CYTOCLUS

PURPOSE:

To establish selection criteria for certain types of phytoplankton particles and perform automatic selection later, based upon those criteria.

PRODUCTS:

A list (Plain text, but readable by EXCEL) of the numbers of particles in each selection set, for all the samples in a directory or a number of directories, and a list in the same format of the total scatter and fluorescence in each selection set.
A list of average particle properties for the particles in one selection set, for all samples in a directory. The selection sets are listed in a separate file.
A list of the properties of all particles, per particle, for the particles in one selection set for one sample.

DEFINITIONS:

Beamwidth: The distance between the points where the intensity of the laser beam is decreased to 13% of its maximum intensity. Normally this distance is about 5 micrometers.
Corespeed: The speed at which the particles move through the laser beam. This is about 2 m/s.
Datapoint: A single value measured on one detector output (forward scatterd light, sideward scattered light etc). Normally the datapoints are spaced 0.5 micrometers apart as there are 4 million datapoints per second, at 2 m/s speed. The datapoints of all detectors are measured simultanuously.
Pulse: The sequence of datapoints as measured during the passage of one particle.
Dotplot: A two dimensional picture where a particle is represented by a dot or small circle that is positioned acoording to the values of two properties of that particle.
Particle plot: A graph of the pulse of forward and sideward scattered light and fluorescence in one or more wavelength bands vs length as measured while the particle passed through the laser beam.
Property: A single value per particle derived from the datapoints as measured while the particle passes though the laser beam.
Sample: One data set measured form a volume of water and recorded as a set of file(s) with a common file name.
Selection set: The set of lower and upper limits for all defined properties within which a particle is considered as beloning to a set.

PROPERTIES:

Classic properties:

The **Average** forward scatter (FWS), sideward scatter (SWS) , red fluorescence (FLR) , "orange" fluorescence (FLO) and "yellow" fluorescence (FLY) are calculated as the total value of all datapoints in that channel devided by the number **N** of dataponts in the particle. Thus : **Average FWS = Σ (FWS) / N**

The reason to use average values instead of totals (as classical cytometers do) is that in a chained colony the average value over the chain is independent of the number of cells in the chain.

Morphological properties:

Length: The estimated particle "body length", that is the length corrected for the beam width. For this correction an **emperical** calculation is used :
1: find the maximum value Fmax in the forward scatter pulse.
2: calculate a threshold as : **T = 0.013 * Fmax**
3: establish the first upward crossing through the threshold level and the last downward crossing through the threshold level. This gives the "raw length" **Lr** from the number of data points in between and the corespeed.
4: The length is then calculated as :

Length = Lr - Beamwidth * (Beamwidth / Lr)

Fill factor : The "fill factor" **F** of a pulse of **N** datapoints **Dn** is defined as :

F = (Σ Dn)^2 / Σ (Dn^2)

For a "box" pulse (**Dn** is constant) this function reaches its maximum value of 1.0**,** for a "raised cosine" pulse (Dn = 1 - COS [k * n]) this function becomes 2/3, and for a “spike” pulse with one high value and the others very low it approaches zero, so it is a measure of how slender the pulse is.

Asymmetry:
The asymmetry is the difference between the "center of gravity" of the pulse and the midpoint of the pulse, devided by half the length of the pulse :
The center of gravity in the length direction of a pulse of **N** datapoints is
CG = Σ (n * Dn) / Σ (Dn), So, the asymmetry **A = ABS{ (2 * CG) / N - 1 }**

Inertia :
The inertia of a pulse is **I = Σ { (n - CG)^2 * Dn }.** To get a value that is independent of the size of the particle this value is devided by the inertia of a "box" of the same length and the average value of the pulse. For a particle that is concentrated in the center the normalised inertia is low, about 1/3 for a "bell shaped" pulse, for "saddle shaped " particle it is about 0.5 to 0.7. Note that for a long chain of cells it will allways approach 1.0 since the moment of inertia of a long stick is about the same if the stick is knobbly or smooth.

Cell number :
The " cell number" **Nc** is an estimate of the number of "humps" in the pulse.This number which is roughly the number of cells in a colony is calculated as follows :
1: get the differences **Δn = D(n)-D(n-1) for n =** 1 **to** N (**D(0) = 0)**
2: The “CellNr” **C** is then found as :

(2πC)^2= (N^2) * Σ(Δn^2) / Σ(Dn^2) - N * (Σ Dn)^2. Actually the "CellNr" approximates the center of gravity of the Fourier transform of the forward scatter pulse.

Apparent sizes:
The FWS size is the "cell" length for more complicated particles. Here the "raw length" **Lr** is based on the total number of datapoints devided by the CellNr.
The "Relative FLR size" is the same estimate, but now based on the red fluorescence pulse, devided by the FWS size. It provides some measure about the length of the chloroplasts within the cells.

Note: The calculations above are mathemathically “rather rough”, but fast and consistent.

TOOLS and STRATEGIES :

The one and only really powerfull tool in an interactive program like this is the brain of the user. What the program offers is “auxilliary”. And will be explained using the following example.

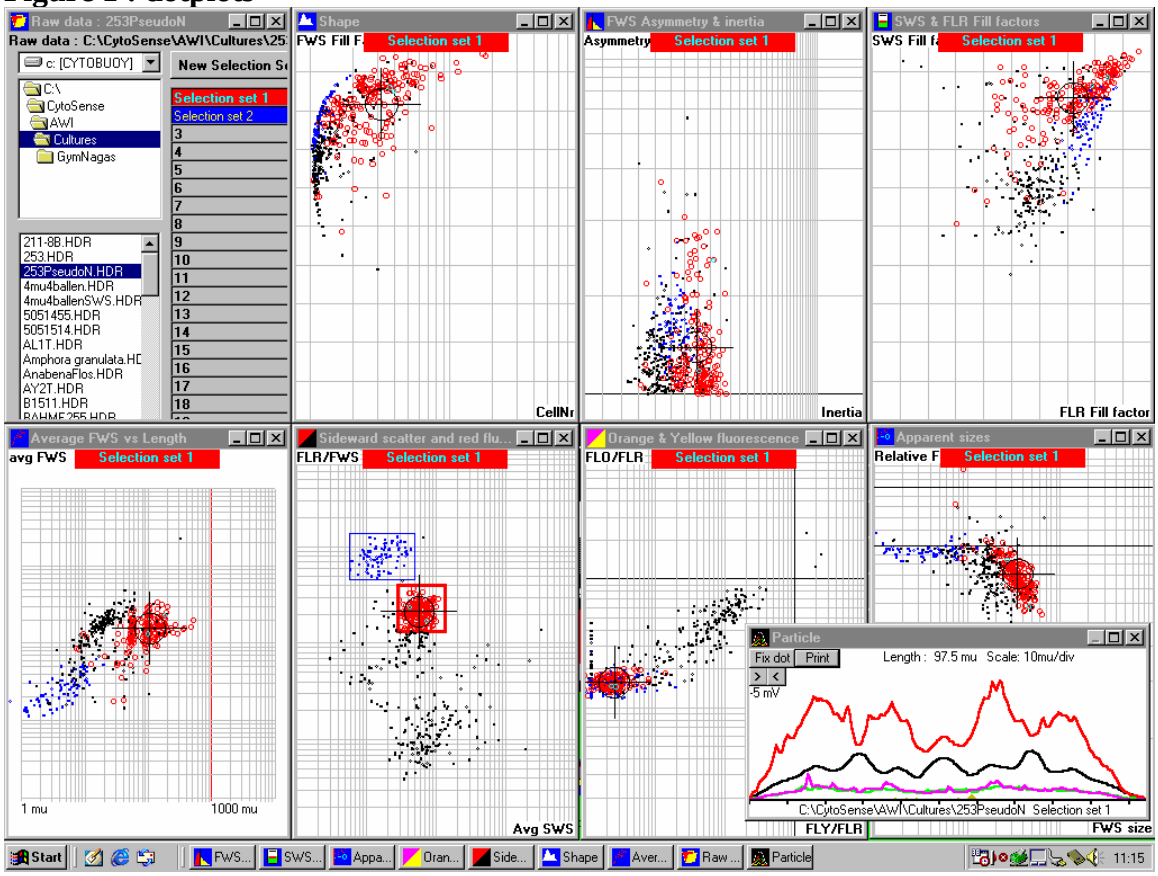
Example:
The example used for this chapter is a data file from a culture of Pseudonitzschia Pungens. The mix in a culture is, of course, simpler than in natural waters but sufficiently rich to show the principles to work with.

Selecting a data file.
The upper left box in the screen shown on the next page is (part of) the main program window. At the left side there are 3 boxes: to select a drive, to select a directory and to select a datafile. Actually the data file is a cluster of five files, but only the name of the first one is shown.

Dotplots.
The main information is in the seven dotplots:
1 : Average forward scatter vs particle length .
2 : The ratio of Red fuorescence and forward scatter vs sideward scatter.
3 : The ratio of Orange and Red fluorescence vs the ratio of Yellow vs Red fluorescence.
4 : Fill factor vs Cell number, both derived from the forward scatter.
5 : Asymmetry vs moment of Inertia, also from the forward scatter.
6 : Sideward scatter fill factor vs Red fluorescence fill factor.
7 : The ratio of the Red fluorescence and Forward scatter “size” vs the Forward scatter “size”, where “size” is the length based on the raw perticle length devided by the Cell number. Every dotplot is a separate “window” that can be moved and resized at will. After rezising all graphs will be adjusted to the new windows by a “left click” in any window. Initially the widows appear “stacked” but a “right click” in a free space in the lower taskbar calls the menue to tile the windows as shown in figure1 on the next page. With a little bit of luck “clusters” of dots can be seen in at least one of the windows.

When dots form a cluster that means that they have at least two, more or less, common properties and probably belong to the same kind of particles. In the example below three distinct clusters are present in the FLR/FWS vs Avg SWS plot.

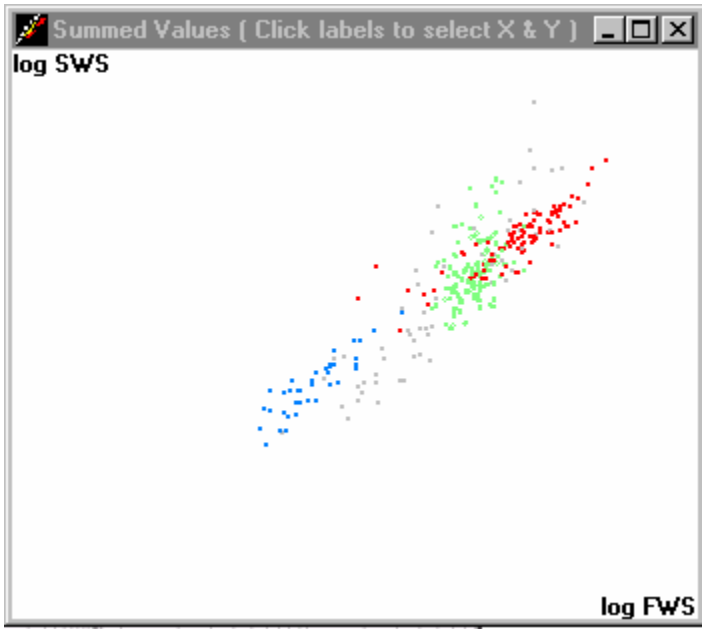
Figure 1 : dotplots



Selecting particles :

To select particles you have to define selection sets. The present set is selection set 0 , which is the set of all particles. Now click the button “New Selection Set” in the upper left window and a color selection window will appear. Choose a color, e.g. red and click OK. The number 1 box below the New Selection Set button will read “Selection set 1” on a red background. As the clusters are most prominent in the FLR/FWS window that is the best place to start. The text in “Selection set 1” is bold, indicating that it is the “active set”. To draw a box around the cluster place the cursor at one corner, depress the left mouse button, move to the opposite corner and release the left button. A red rectangle appears and all dots inside that box become red. The “bulls eye” is the center of gravity of the selected dots. There appears also a window with the signal shapes of the first particle in that selection. Creating a new, blue set and a box around the upper cluster in the FLR/FWS and then clicking on the name Selection set 1 yields the screen as above. The black dots are all other particles.

The auxilliary dotplot



Classical flow cytometers measure the integrated values of forward and sideward scatter and one or more fluorescence wavelengths. The associated software makes usually logarithmic dotplots as above. With 5 parameters there are 10 possible combinations, another screen full of windows. So, only one is shown. To select what is shown scroll through the possibilities for the X- and Y axis by clicking on the labels.

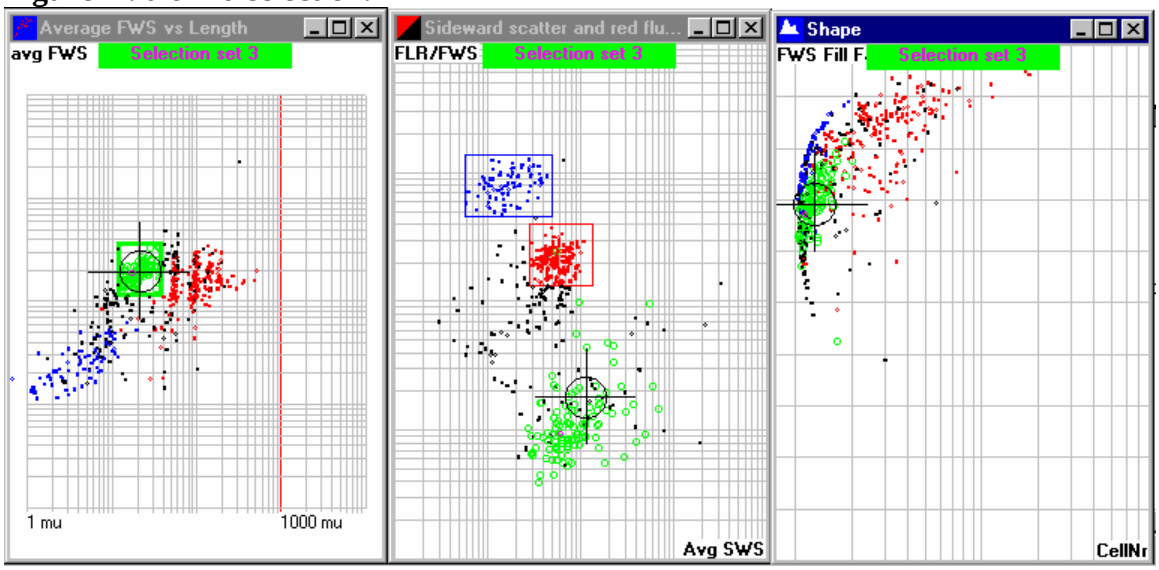
Some times this representation shows clusters more clear than the plots of more or less normalised values in the main Cytoclus program, but selection on simple limit values is allmost impossible. If you check the "Show auxiliary dotplot" box in the main window then a dotplot as above is shown, with selected particles in color. You can not select a region here, but you can select individual individual particles by clicking on the dots, look at the pulses and mark the positions in the other plots. To do so click the "Fix dot" button in the "Particle " picture.

What does it show ?

The red box is drawn thick, that set is the "active set", so the pulse in the Particle window is a member of that set. Further, red and blue particles are also clustered together in other windows. That confirms the notion that the red particles are one kind and the blue ones another kind. In the average FWS vs Length plot the blue ones are among the smaller particles and the red ones are the group of large particles. Also, in the "Shape" window the blue particles are concentrated on the crescent at the left side with CellNr between 1 and 1.5. This shows that they are simple single cells, whereas the red particles are at CellNr's up to about 20, they contain multiple cells. In the FWS vs length plot

there is a clear cluster of other particles left in between the red and blue. Creating a new, green selection set 3 around those locates them in the low region of the FLR/FWS window and just right of the “simple cells” in the shape window at CellNr’s between one and two. They are still single cells, but of a more complicated shape.

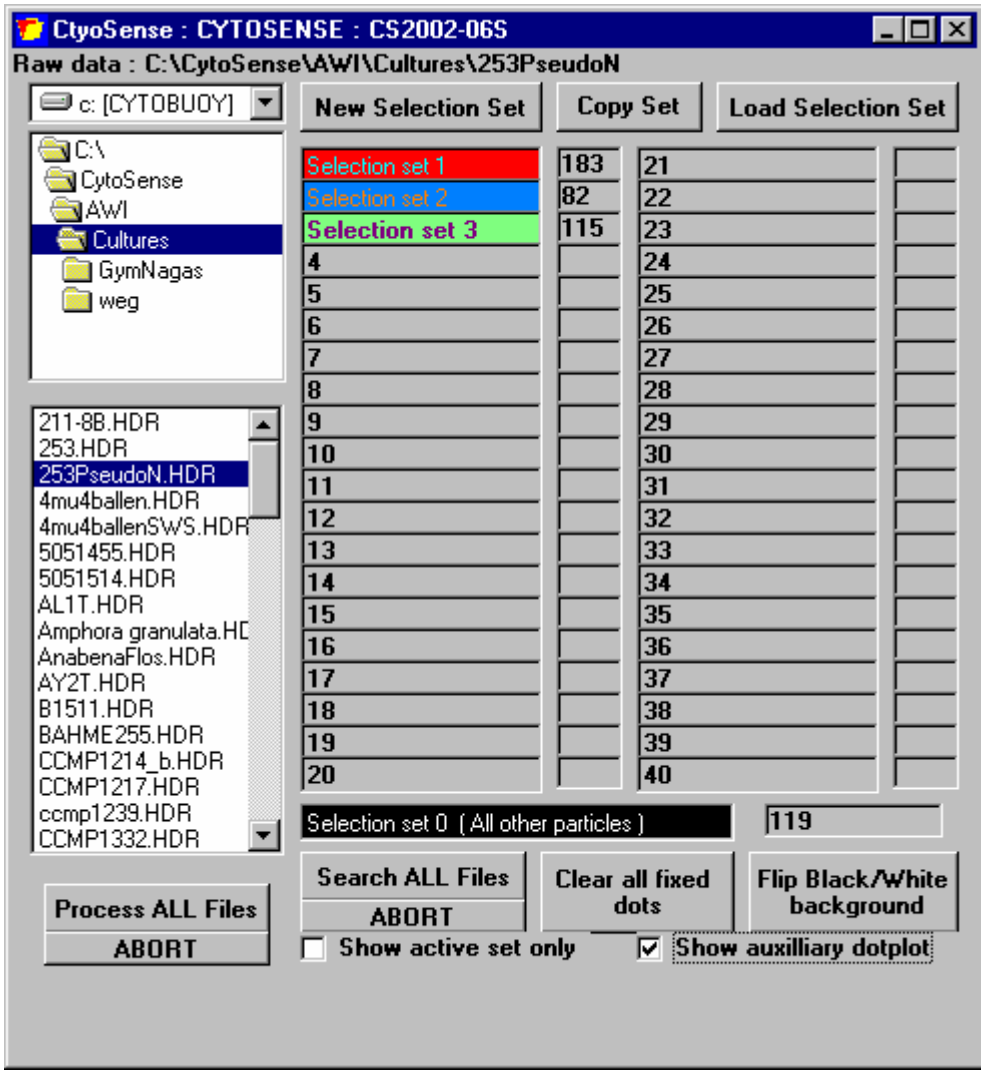
Figure 2 : a third selection.



Refining the selection sets.

The three sets as they are now are not yet very specific since the are each based on one pair of properties only. To include other properties in a set make it the active set by clicking on the name in the setslist. Which set is active is shown by thick borders and the name in all dotplots. The idea is to make boxes around all visible clusters of one set. Now the green set exhibits a cluster in the FLR/FWS window, but also some green “outlyers” there. To judge if these outlyers are like the other cells in the cluster or not one can view the pulses of a specific particle by placing the mouse pointer on the dot and clicking the right button. In natural samples with many different species those clusters are some times “drowned” among the other particles. To make them visible check the checkbox “Show active set only”. (see fig. 3). All boxes of the active set can be redrawn at any time. Another tool to edit a selection set is to click on the set name in any of the dotplots. This makes a window with a table of the lower and upper limits of all properties.(fig.4) These values can be changed numerically in that window and made effective by clicking the button “Apply changes” in that window. This can be usefull for instance if one wishes to leave no empty space beween adjacent boxes in a dotplot, or make a very small change to a limit. Another use of this window is to “remember” a set before you make changes with the mouse . This does not update the values in the table until you click on the name in a dotplot again. So, if you do not like the result of the mousework, you can return to the values in the table by clicking “Apply changes” there. When you click on the color button you can change the color of the set. This is effective immediately.

Figure 3 main window.



The main window now looks like this, with 3 selections containing 183, 82 and 115 particles and 119 particles left in the set of all other particles. Selection set 3 is the active set. The sets are now named Selection set 1, 2 and 3. You can change the name of a set when you place the cursor on its name and click.

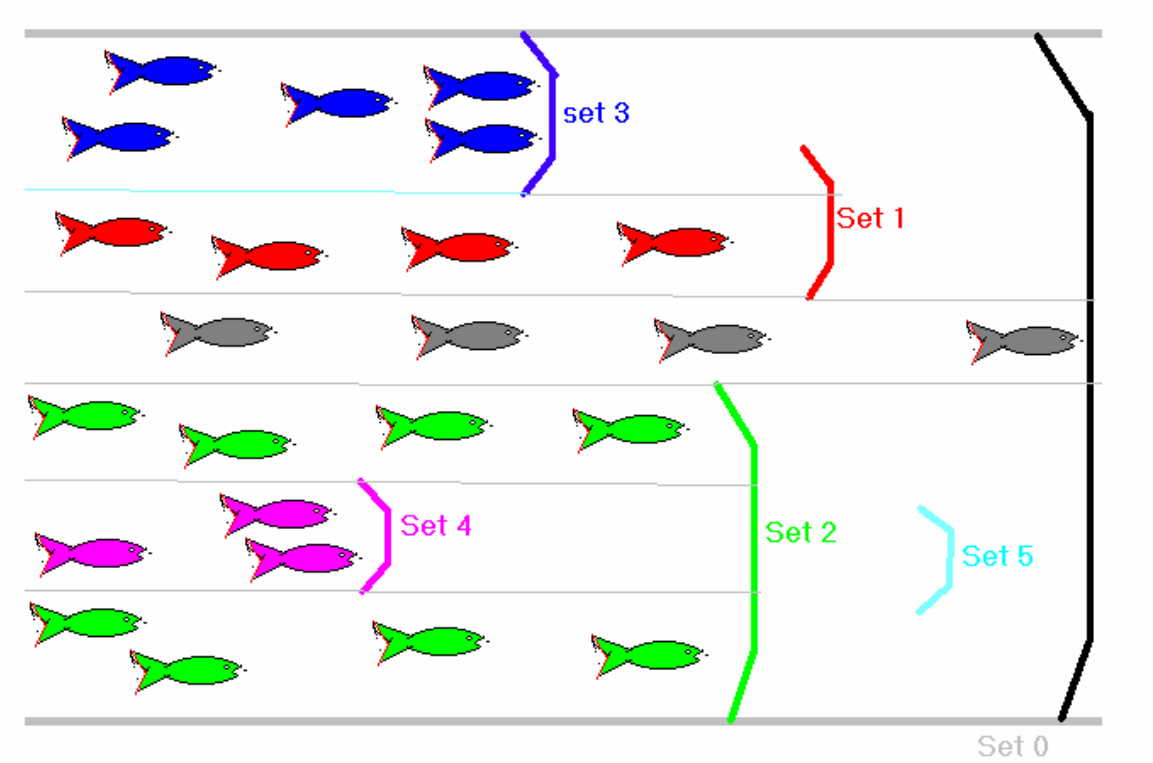
Sporadic particles.

To have a cluster you need at least a reasonable number of particles. But you can only see the data of one sample at a time. The “Particle” window with the pulses has a button “ Fix dot “. When you click this button then a black/white circle is placed around the dots belonging to that particle. These circles stay in the dotplots intil you click “Clear all fixed dots” in the main window. This allows to assemble a set of clusters from multiple samples.

Order of selections, subsets.

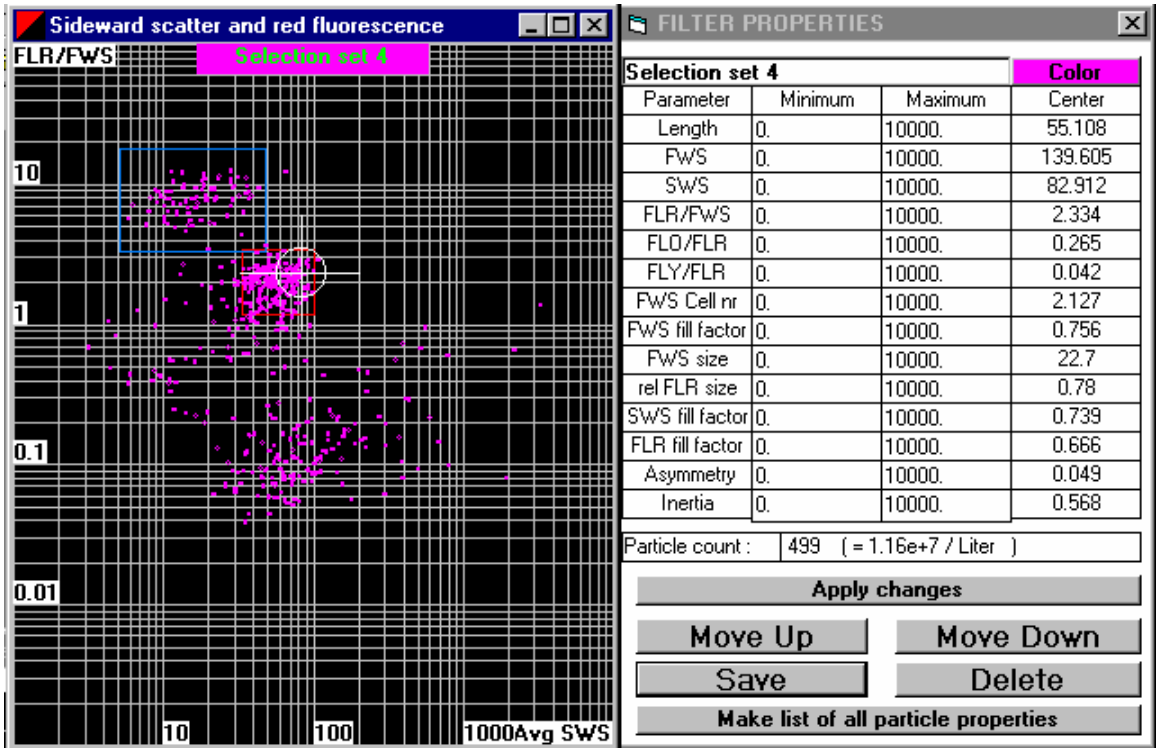
A particle can be assigned to one selection set only and selection is from the bottom up The top is selection set 0, that is the set of all particles When a particle is assigned to a selection set then it is removed from the sets above that set and assigned to the last set that it fits in. The selection sets can be regarded as a series of nets in a stream, that may partially or completely overlap.

Sets and subsets.



The “end net” is selection set 0 and catches every thing NOT catched in the other sets. Set 3 partially overlaps set 1, set 4 is a subset of set 2 and since it is before (below) set 2 what goes in set 4 is excluded from set 2. Set 5 is also a subset of set 2, but fishing “behind a net” it catches nothing. Note that this is a single parameter picture. A real overlap exists only in sofar as it overlaps in all parameters.
Example : make a new selection set (which is automatically the active set) and click on any dotplot to refresh the plots. Surprise: all 499 dots are assigned to the new set, and all sets above it are empty !. What happened ? The new set starts with the widest limits possible so all particles fit in that set and, since it is the last set, are assigned to set 4. Now click on the set name in a dotplot to call the “Filter properties” table of the new set and click the button “Move up” there.

New selection set, filter properties window.



Selection set 4 will move up in the list and swap places with set 3, so that set 3 is now the last one and regains its 115 particles. Now, with set 4 active, make a box around some particles not in sets 1, 2 or 3 and also sets 1 and 2 will regain their particles.

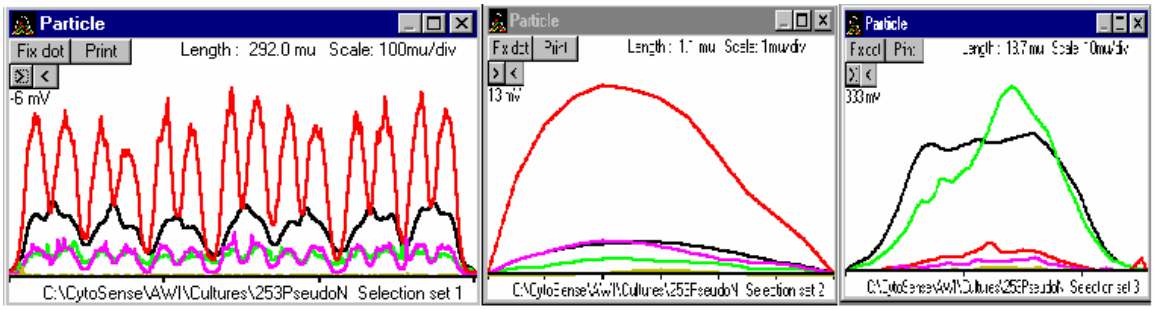
Subsets: Sometimes you select a cluster in one dotplot and see that one of the other plots shows two or more distinct “sub” clusters among the newly selected particles. So, they are not of the same kind and you can split your selection further. Click the button “Copy set” in the main window and you will get a copy of your selection set. Then make a box around one of your “sub” clusters and you will have a subset containing only the particles in that cluster. That it is a subset means that it falls within the set that you copied. To make it an independent set change the parent set that you copied so that it contains only the other cluster. Sets are independent (have no overlap) if their content stays constant if you move them up or down.

What is what ?

Our example is the result of a sample from a culture of Pseudonizschia pungens. We now that it is in there and that it is a chain forming diatom. Nevertheless we found three kinds because there is some contamination in the culture.

Scrolling with the “>” and “<” buttons in the particle pictures through particles in the the selection stets. We see pulses as below :

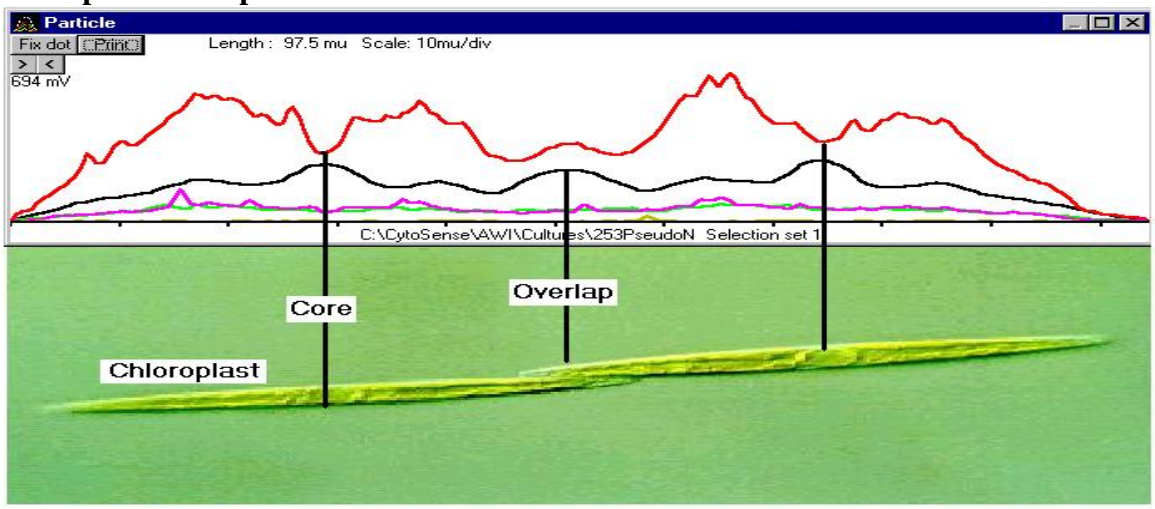
Pulses from the three sets



Selection set 1 selection set 2 selection set 3

In selection set 1 we find pulses like the one above, which is a profile of a neat 7-cell chain, selection set 2 has pulses of very small single cells, set 3 of larger single cells. Selection set 1 is obviously the Pseudonitzchia. The picture below shows what the cells look like and how that is connected to the pulse profile of 2-cell chain in set 1:

Pulse profile and particle

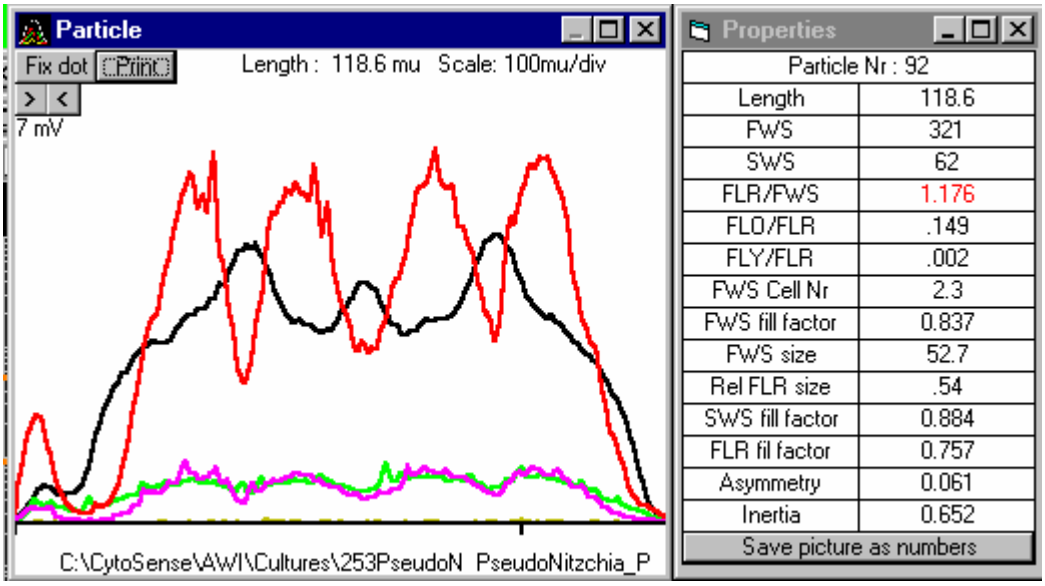


The red line in the pulse is the red fluorescence signal that correponds to the chlorophill in the chloroplasts. The dip in the red fluorescence together with the peak in the forward scatter (black line) result from the cell core passing through the light beam. In the center the overlap where the cells are connected is visible in the fluorescence and in the forward scatter. Of course, knowing what the main content of a sample is and how it looks makes it easy to identify a cluster in the data. The other way around, constructing a possible cell shape from the pulses and then concluding what it might be is not easy. Two remarks must be made here :

1 : The flow pattern in the Cytosense instrument is such that particles are oriented with the long axis along the direction of measurement. But particles go head first or tail first randomly. So mirrored pulses are not different particles.

2 : When flat or rod-like cells stack side by side in a colony the long axis of a sufficiently long colony is perpendicular to the long axis of the individual particles. This can make the pulses from individual cells look quite different from the same cells in the colony.

Why is this particle not selected ?

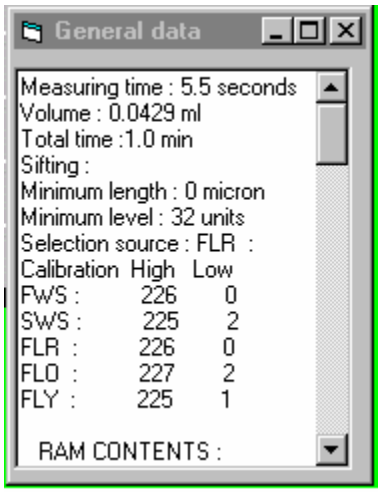


The particle above looks pretty well like P.Pungens, but it was not selected. If you click right in the “Particle” window then a table of the particle properties appears and the properties that fall outside the limits of the active selection set are in red. So, here the FLR/SWS ratio falls outside the Psudonitzschia_P selection set, probably because of the oddity at the left side of the pulse.

Save picture as numbers :
The button at the bottom of the properties window lets you make a file of all datapoints in the pulse. It is a text file that can be imported in EXCEL.

General data :
There is also a “General data” window that shows some data about how the sample was processed in the Cytosense. Topline: effective measuring time and processed volume. Next : the gross time that the process has been running, including the preprocessing in Cytosense. Then the “sifting” criteria, if any were applied. So, for this sample only particles with a minimum red fluorescence (FLR as selection source) of 32 units were selected, with no minimum (0) requirement for the length.

Then follow calibration results that are used by this program : the “high” results are also an indication of an internal temperature in the unit. Values over 250 mean that it was close to the maximum operating temperature. The “low” values are offsets and should be less then about 10. There after follws a list of 64 RAM memory places. These numbers become only interesting when some trouble is suspected.



Saving and reloading selection sets.
One way to tackle the problem of identifying particles is to assemble a library of selection sets based upon cultures and microscopy. Selection set 1 in this example can be resonably renamed “Pseudontzchia_P” and saved as such, using the “Save” button in the “Filter properties” window, and loaded later using the “Load selection Set” button. Both buttons call the standard windows dialog windows.

Note: The “Delete” button in the “Filter Properties” window deletes a selection set from the sets used in the program, not from any library.

When exiting from the program the question is : “Save all selection sets ?”. If you say “yes” then all sets in use will be saved (as plain text files) plus the list of sets in use as SetsList.TXT. When starting the program the question is : “Reload Selection sets ? “. If you answer “yes” then the program will look for the SetsList file in the directory that you choose and reload the listed selection sets in the same order as used the last time.

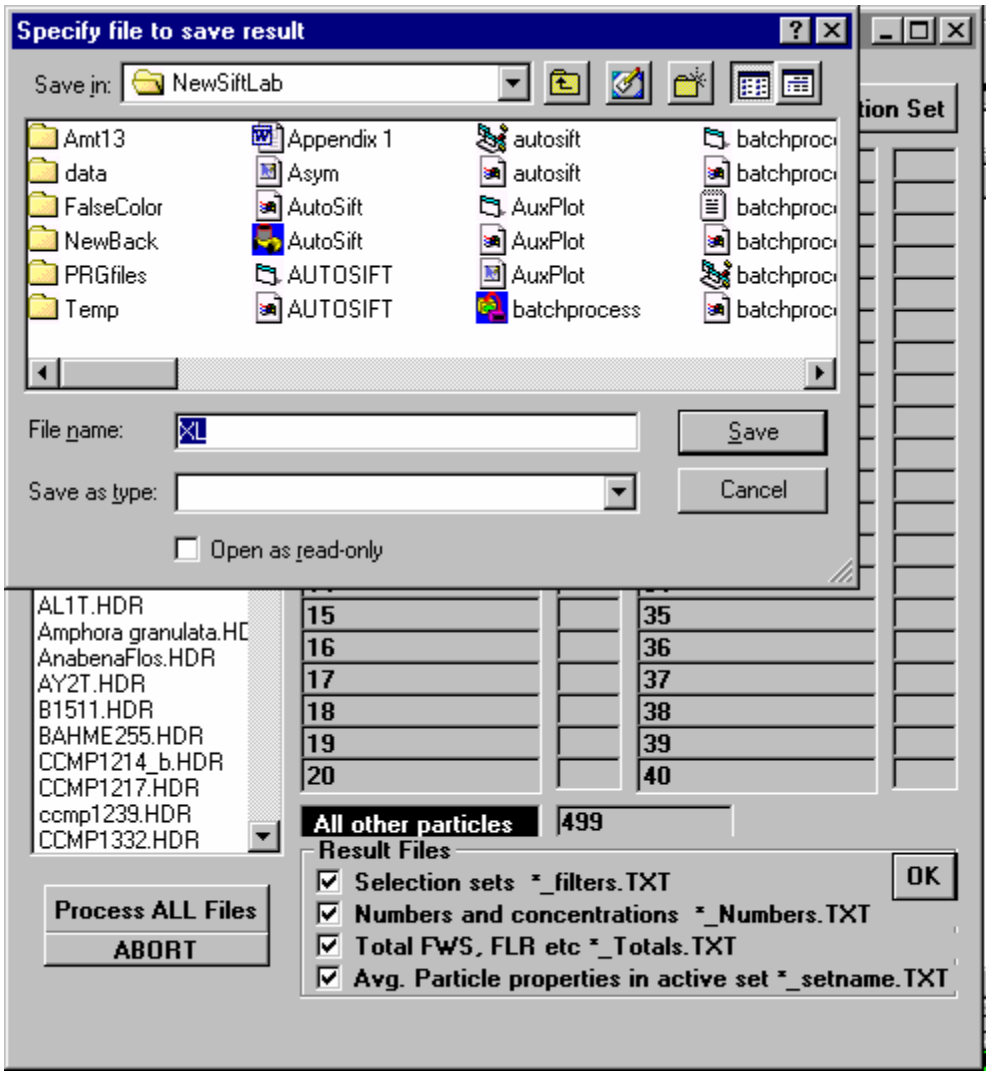
Searching through files : Particularly when looking for sporadic particles with one or two or none in a sample you can use the button ”Search ALL Files” to search through a whole directory for particles fitting in the active selection set. When it finds some it stops and you can view the pulses and fix dots. Click the search button again to resume. “ABORT” stops the search.

Process ALL files : use this button to make text files, readable by EXCEL.

You can produce the following files :
Name_Filters.txt, a description of all used selection sets
Name_Numbers.txt, a list of relevant instrument settings and of numbers and concentrations per sample and per selection set.
Name_Fluor.txt, a list of total red, orange and yellow fluorescence per sample and per selection set.

Name_active selection set.TXT: a list per sample of the average properties of the particles within the active selection set.

Click on “Process ALL Files” and the “Result Files” box appears. Here you can choose which files you want to make. Then click the “OK” button and the “Specify file to save result” dialog appears to choose a directory for saving and to type a Name for the files.



Note : When you continue in a new directory, but keep the Name the same then the files are NOT overwritten, but appended, so you can combine directories in a single report.

“Listmode” file of selected particles :
The “Filter properties “ window has a button “**Make list of all particle properties”.** This button lets you make a list of the particle properties, per particle, of all the particles in the active selection set. It is saved as Name_selectionset.TXT.

Manipulating Windows:

There are many windows generated by this program. Initially these are all “stacked” but it is convenient to view at least a selection together, tiled on the screen. When you click right on an empty place of the taskbar that shows the icons then you can choose the option “Tile windows vertically” or “Tile windows horizontally”. This will tile all visible windows, so first remove windows that you do not want to see permanently.

Note: Most windows in this program have processing software attached so they have to stay active. You can not close them but must minimise them instead and then can recall them by clicking on the icon. A window that you recall after tiling the others will appear on top.

Selecting outside windows:

Very small or exceptionally large particles may fall ouside some dotplots. Note that when you draw a rectangle for a selection set that you can go with the mouse pointer **outside** the picture to set a limit beyond the range of the plot. In the Filter properties window you can also set limits beyond the range of the dotplots.