data.
Close all figures	: to close all open figures

6. Start another live project or location?

You can start the LIVE procedure for another new location too, but you can't run at two locations simultaneously (there is a trick you can, ask TRP).

To start for a new location/project , 'Stop' running LIVE processing.

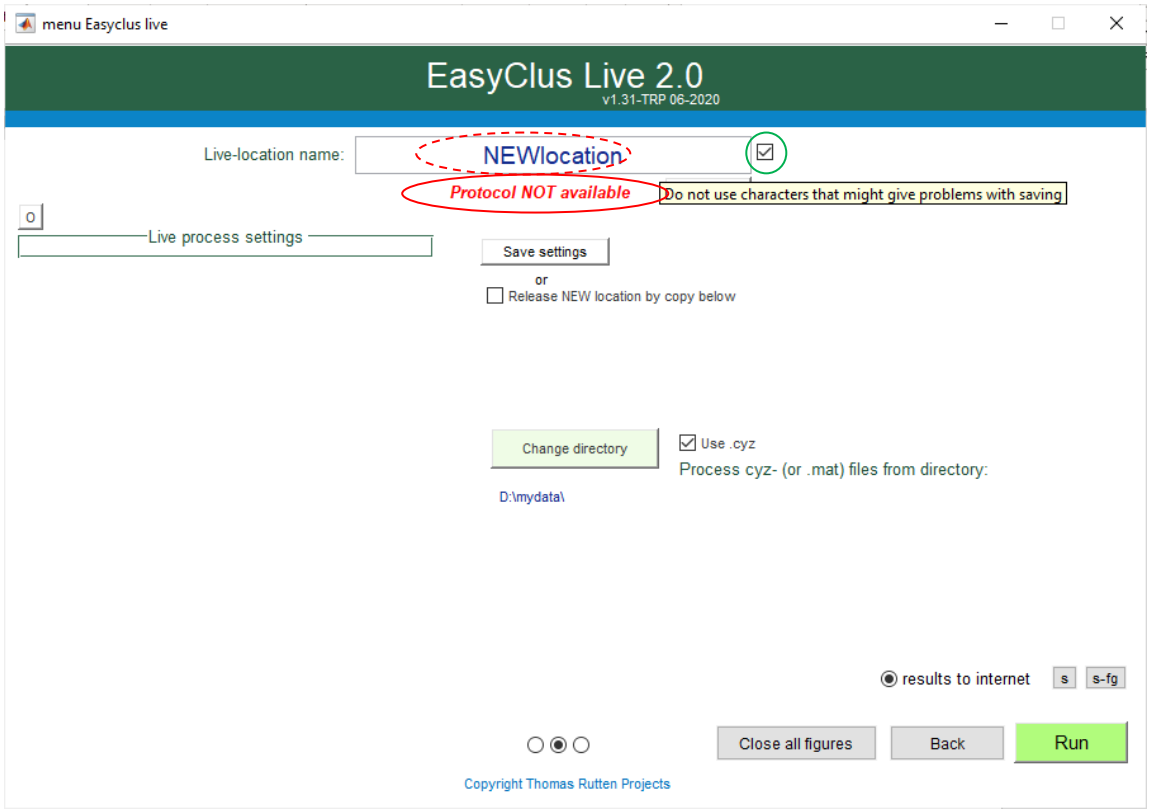
You have two options:

1) Start another new location with default settings
   Just change the live-location name (and if necessary the directory of FCM-files)

2) Start another location with non-default or default settings
   Change the live-location name (and if necessary the directory of FCM-files)
   Tick 'Release NEW location' checkbox and the popup menu appears.
   Select a previous location name in the popup menu to copy the settings
   Activate the new location with the popup menu settings by the 'OK & Release' button

6.1 Start another new location with default settings

- Tick the checkbox (green encircled) and type your desired new location name. Prevent to use characters which might give problems if used in filenames for saving.
- Change the directory, if necessary, where the LIVE cyz-files will be stored.



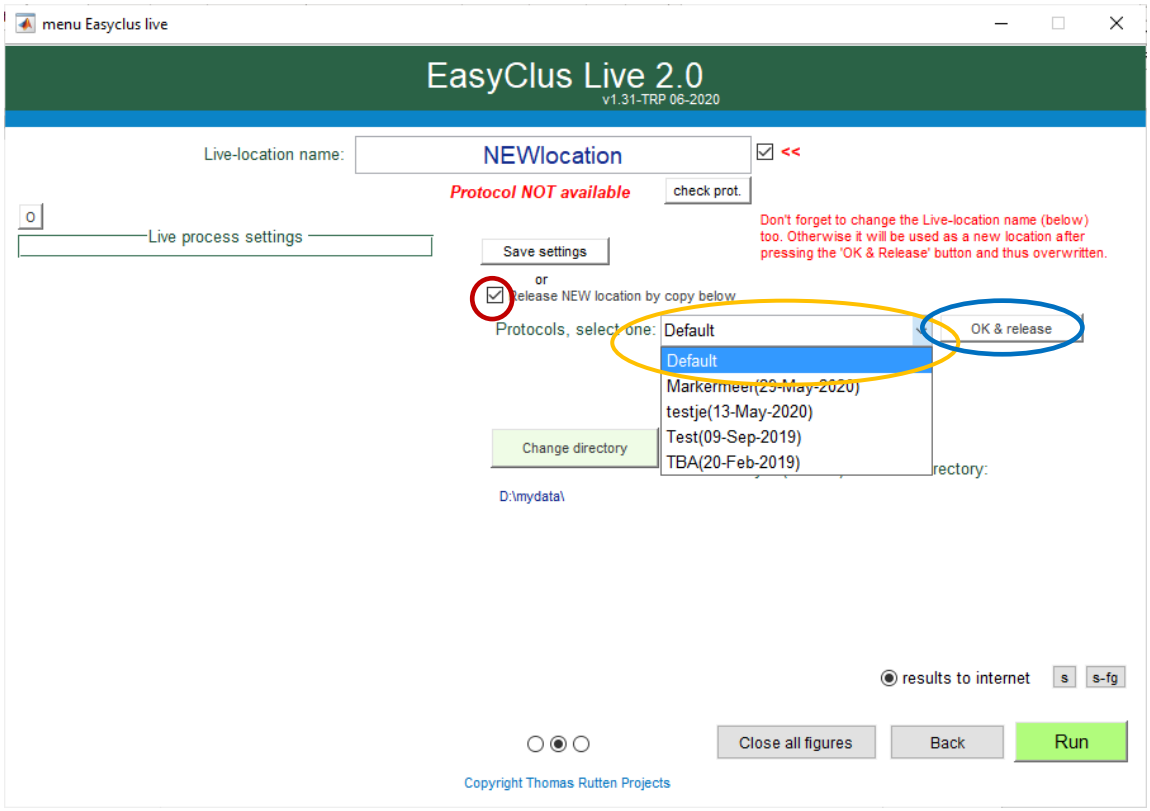
- Press the 'Run' button to launch the new location.

Only in a few cases, a question will be asked once, such as 'results to internet'(The FTP settings should be filled in).
Be aware that users need to set up the directories of the server themselves if they use ftp and this is not automatically done by EasyClus LIVE. E.g. in case of fytoplankton.nl users,

TRP needs to activate the new location on this server and put the website files to this new location.

6.2 Start another location with non-default or default settings

- Tick the checkbox (green encircled) and type your desired new location name. Prevent to use characters which might give problems if used in filenames for saving.
- Change the directory, if necessary, where the LIVE cyz-files will be stored.

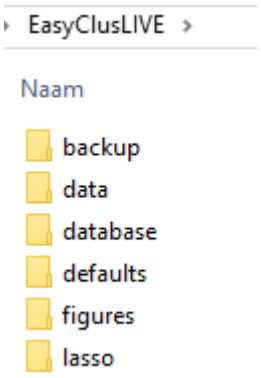


- Tick 'Release NEW location' checkbox (red encircled) and the popup menu appears.
- Select a previous location name in the popup menu (orange encircled) to copy the settings
- Activate the new location with the popup menu settings by the 'OK & Release' (blue encircled) .
- Press the 'Run' button to launch the new location.

This option copies all the settings used in the location popup menu, except the filled database (empty database is started). This option is introduced if you have user specific settings other than the default settings. All settings of a new location are saved using the new location name.

7. Where are the results?

All LIVE results, data and settings are put in a EasyClusLIVE directory of your computer.



All generated data in directory \EasyClusLIVE\data
All generated databases in directory \EasyClusLIVE\database
Location specific settings are in directory \EasyClusLIVE \defaults
All generated figures in directory \EasyClusLIVE\figures
All selectionsets or lasso's, which should be used in the clustermethod LASSO should be put in directory \EasyClusLIVE\lasso
Directory \EasyClusLIVE\backup is not automatically filled, but cab be used by the operator

8. Learned from other LIVE measurements

1. Settings PMT voltages HS-LS not ok (LS should be 100x lower max than HS).
   Reset the PMT voltages in such a way that 5000 mV in HS is about 50 mV in LS.

2. Preconcentration not performed, but should be performed.
   Set on in USB settings.

3. Trigger too low, many noise, less real particles of interest.
   Reset your trigger. E.g. set a higher mV level. Even better is to find out when you're measuring in the noise by decreasing the mV until the moment that the concentration increases extremely. Set your trigger level 10 mV above this level.
   Use smart triggering as well e.g. the combination of SWS triggering (first trigger) and FL Red HS triggering (only red fluorescence).

4. Flush time in between analyses too short (flush time 50 > 80).
   A flush time of 50 seconds might be rather short leading to cross contamination. Just flush 30 seconds longer (=80s to be set in CytoUSB)

5. Very high particle rate (part./s) and subsequently high chance on coincidence.
   Coincidence means two particles in the field of view of your detection and in the laser beam, giving a one particle signal and count. Decrease the sample speed or if not possible dilute the sample.

6. Memory Block size quite low, and as a consequence low effective used analysis volume due to dead time (AUTO set now?).
   Probably AUTO set now, but check the effective analysis volume towards the pumped volume. If they are very different, the block size memory should be increased.

7. Alignment problems. Ratio FWS -L-R curvature detection far from value 1 and/or fluctuates.
   The curvature FWS L-R ratio should be around 1, meaning that the signal on the left and the right is (almost) similar. If that is not the case, check for air bubbles, check with a lower sample speed, check the FWS L-R alignment, or at the end check the overall flow cell towards laser alignment. Is your core really positioned in the center? If not, realign.

8. Most particles of the Imaging in Flow images are small particles
   Statistically we have more small than large particles (more insects than elephants), so if we randomly make images, we have a lot of small towards large particles. We would like to change the triggering, but we have to keep the triggering of imaging in flow similar to the usual triggering for mutual comparison. Use a preset-preselection of only bigger particles for the triggering of the imaging in flow or use smart grid triggering (only a maximum amount per grid).

9. Maintenance Instrument
   A CytoSense is a unique high qualified instrument compared to other 'online' instruments. Why? This instrument can run for months without maintenance, while a lot of instruments can't, because their inner flow cell walls get dirty/foul. This fouling process does take place in a CytoSense only very slowly, because the walls are not in direct contact with the sample. Despite this, it is recommended to maintain the instrument at least once a year or if used 24 hrs a day, each half year.

10. Results to internet (FTP) problems?

It is very likely that the firewall is blocking Matlab. FTP cannot take place with this firewall. To 'unlock' the firewall or to give Matlab permission to ftp do go to the Windows configuration panel - security settings – Windows Firewall – Give permission to program to pass windows firewall – programs list to give permission - put checkbox on of running Matlab.