

Date : 4 November 2025
By : Thomas Rutten
Ref.nr : TRP 2025.030

Determining cyanos from flow cytometry data using EasyClus

Introduction

There are various methods in EasyClus to filter blue-green algae or cyanobacteria from the data and to determine their numbers, biomass or other characteristics.

1. Cyano's module via ratio
2. Database - via lasso-methode
3. Database – via rules (but not only cyano's but also other species in database)
4. Database – via hybrid (but not only cyanos but also other species in database)
5. FIX-zone – draw ratio lines or lassos yourself
6. Clustering DESIGN via ratio's in clustering process

1. Cyano's module via ratio

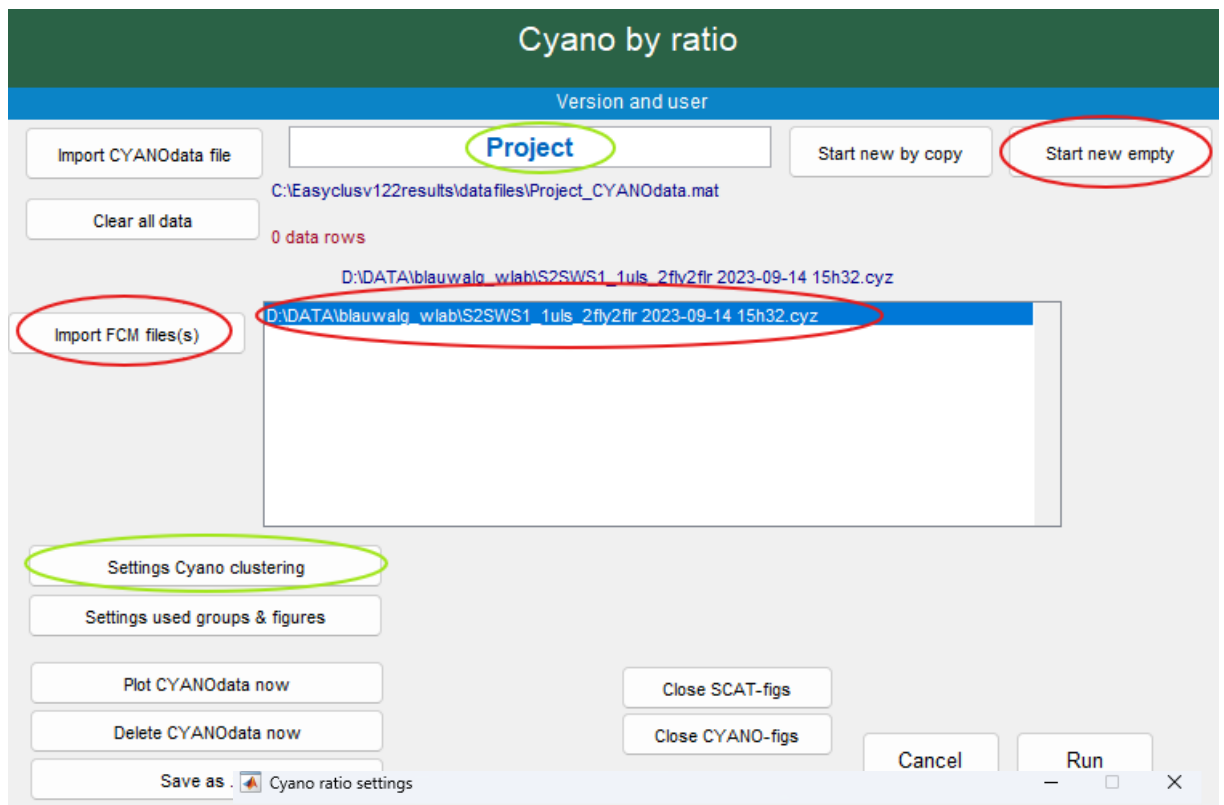
The simplest method works via the Cyano module. The Total FL Orange/ Total FL Red ratio is used to recognize the cyanos and possibly supplemented with a second criterion. The phycocyanin, which is characteristic of cyanos, gives orange fluorescence, which other algae do not or different in intensity. Cryptophytes also give orange and especially yellow fluorescence due to their phycoerytrin pigment, but at a different level than the phycocyanin. The blue laser is actually not optimal for phycocyanin (and phycoeritrin) excitation, but it is sufficient to distinguish it from other types.



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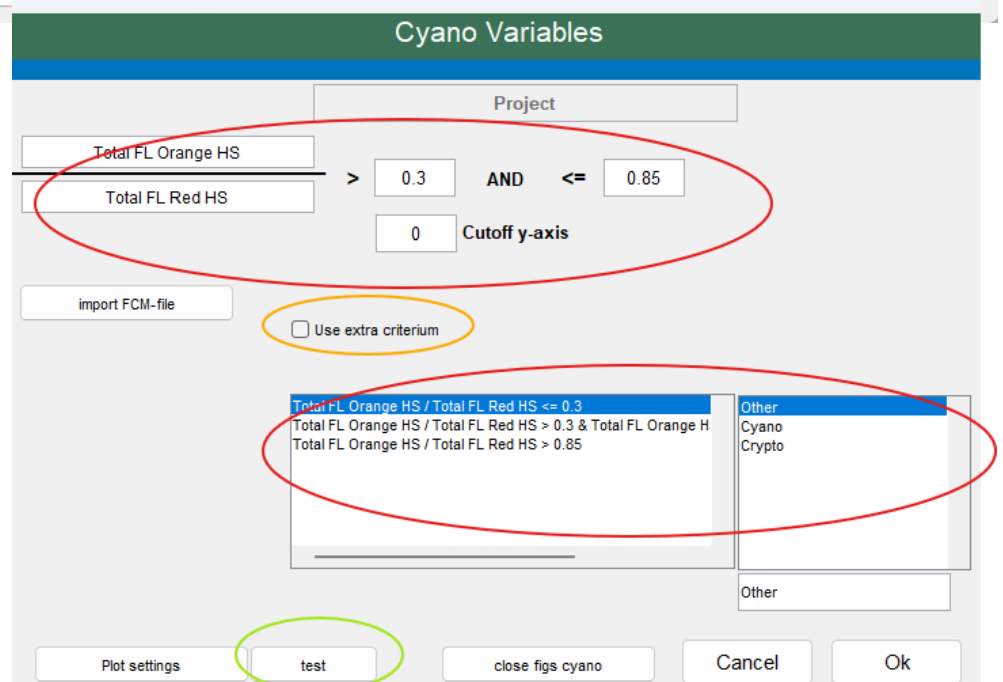
A menu will appear where you can define a project name (e.g. a location). This project name is used to put multiple files together, for example.

We choose a file to set and test the definition of the settings. This only needs to be done once as long as the instrument detectors and levels are not adjusted. Later, more files can be added to the menu and executed in one go, with all results being saved in the 'project' name.



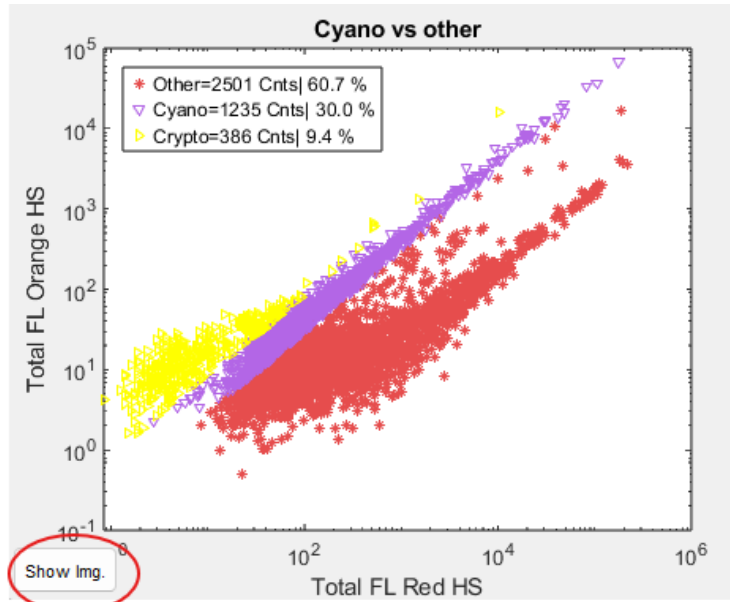
We set up via 'Settings Cyano clustering', the following menu appears :

The ratio of lower and upper ground can be adjusted. Check with the 'Test' button whether the Cyano cluster is correctly defined.

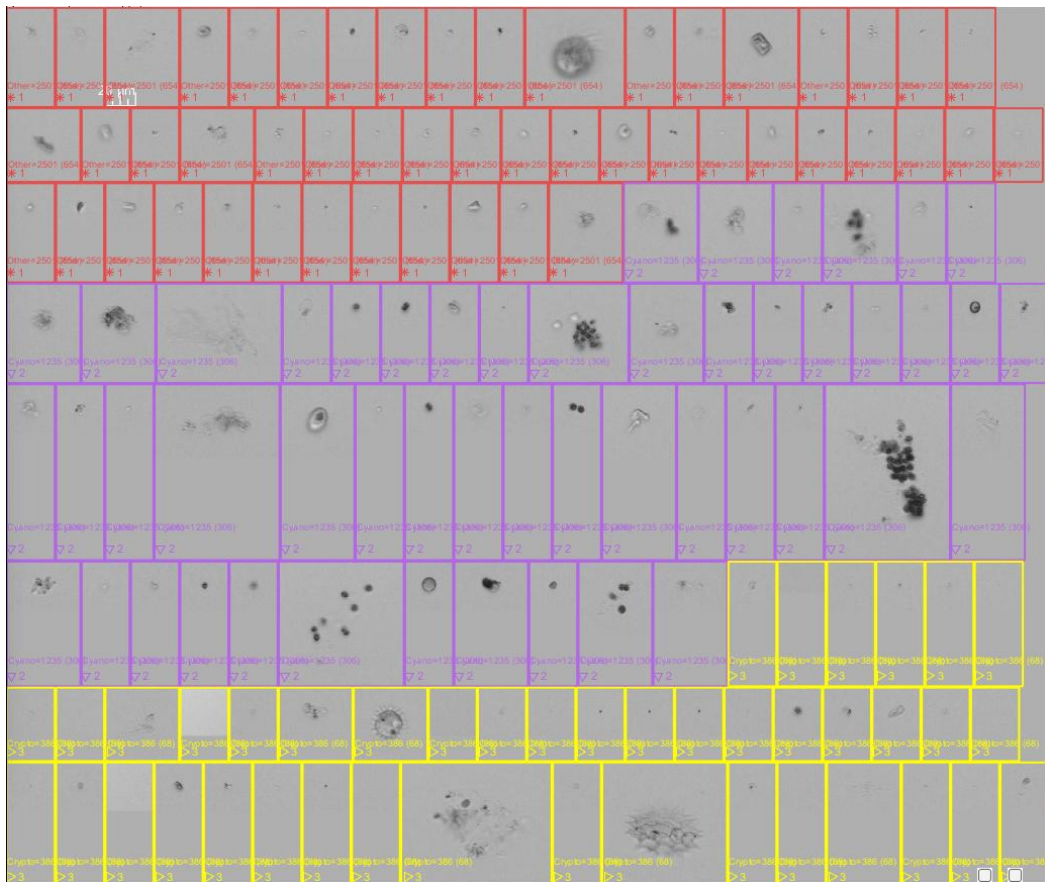


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Consider using photos of particles within the clusters to determine the accuracy of the classification.

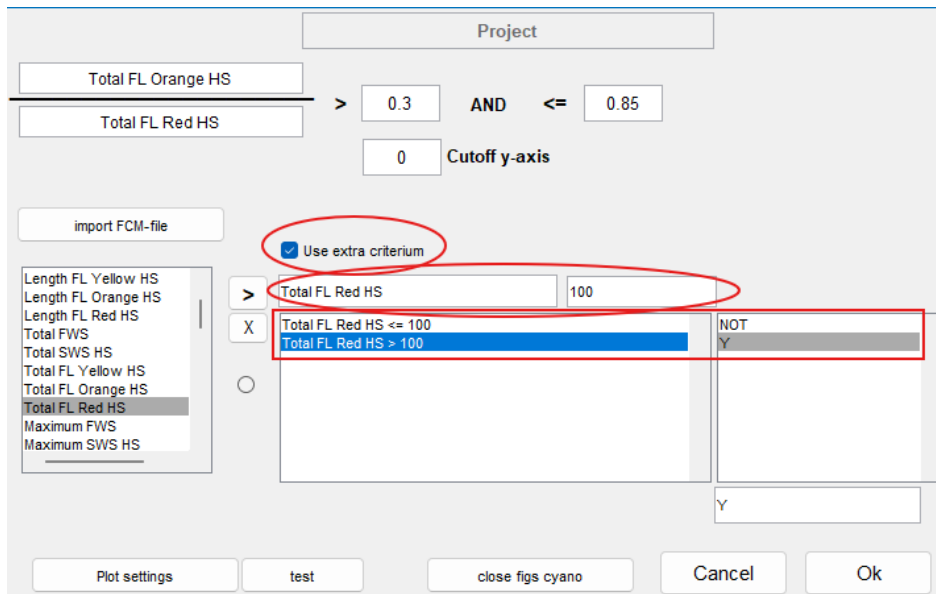


The (purple) cyano cluster contains cyano particles, but also aggregate-silt cloud particles.

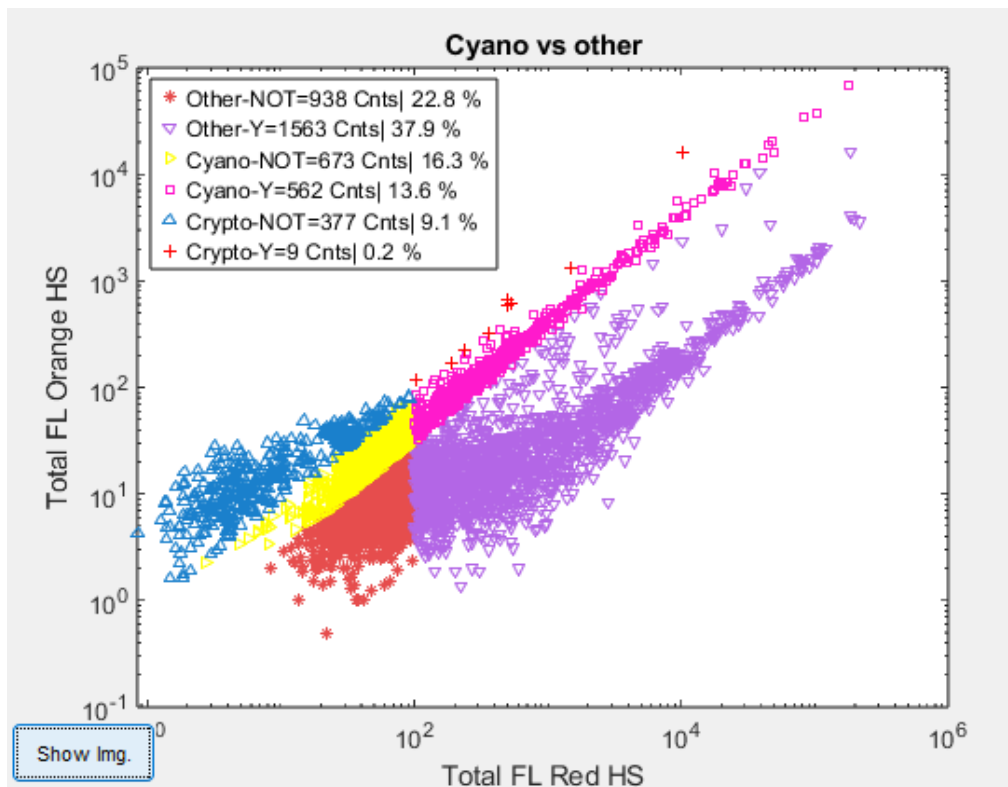


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If necessary, a second criterion may be set and used.

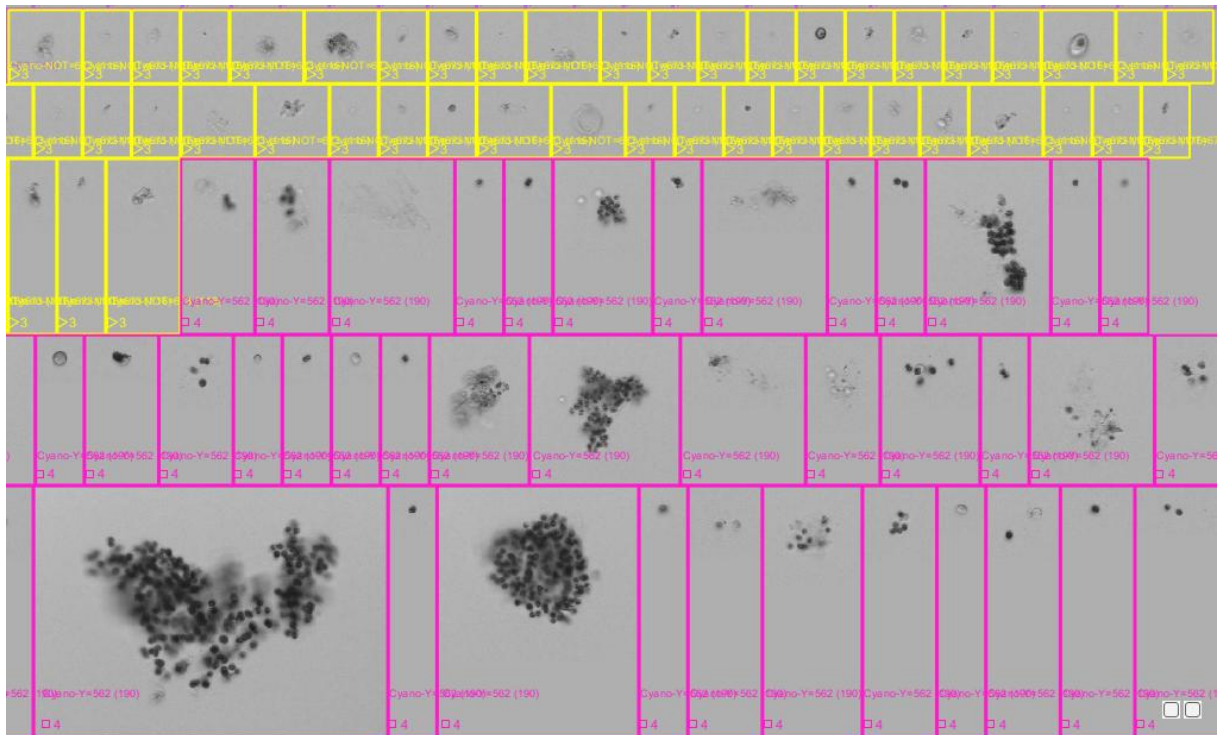


All particles with Total FL Red values ≤ 100 are called 'Cyano_NOT' and all particles > 100 'Cyano_Y'. We test the additional criterion setting.



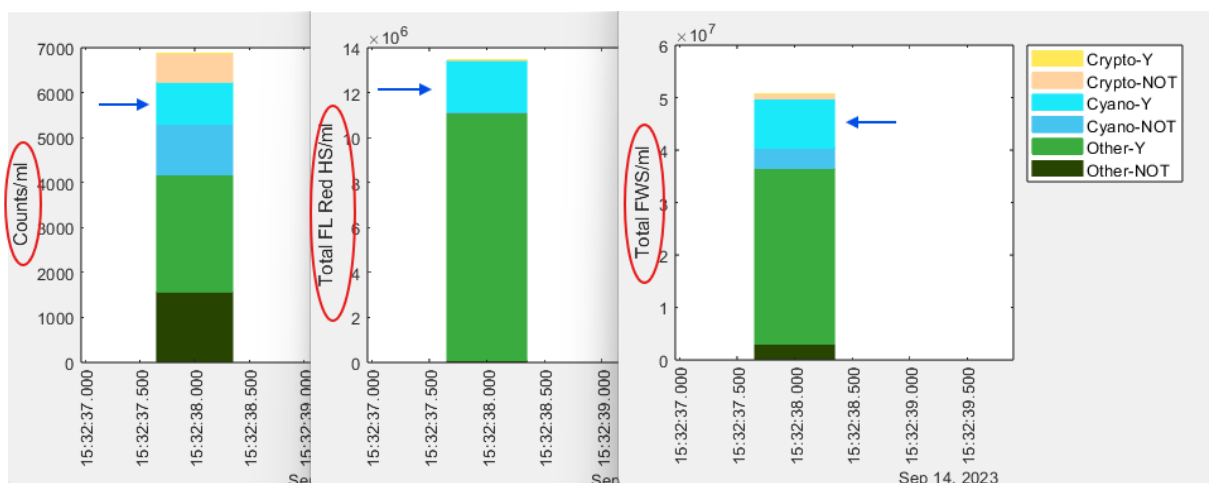
The result looks better, even if we look at the photos within the clusters.

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The method is now set (press Ok button to confirm this and close the menu) and cyano's can be calculated in this and in other files (via Run).

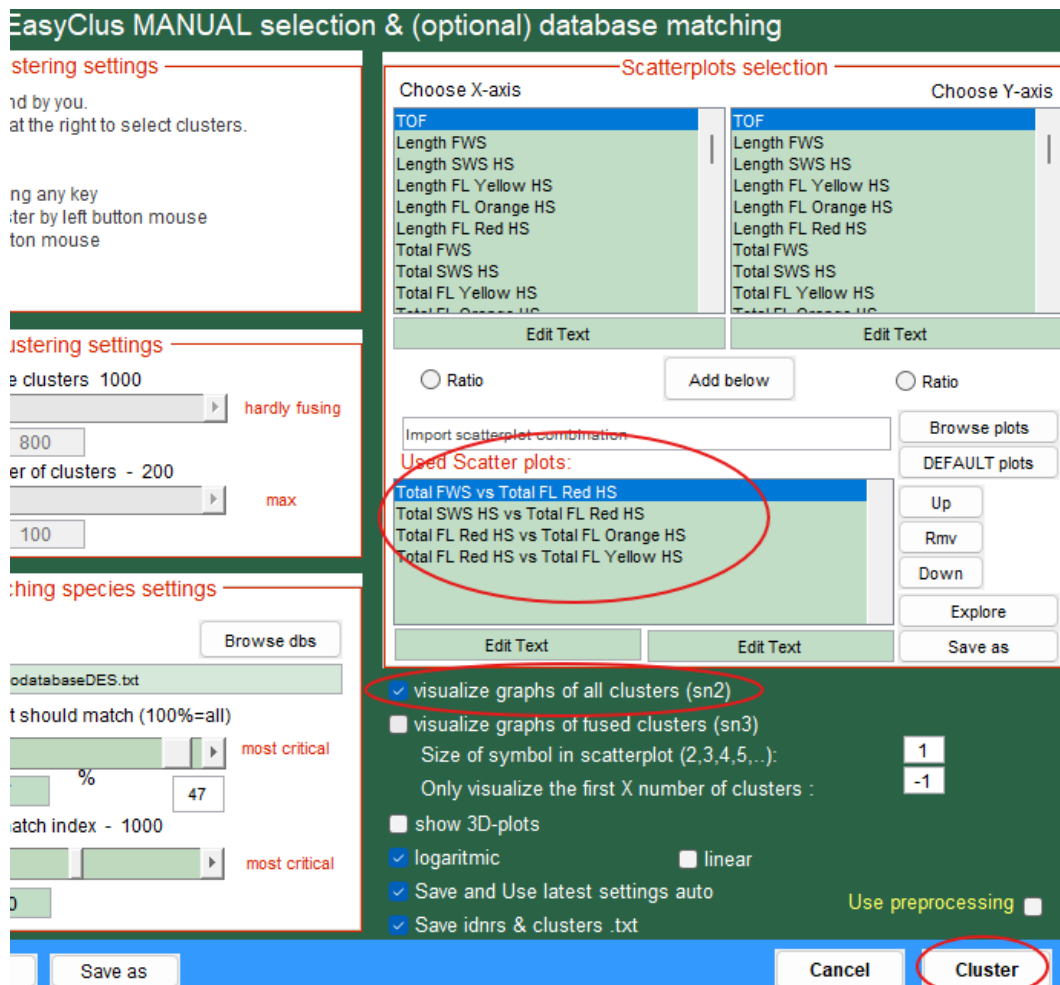
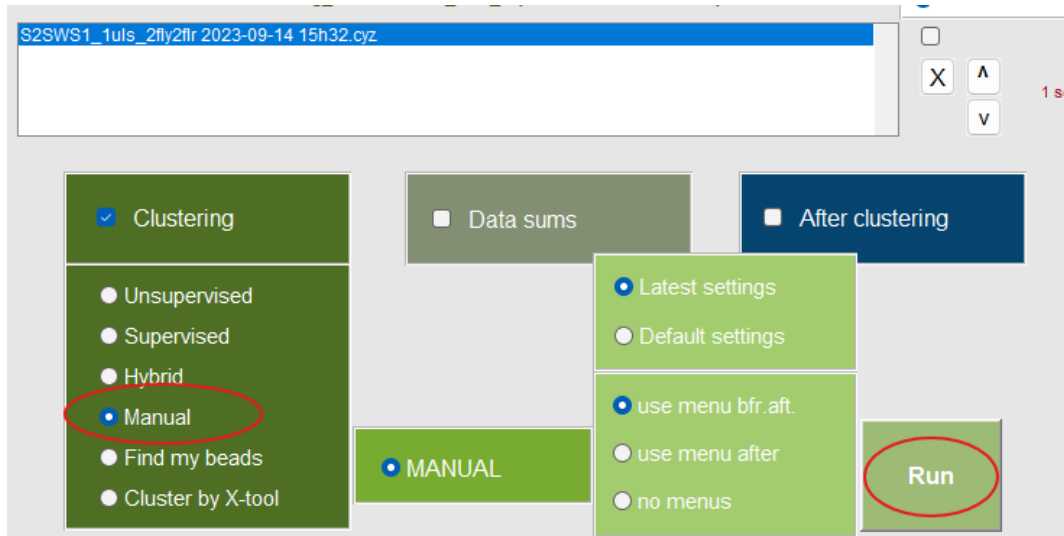
You will be asked (once) which figures need to be made (click on 'AUTOset' the first time). This can then be adjusted via the 'settings used groups & figures' button.



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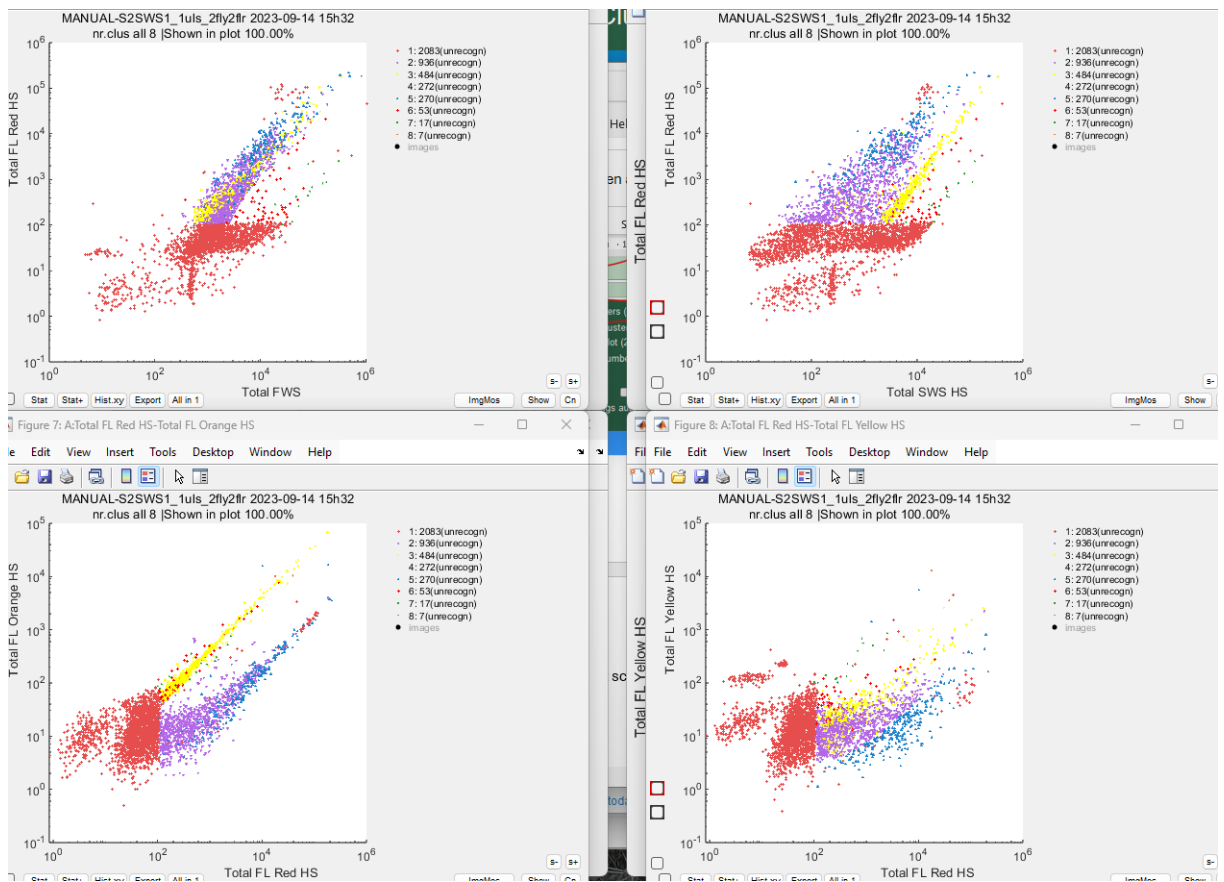
2. Database - via lasso-methode

Lassos or selection sets are drawn around the cyano-particles in EasyClus or in CytoClus. The selection is used to cluster by the LASSO-method. The selection can also be used to store the selected particles in the database.



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Start (manual) clustering, use at least three different scatterplot combinations and save manual cluster results.

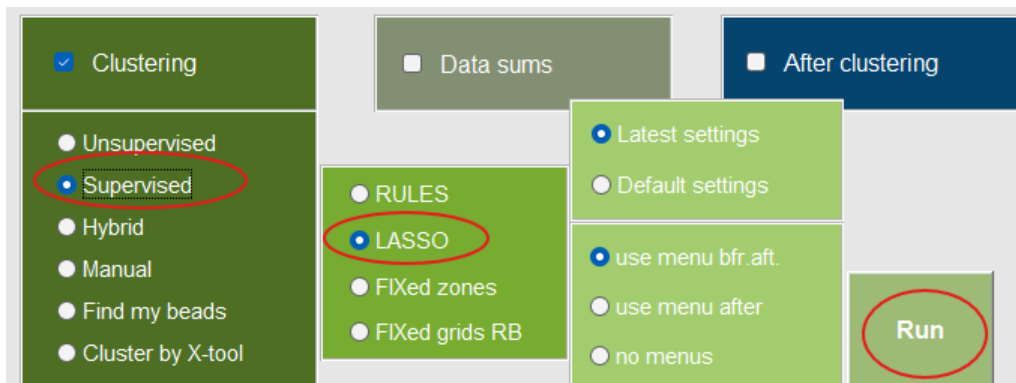


2results > selectionsets > manual Zoeken in manual

Sorteren Weergeven Details

Naam	Gewijzigd op	Type
selectionmanual_29-Oct-2025_15u47_Cyano.bt	29-10-2025 15:47	Tekstdocun

Use LASSO clustering for automatic clustering on basis of freshly made selection sets in many files



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menusettings_supervised

EasyClus semi supervised lasso clustering

Selectionsets/lasso's to be used for clustering

Choose selection sets/lasso's: Only unique-> results\selectionsets>manualselectionmanual_29-Oct-2025_15u32_cyano.txt

SELECT NEW LASSO's

Up, Down, Import file, Save as

Clustering settings

Expand % lasso >>lasso's become larger<<>>

original size: 100 %

Nr of lasso's to use: 4

smaller, larger

Matching criteria for overlapping clusters

lasso's based on database... lasso's based on select.sets

Database used/file: C:\Easyclusv122\results\databases\phytodatabaseA_29-Oct-2025_15u33.mat

% FCM parameters that should match

less critical, most critical

Use ignore dbs d... 99 % 48 Nr of col.

500 - Similarity match index - 1000

less critical, most critical

use weighing 500 use labels Sett.

Scatterplots for visualization

Choose X-axis: TOF, Length FWS, Length SWS HS, Length FL Yellow HS, Length FL Orange HS

Choose Y-axis: TOF, Length FWS, Length SWS HS, Length FL Yellow HS, Length FL Orange HS

Ratio, Add below, Browse plots, DEFAULT plots, Up, Down, Explore, Save as

Scatter plots for visualization:

- Total SWS HS vs Total FL Red HS
- Total FL Red HS vs Total FL Orange HS
- Total FL Red HS vs Total FL Yellow HS

Do Biovol.-img. relations

- Show Sc.plots all clusters (sn2)
- Show Sc.plots merged clusters (sn3)
- Show label box

Size of symbol in scatterplot (2,3,4,5,...): 5

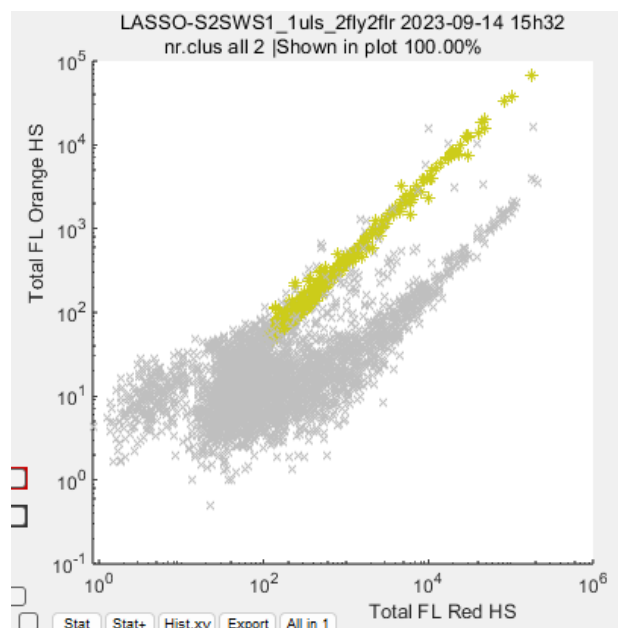
Only visualize the first X number of clusters: -1

logarithmic linear

Save idnrs & clusters .txt

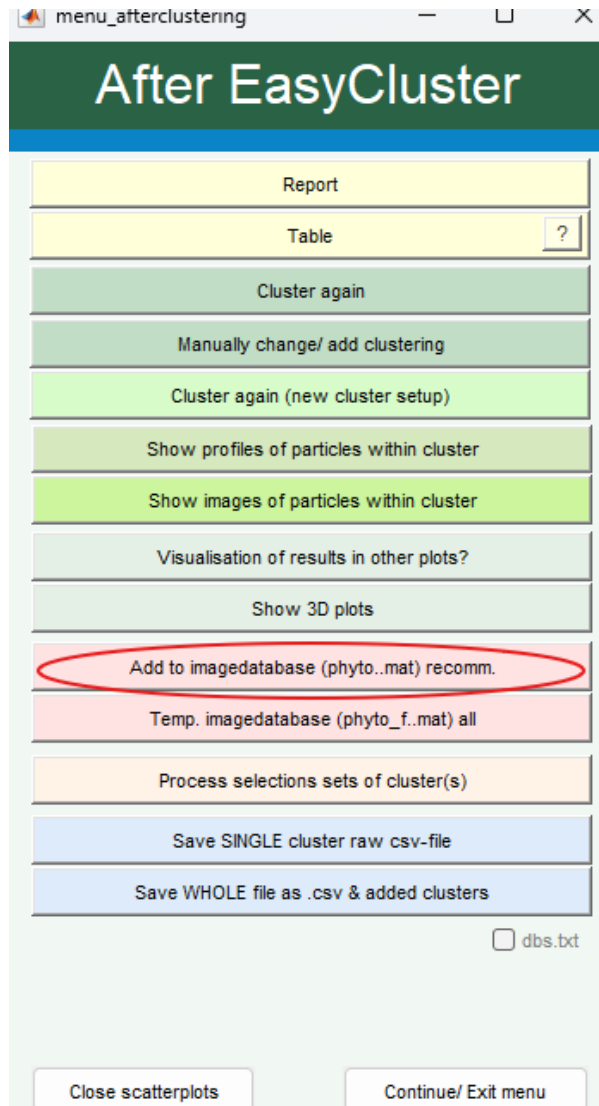
Use preprocessing

load settings, Reset, Save as, Cancel, Cluster



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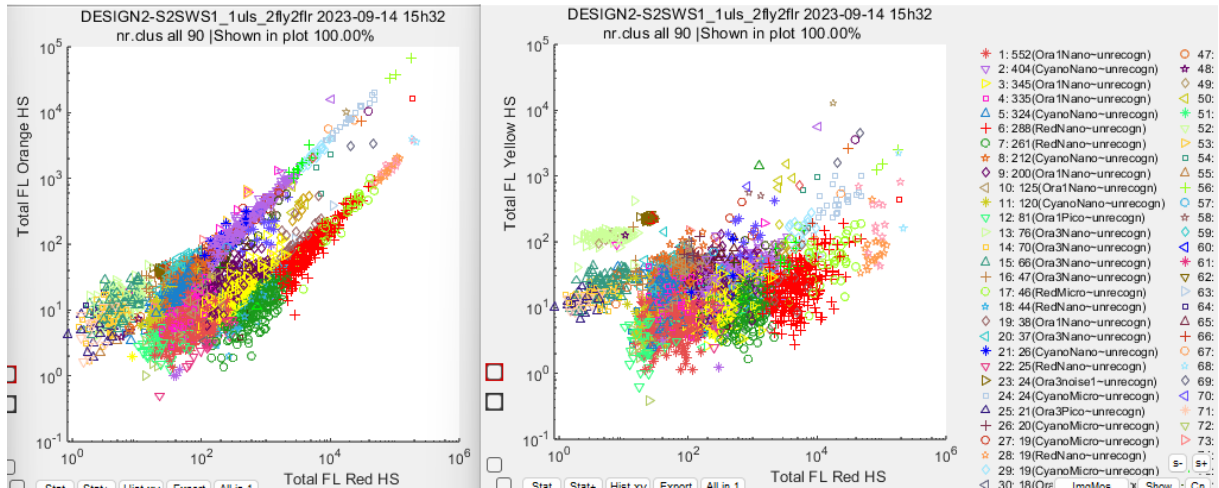
And/or add cluster (nr 3) to a new database as well by:



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3. Database – via rules (but not only cyano's but also other species in dbs)

This supervised cluster method uses a database with multiple species (e.g. >15) that represent a large proportion of the particles in the scatterplots and not just cyano particles. Using the UNsupervised method, we need a database which we have previously filled with different species.



And added to a database or created a new database.

After EasyCluster

This is still done in the UNsupervised DES2 method

EasyClus Cluster(s) to Database

Report

Table

Cluster again

Manually change/ add clustering

Rename merge clusters by list

Show profiles of particles within cluster

Show images of particles within cluster

Visualisation of results in other plots?

Show 3D plots

Add to imagedatabase (phyto_.mat) recomm.

Temp. imagedatabase (phyto_f_.mat) all

Process selections sets of cluster(s)

Save SINGLE cluster raw csv-file

Save WHOLE file as .csv & added clusters

Clusters result filenaam: C:\Easyclusv122results\databases\phytodatabase_04-Nov-2025_16u03u26.mat

Clusters found

- 1 : Ora1Nano-CI-0001
- 2 : CyanoNano-CI-0002
- 3 : Ora1Nano-CI-0003
- 4 : Ora1Nano-CI-0004
- 5 : CyanoNano-CI-0005
- 6 : RedNano-CI-0006
- 7 : RedNano-CI-0007
- 8 : CyanoNano-CI-0008
- 9 : Ora1Nano-CI-0009
- 10 : Ora1Nano-CI-0010
- 11 : CyanoNano-CI-0011
- 12 : Ora1Pico-CI-0012
- 13 : Ora3Nano-CI-0013
- 14 : Ora3Nano-CI-0014
- 15 : Ora3Nano-CI-0015
- 16 : Ora3Nano-CI-0016
- 17 : RedMicro-CI-0017
- 18 : RedNano-CI-0018
- 19 : Ora1Nano-CI-0019
- 20 : Ora3Nano-CI-0020
- 21 : CyanoNano-CI-0021
- 22 : RedNano-CI-0022
- 23 : Ora3noise1-CI-0023
- 24 : CyanoMicro-CI-0024
- 25 : Ora3Pico-CI-0025
- 26 : CyanoMicro-CI-0026
- 27 : CyanoMicro-CI-0027
- 28 : RedNano-CI-0028

Selected to put to Database

non-species

- Ora1Nano-CI-0001
- CyanoNano-CI-0002
- Ora1Nano-CI-0003
- Ora1Nano-CI-0004
- CyanoNano-CI-0005
- RedNano-CI-0006
- RedNano-CI-0007
- CyanoNano-CI-0008
- Ora1Nano-CI-0009
- Ora1Nano-CI-0010
- CyanoNano-CI-0011
- Ora1Pico-CI-0012
- Ora3Nano-CI-0013
- Ora3Nano-CI-0014
- Ora3Nano-CI-0015
- Ora3Nano-CI-0016
- RedMicro-CI-0017
- RedNano-CI-0018
- Ora1Nano-CI-0019
- Ora3Nano-CI-0020
- CyanoNano-CI-0021
- RedNano-CI-0022
- Ora3noise1-CI-0023
- CyanoMicro-CI-0024
- Ora3Pico-CI-0025
- CyanoMicro-CI-0026
- CyanoMicro-CI-0027

Species/types found in database

Listbox

Import database

New empty database

Counts stored in database

1 % add % of found counts of each

200 but at least minimum nr of counts per cluster

only data WITH images

use sharpness

smart largest selection on

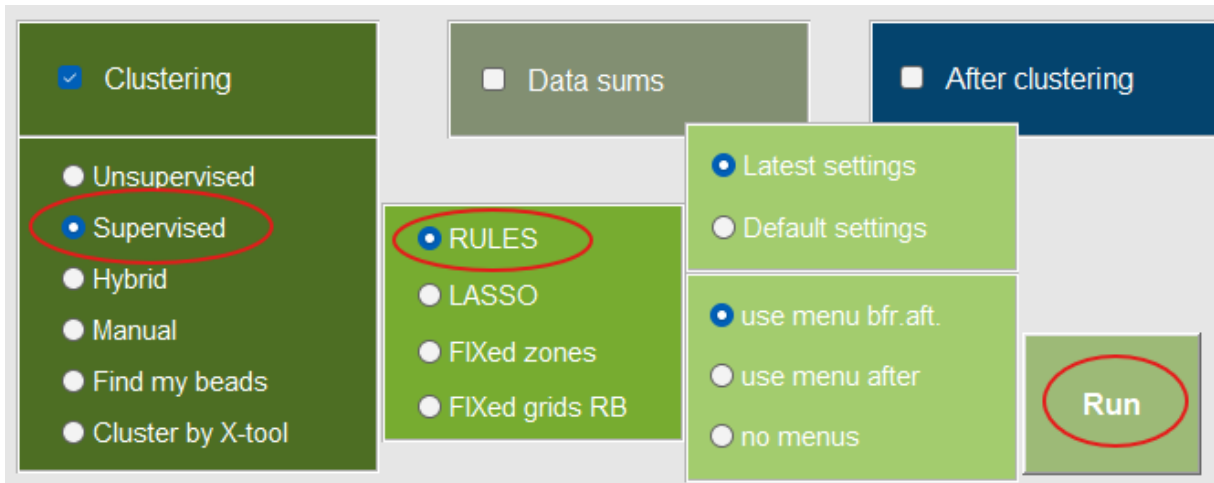
use VIRTUAL images

Database name

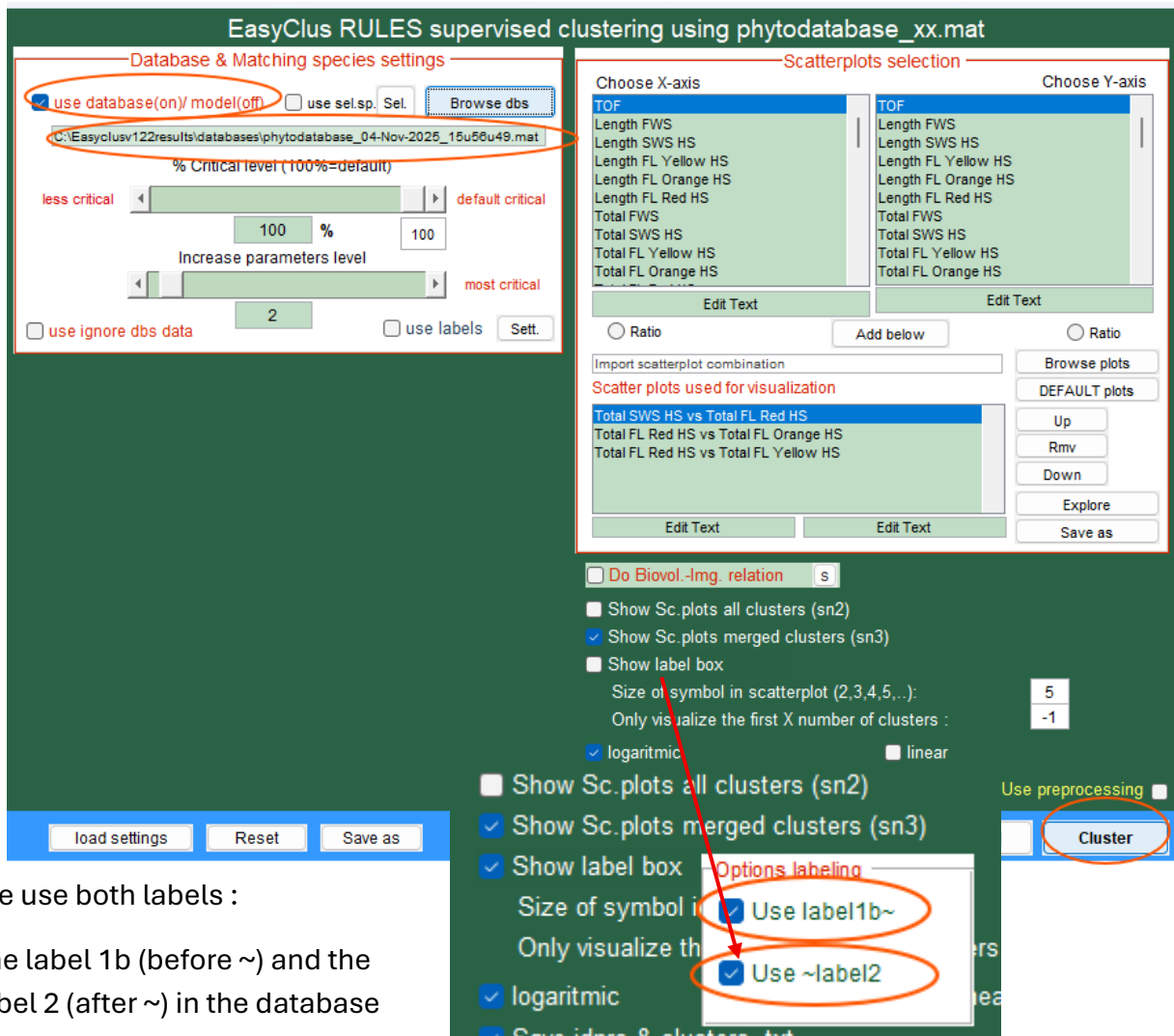
Cancel Ok & Do

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Only clusters with more than 10 counts (up to and including Cl-38) have been added to the database. Now that there is a database, we can use it for the supervised RULES clustering



We import the databse containing previously defined cyano clusters and start clustering with the database

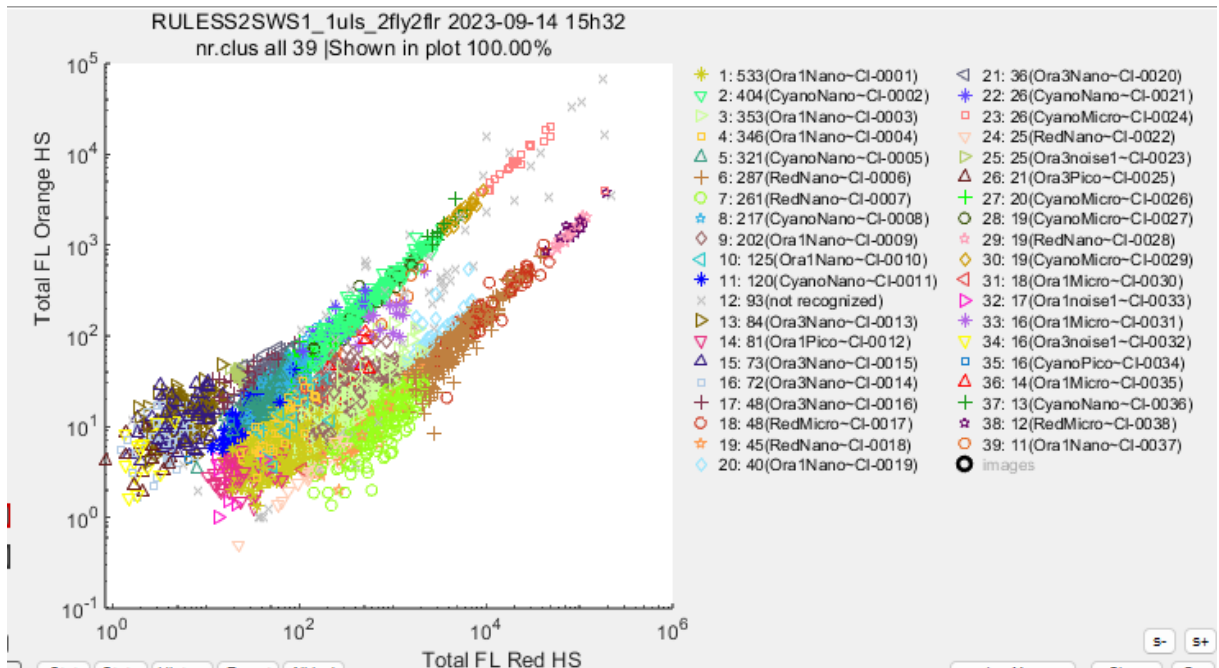


We use both labels :

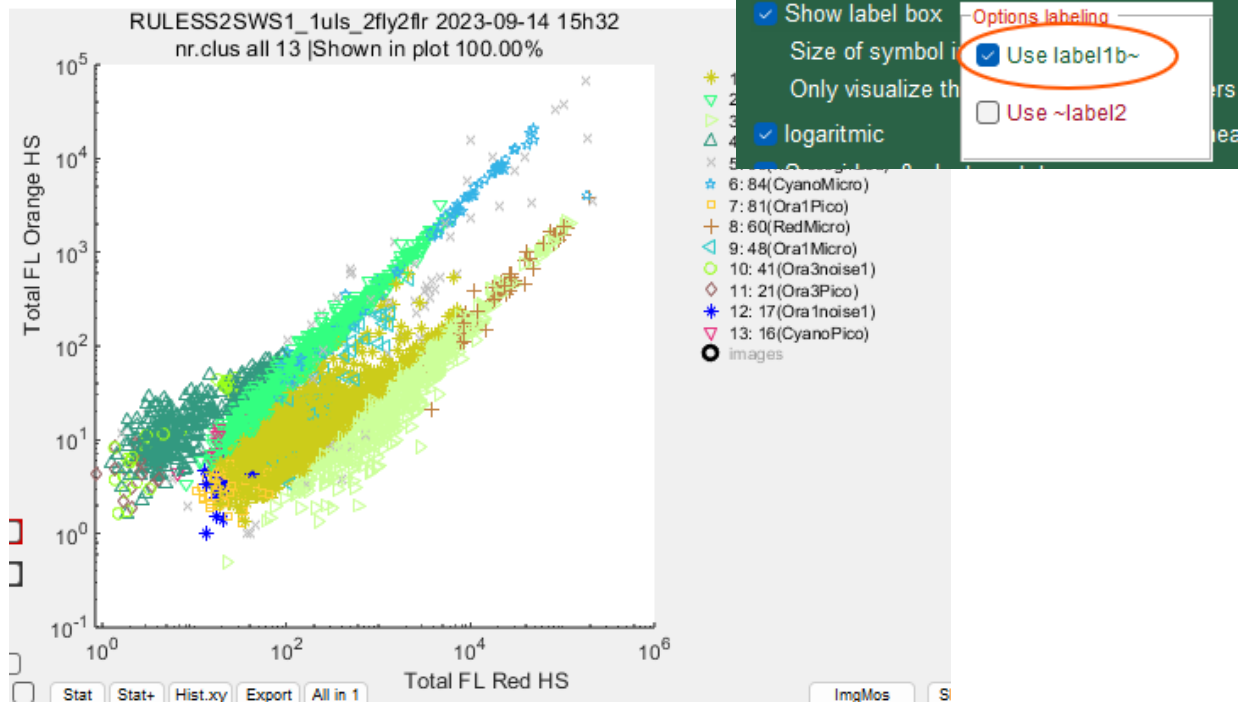
The label 1b (before ~) and the label 2 (after ~) in the database

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The supervised cluster results RULES with both labels



The supervised cluster results RULES with only label1b (before ~) in the same database



If desired, the database can also be adapted and expanded with more species/types of algae.

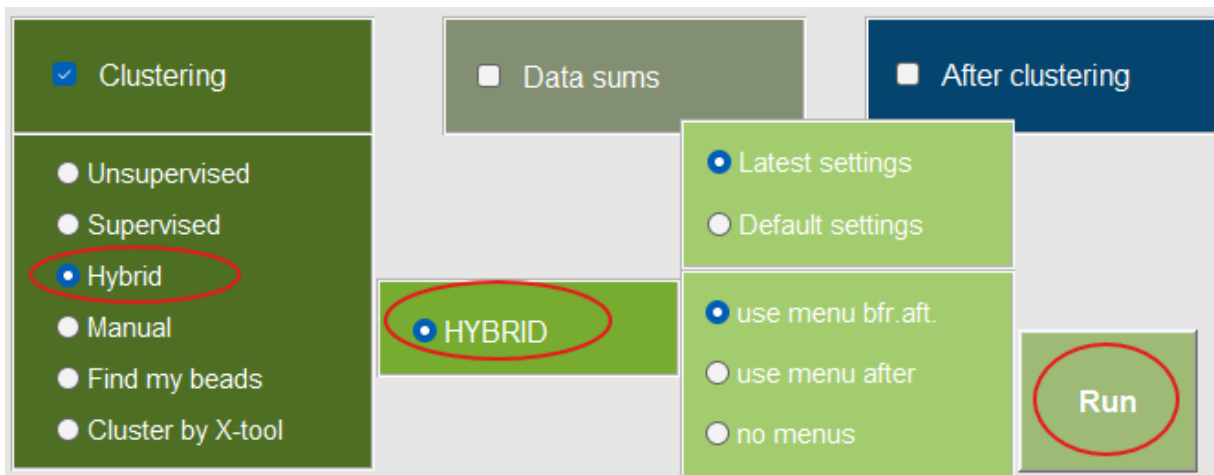
4. Database – via hybrid (but not only cyanos but also other species in dbs)

This method is mainly used when a database must be built up in a large series of samples, which contains species from the entire measurement series.

The hybrid method collects unique clusters and automatically adds them to the database. After the database and/or the model has been filled (last sample has been measured), the names of the clusters found are given by a specialist for each cluster/group, including the cyano species.

One can also use an existing database (such as in RULES), where it is expected that more particles will be assigned to a species from the database.

Then the HYBRID method is used with the database



In the example below, we use a database (the same as in RULES), which is not auto-filled (see the settings below) but is used for the HYBRID clustering matching method.

First, there will be unsupervised clustering with combinations Total SWS-Total FL Red, Total FL Red- Total FL Orange, Total FL Red-Total FL Yellow at grid 55 and neighbouring distance 3. Particles within the clusters are matched with the database and clusters are assigned to the best matching species from the database.

Label1b~label2 are used in combination (according to the setting).

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EasyClus HYBRID un- & supervised clustering using phytodatabase_xx.mat

Clustering settings

Grid Resolution

neigh linked to grid

55 more clusters

Neighbouring distance

3 less clusters

always separate clusters if white space in between

AutoAdd clusters to parsset rules and/or database

AutoAdd clusters to model Start New empty

AutoAdd clusters to database Start New empty

Save background data on

Database & Matching species settings

use database(on)/ model (off) use sel.sp. Sel. Browse dbs

C:\Easyclusv122results\databases\phytodatabase_04-Nov-2025_15u56u49.mat

Use ignore dbs data % Critical level (100%=default)

less critical default critical

100 % 100

Use simil after rules

Increase parameters level

most critical

use weighing 1 use labels Sett.

Scatterplots selection

Choose X-axis

TOF
Length FWS
Length SWS HS
Length FL Yellow HS
Length FL Orange HS
Length FL Red HS
Total FWS
Total SWS HS
Total FL Yellow HS
Total FL Orange HS

Choose Y-axis

TOF
Length FWS
Length SWS HS
Length FL Yellow HS
Length FL Orange HS
Length FL Red HS
Total FWS
Total SWS HS
Total FL Yellow HS
Total FL Orange HS

Ratio Ratio

Add below Browse plots

Import scatterplot combination

Used Scatter plots:

Total SWS HS vs Total FL Red HS
Total FL Red HS vs Total FL Orange HS
Total FL Red HS vs Total FL Yellow HS
#Length FWS vs .26;3,2U,noise,pico,nano,micro
#Length(20%) FWS vs 20. micro

Do Biovol.-Img. relation

visualize graphs of a

visualize graphs of f

Show label box

Size of symbol in sca

Only visualize the first

logarithmic Save idnrs & clusters

Label options

Use label above for: 'not recognized' other

Merge how:

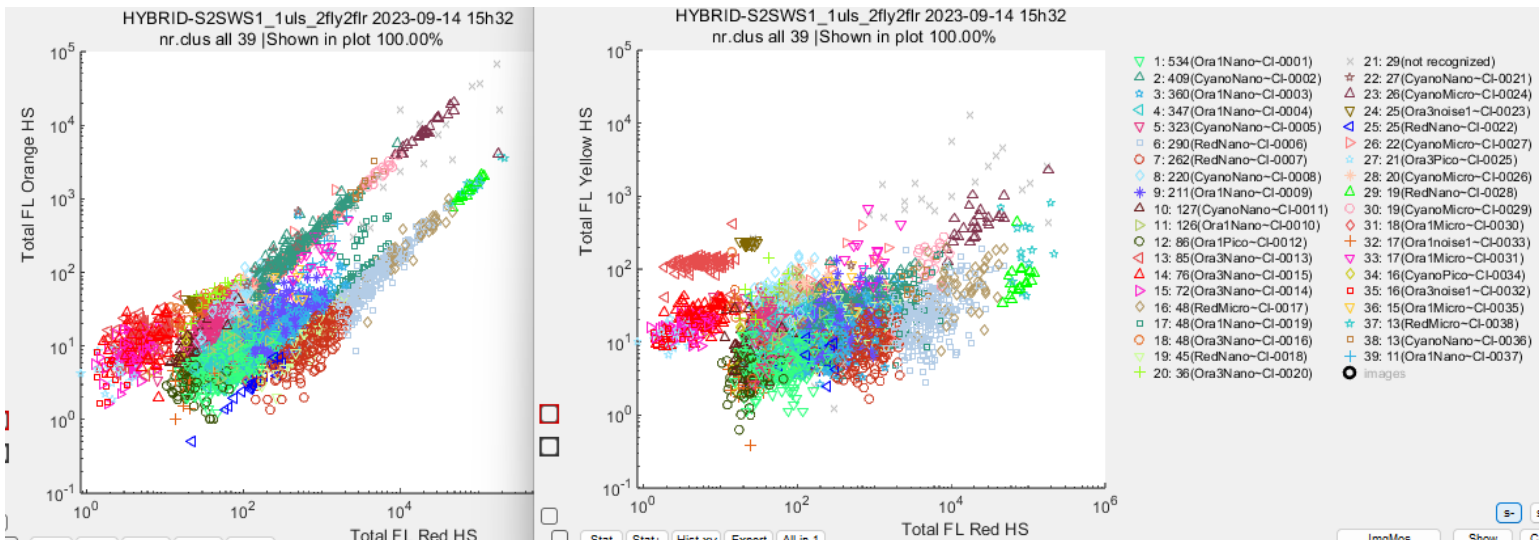
Unrecogn.: Use label1
 Unrecogn.: Use label1~label2
 Unrecogn.: Use label2

other: Use label1~
 other: Use label1~label2
 other: Use ~label2

Merge more:

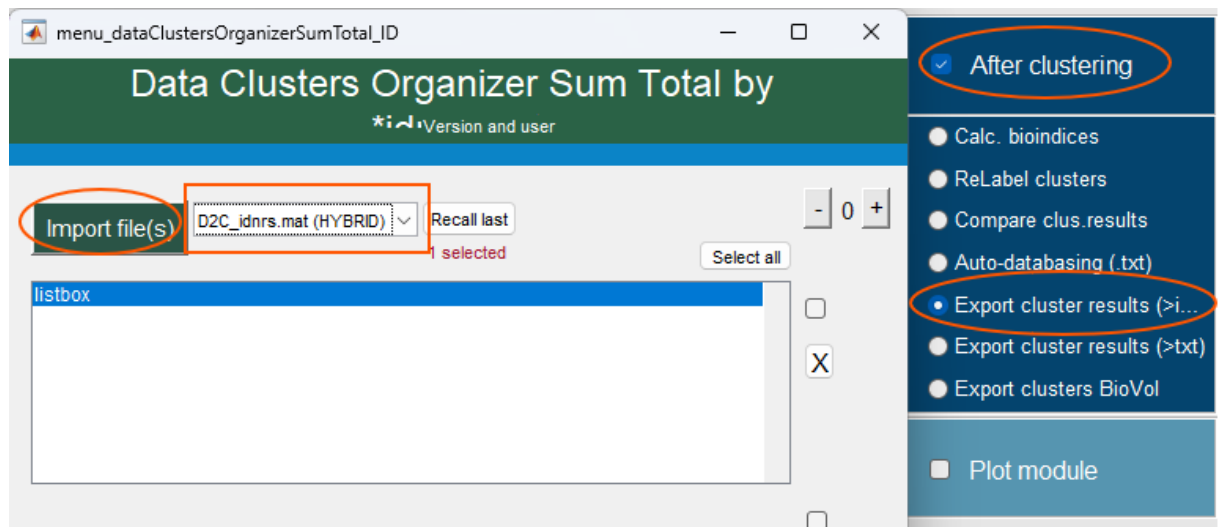
load settings Reset Save as Cancel Cluster

The HYBRID cluster results are shown in the scatterplots



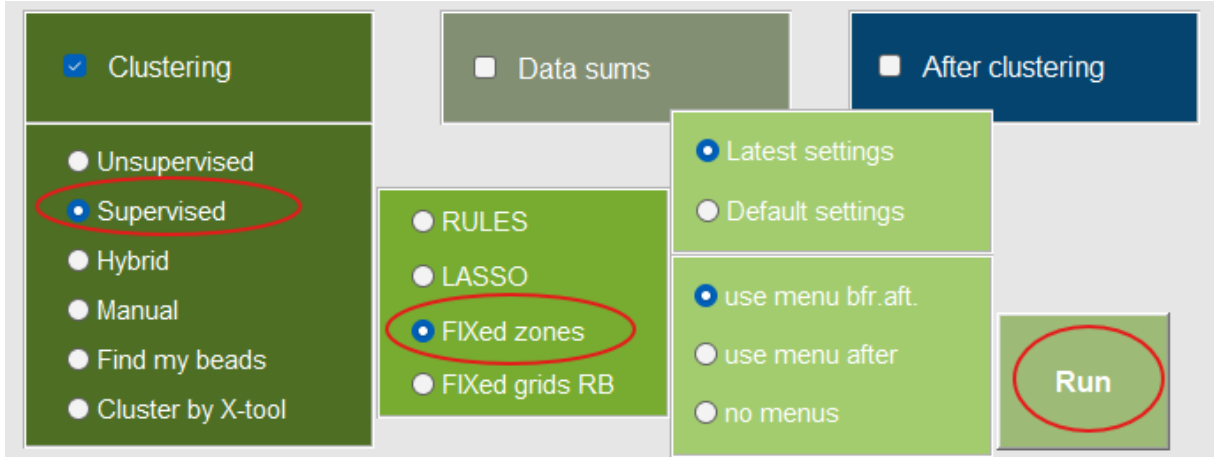
The HYBRID cluster results can be put together in a table with the 'After clustering' module. To import the HYBRID cluster results, choose the option 'filename_D2C_idnrs.mat' files.

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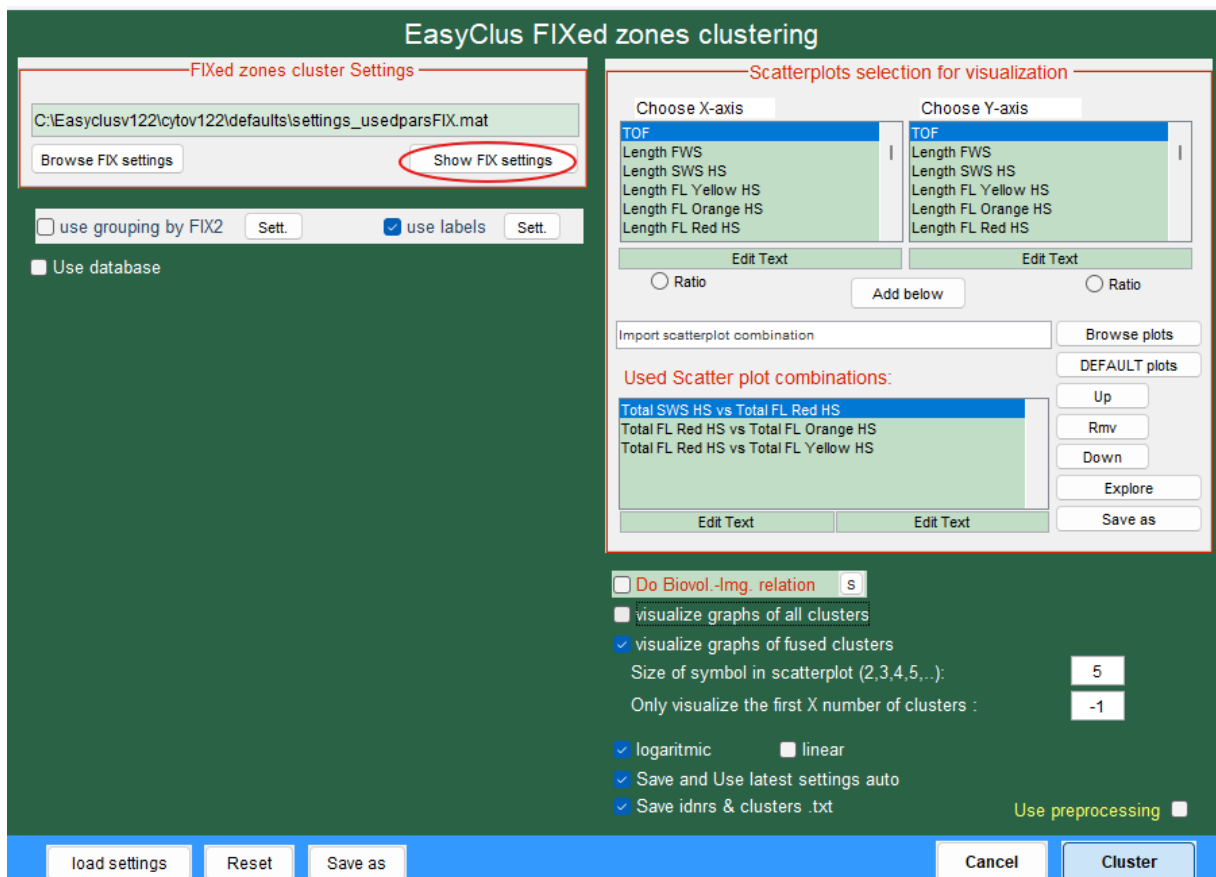


5. FIXEd zone method – define zones yourself by ratio lines and/or lassos

The FIXEd zone method is a very fast method in which (once) zones are defined in the scatter plots. Each zone per scatter plot is given a name. The composite zones from

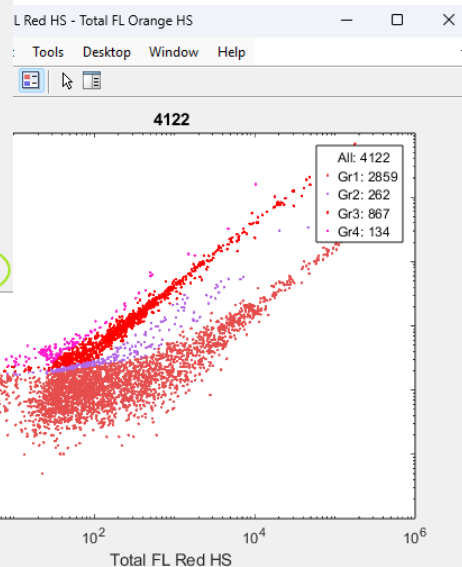
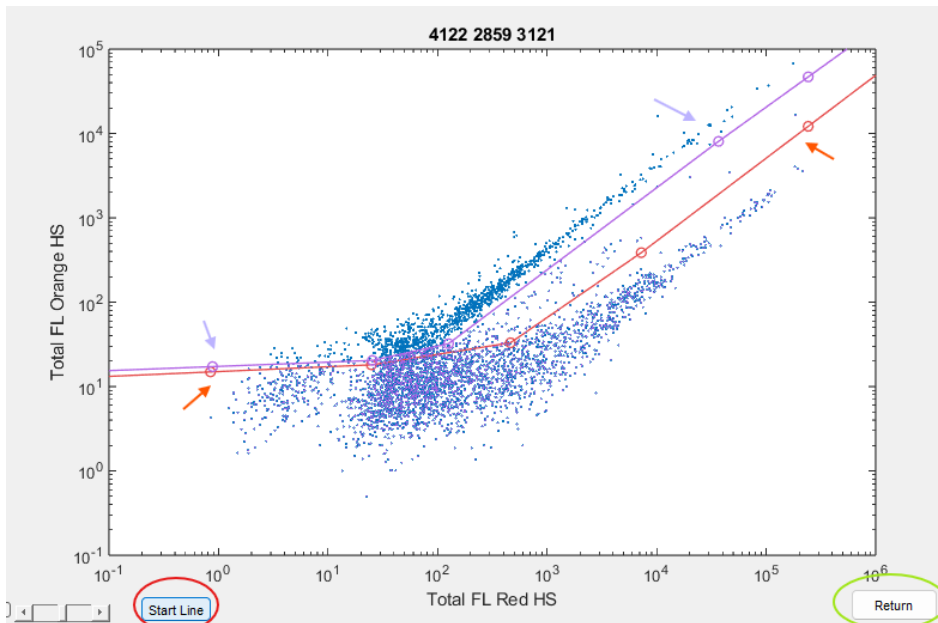


different criteria/zones together form unique clusters and names per zone. The 'Show FIX settings' is used to define the zones, usually this is done once and is only adjusted afterwards.



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The zones are defined by hand (from bottom to top). All particles below, between, above the lines per plot are in a separate zone.



Lassos can also be defined, for example for reference spheres, which are more difficult to define by a line zone.

menu_settingsFIXcellclustering_FIX3L

Settings for FIXed zones Clustering

Length FWS	26.2, 20
L1 multiline: Total FL Red HS_vs_Tota	7.9, 17.5, 27

import FCM file

Delete

Edit above

Add multiline

Add lasso

Import settings

Save settings as

Test

Theoret. nr of clusters: 16

Show clusters & def. labels

clusnrns explain

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Cancel

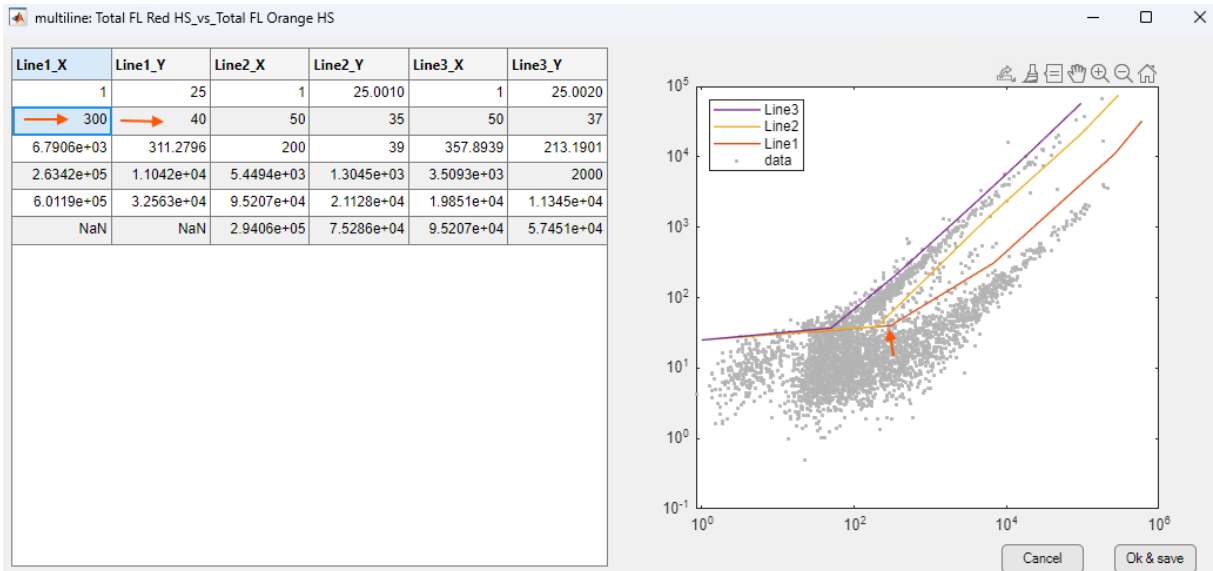
Save & Exit

The multiline zones can be fine-tuned if necessary, via the 'edit above' button (see above). Lines may be almost on top of each other, but the highest line must always be just above

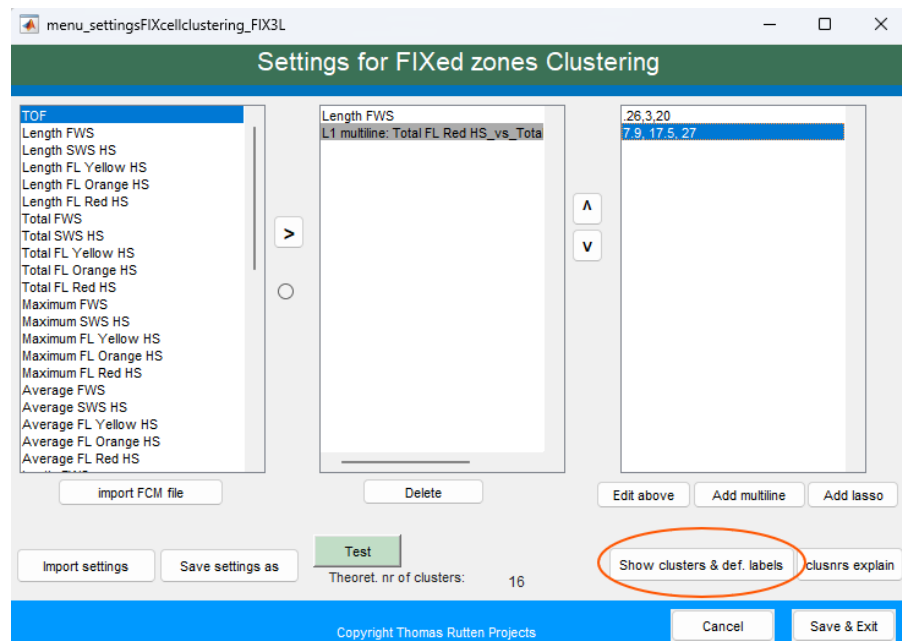
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the next other line(s). This applies to every line. A multiline may never cross another multiline. The lines are extrapolated at the front and back automatically by the software.

Editing multi-lines to make them even more precise in relation to each other is possible by adjusting the number values via the table but make sure that the top line always remains the top line.



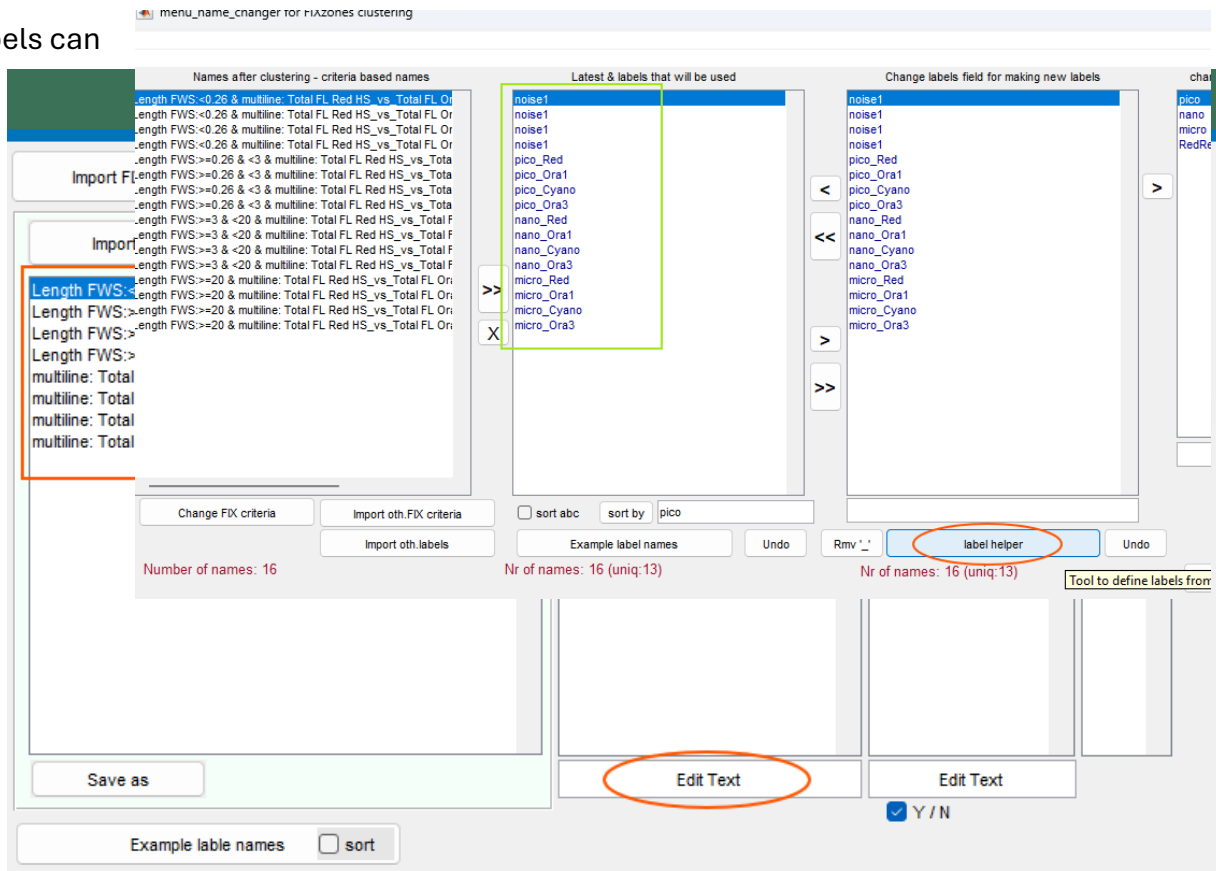
Each zone corresponds to a given name, which can be viewed/defined via the button 'Show cluster & def(ine) labels'.



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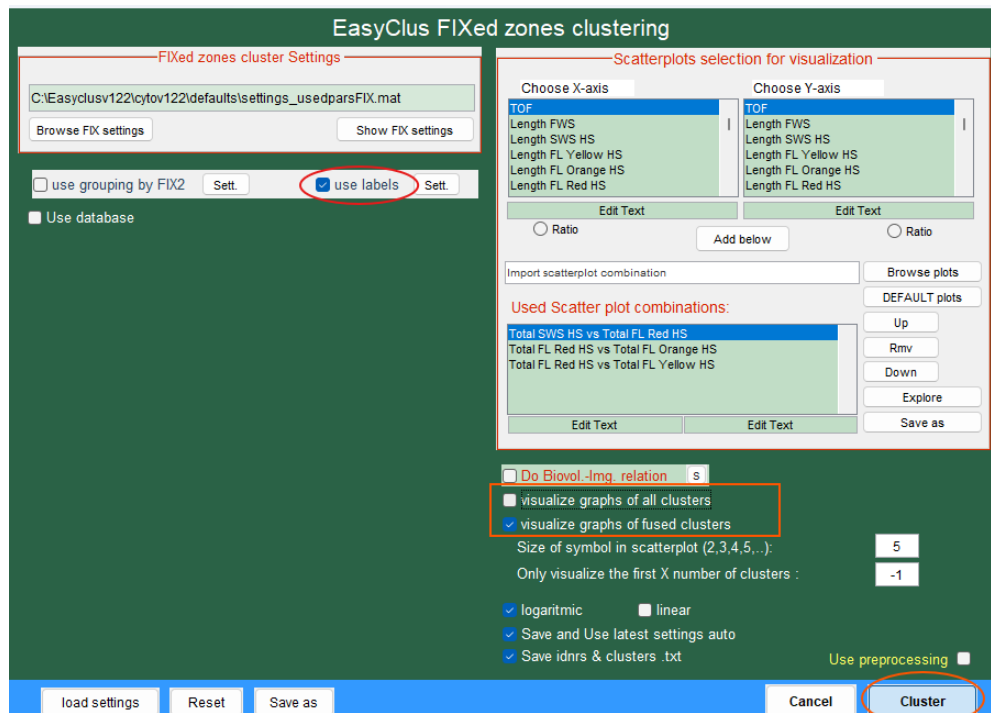
In the green square the names used.

Labels can



be defined via the 'label helper'

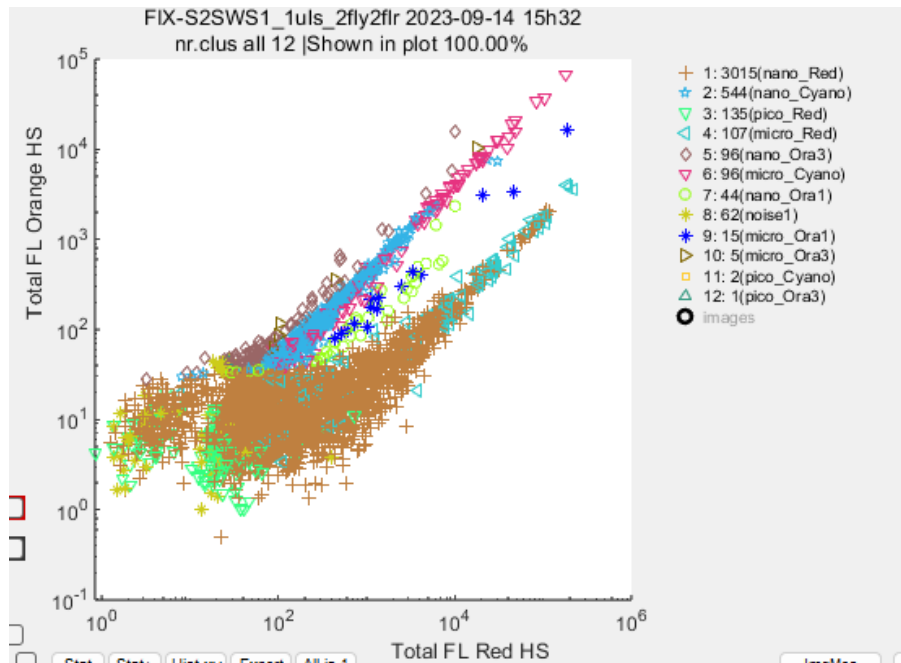
The FIX settings are ready, clustering can take place. Specify that the labels should be used.



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After pressing the 'Cluster' button, clustering is done according to the FIXed zone method (see below)

The cluster results in a scatterplot.



Via the 'After clustering' option, all cluster results 'filename_FIX_idnrs.mat' of a series of files can be put together in one table.

Data Clusters Organizer Sum Total by
*i Version and user

Import file(s) DE2_idnrs.mat (DESIGN2) Recall last

DE2_idnrs.mat (DESIGN2)
DE2_B_idnrs.mat (DESIGN2 +)
RUL_idnrs.mat (RULES)
D2C_idnrs.mat (HYBRID)
D2C_B_idnrs.mat (HYBRID +)
LC3_idnrs.mat (LASSO)
FIX_idnrs.mat (FIXed zone)
FIXgrRD_idnrs.mat (FIXed grid Rainbow)
MC1_idnrs.mat (MANUAL)
AC2_idnrs.mat (DESIGN1)
GO4_idnrs.mat (GO)
REF_idnrs.mat (REFERENCE)
PSA_idnrs.mat (PULSE SHAPE)
_idnrs.mat (OTHER)

Select all

After clustering

Calc. bioindices

ReLabel clusters

Compare clus.results

Auto-databasing (.txt)

Export cluster results (>i...)

Export cluster results (>txt)

Export clusters BioVol

use export as .xlsx

auto merge select. fil...

Merge & Save VAL*

Run

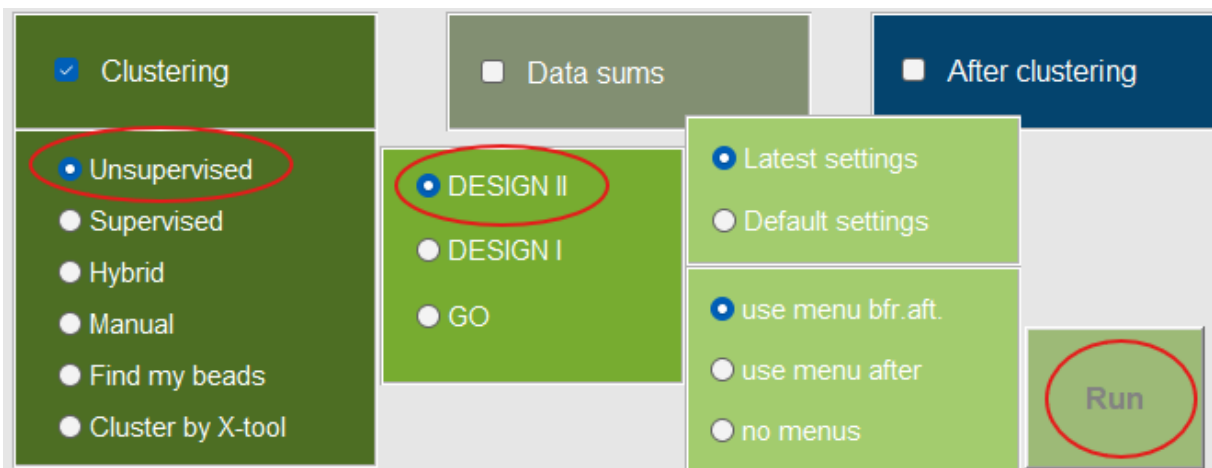
Back

6. Clustering DESIGN via ratio's in clustering process

This method is quick and easy to set up and has the advantage that it can be used in combination with a database (based on cluster averages), so also species outside the cyanos. A second advantage is that a distinction can be made/added between cyano groups, for example based on length. A third advantage is that biodiversity information also remains available because the unsupervised clustering classifies all particles with the same optical properties into clusters.

All particles are assigned a (pre)name, from which the main group (e.g. cyano) classification becomes clear.

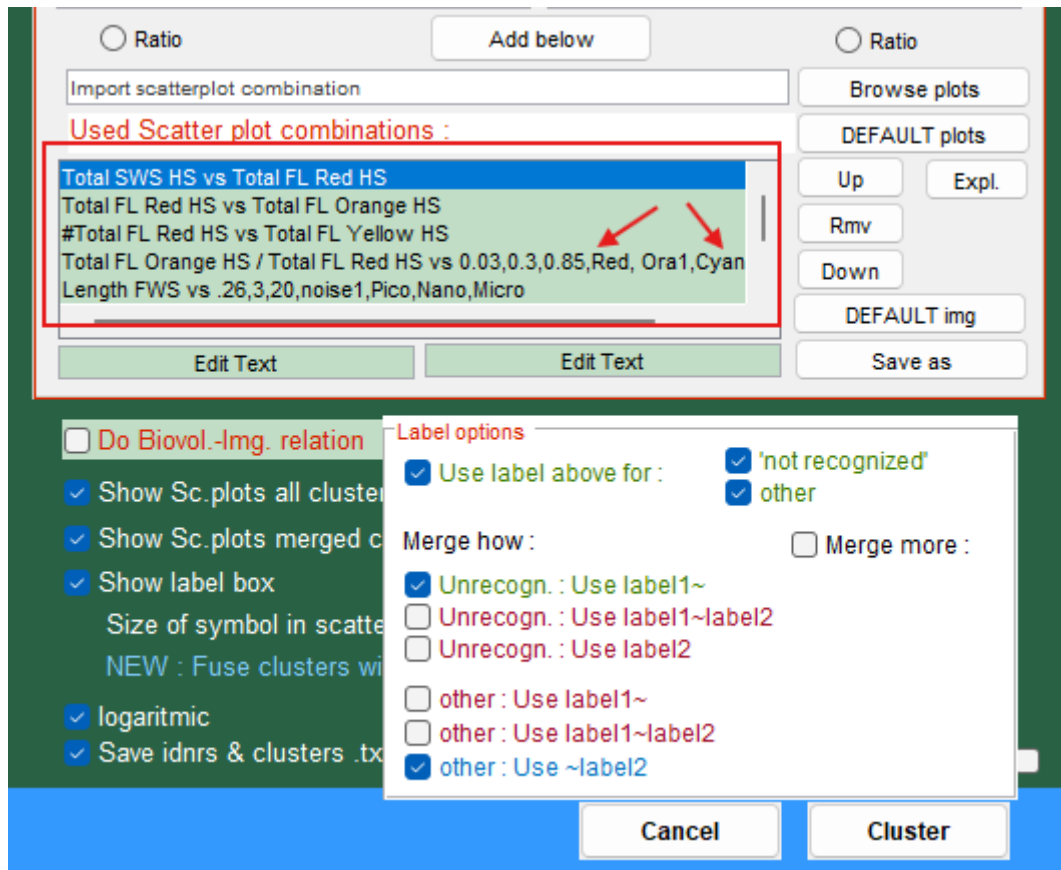
The disadvantage of the method, but this also happens with the other methods, is that the settings must be re-adjusted when ratios change, although this can be set relatively quickly and easily in this method.



In the example below, in addition to unsupervised clustering in scatterplots, Total SWS vs Total FL Red and Total FL Red vs Total FL Orange (#Total FL Red vs Total FL Yellow becomes inactive here by applying #) some extra criteria are introduced. The strict boundaries are defined on the ratio of Total FL Orange/ Total FL Red (left) with the addition of 0.03, 0.3, 0.85, Red, Ora1, Cyano, Ora3. This means that the O/R ratio < 0.03 becomes 'Red', ≥ 0.03 & < 0.3 becomes 'Ora1', ≥ 0.3 & < 0.85 becomes 'Cyano' and everything ≥ 0.85 is called 'Ora3', whereby clusters from the unsupervised clustering are split up based on these criteria.

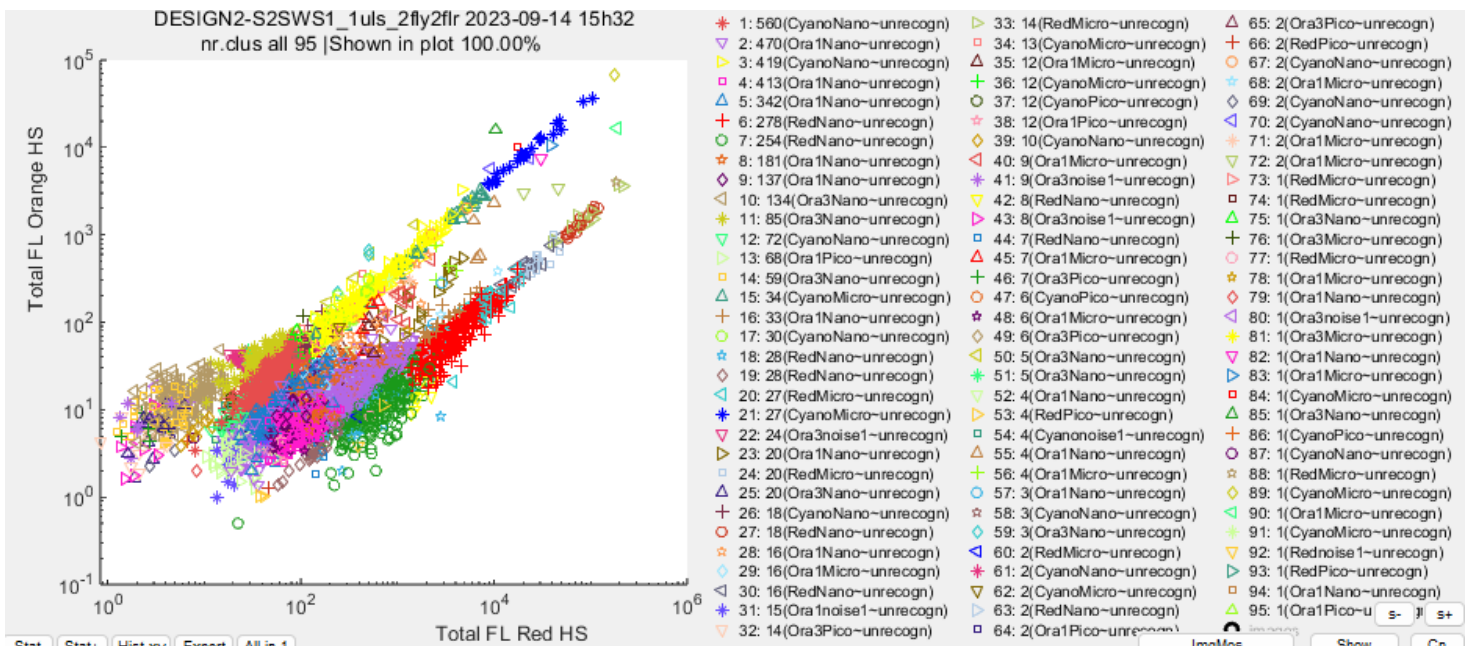
In addition, there is another length particle distinction by the application of Length FWS, 0.26, 3, 20, noise, pico, nano, micro. For example, particles ≥ 3 & < 20 are called 'nano'. This is done based on individual particles (and not on the basis of a cluster average) so that all particles in the cluster 'nano' have a length between 3 and 20 μm .

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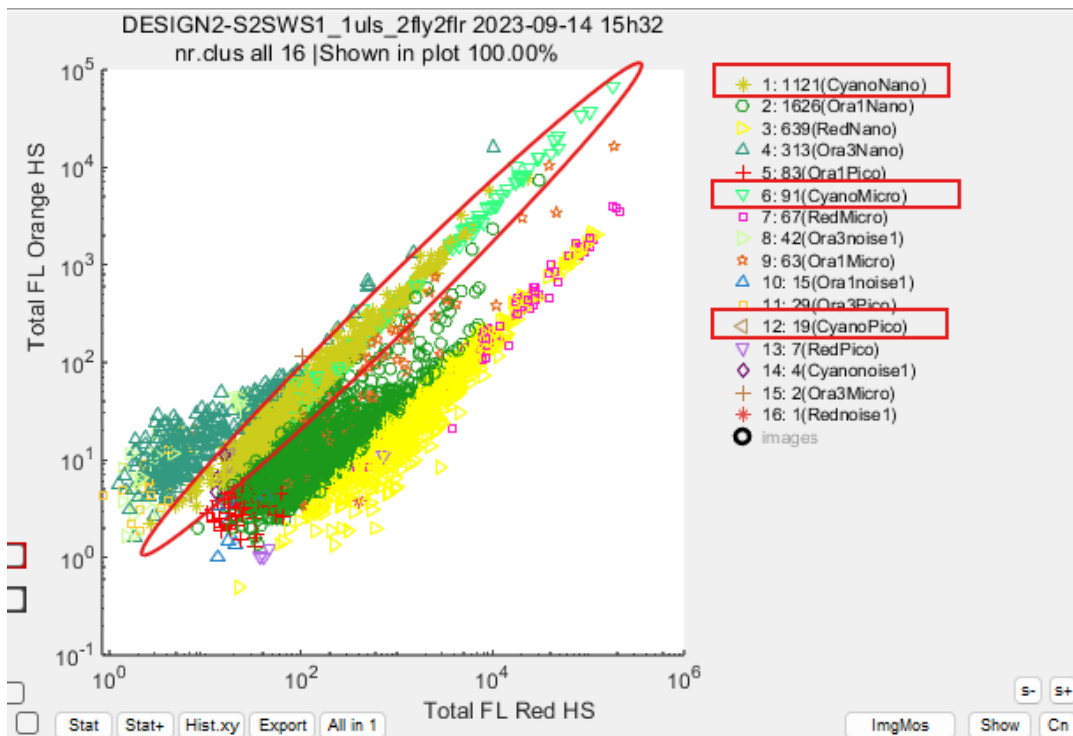


The window (above) in white is used to indicate whether the 'label1' names ('Red', 'Ora1' etc. 'pico', 'nano' etc.) should be given to clusters that are not recognized by the database or to all clusters or to use both names at the same time. A sample (with cyano particles) was processed with these settings and that led to the scatter plot below. The (sn2) clusters are all clusters that have arisen from unsupervised clustering and splitting according to the specified criteria and producing clusters with the same name.

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After merging the clusters with the same name (the sn3 clusters), there are 16 clusters with a unique name. The total counts of the sn3 clusters below are equal to the total number of particles of the 95 sn2 clusters (top).



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The cluster results per file can be collected with the 'filename_DE2_idnrs.mat' files from the EasyClusvxxxresults\cluster folder and put together by in a file, for example, a .txt or .xlsx file via the 'After clustering' tool of EasyClus (see below).

