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Flow cytometry data handling and analysis

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Principle of Flow Cytometry

Fluidics

- Cells in suspension
- Cells flow in single-file

Optics

- Intercepted by light source(s) (laser)
- Scatter light and emit fluorescence
- Signal collected, filtered and

Electronics

- Converted to digital values
- Storage on a computer

Data display and analysis

Let’s start from the very beginning

Data acquisition process in flow cytometry

- Comprises all the operations required to measure one or several specified characteristics of particles (cells)
- Conversion of the data to a numerical form for manipulation and storage (by a computer *)*.

Data analysis in flow cytometry

• Includes any operations used to convert measured values of the physical characteristics into information about the (biological) characteristics of some or all the particles (cells) in the sample.

•Methods depend about the data acquired and about what the experimenter wants to now.

Some Flow Cytometer Companies

- Advanced Analytical Technologies, Inc. (USA)
- Agilent Technologies (USA)
- Apogee Flow Systems (UK)
- BD Biosciences (USA)
- Beckman Coulter (USA)
- BioDETECT AS (Norway)
- Bentley Instruments (USA)
- Chemunex SA (France)
- CytoBuoy b.v (Netherlands)
- Cytopeia (USA)
- DakoCytomation (USA)

- Delta Instruments bv (Netherlands)
- Fluid Imaging Technologies, Inc. (USA)
- FOSS Electric A/S (Denmark)
- Guava Technologies, Inc. (USA)
- Howard M. Shapiro, M.D., P.C. (USA)
- iCyt- Visionary Bioscience (USA)
- International Remote Imaging Systems (USA)
- Luminex Corporation (USA)
- NPE Systems, Inc. (USA)
- One Lambda, Inc. (USA)
- Partec GmbH (Germany)
- Union Biometrica, Inc. (USA)

Listed from Practical Flow Cytometry 4th Edition (H. Shapiro)

Data Format … Toward a Standard?

- Need to provide a **clearly defined** and **uniform file format** that allow data collected by one instrument to be correctly read for analysis by other software on another computer.

↓

- Data stored and saved under a Flow Cytometry Standard (.FCS) file

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From Flow Cytometry Standard (FCS) 1.0 to 3.0 …

FCS 1.0	**1984**	Murphy and Chused (Cytometry 5:553-555)
FCS 2.0	**1990** FCS 1.0 revised by the Data File Standards committee	Society for Analytical Cytology - now called ISAC- (Cytometry 11:323-332)
FCS 3.0	**1997** FCS 2.0 revised →Handle data files > 100 MB →Support UNICODE text for keyword values	Seamer *et al* (Cytometry 28:118-122)

Structure of a FCS file

- **Structure in 3 or 4 segments**
  - **Header:**
    - Identify the file as an FCS file and specify the version of FCS used
    - Contain numerical values identifying the position of the following TEXT segment.
  - **Text:**
    - Several Keywords and numerical values used to describe the sample and the experimental conditions
  - **Data:**
    - Numerical values in a format specified in the TEXT segment
  - **(Analysis:** Optional**)**
    - Same structure as the Text segment
      - Example : Results from cell cycle analysis

Example of FCS file

Header

```
FCS2.0          256     2419     8192     22640
```

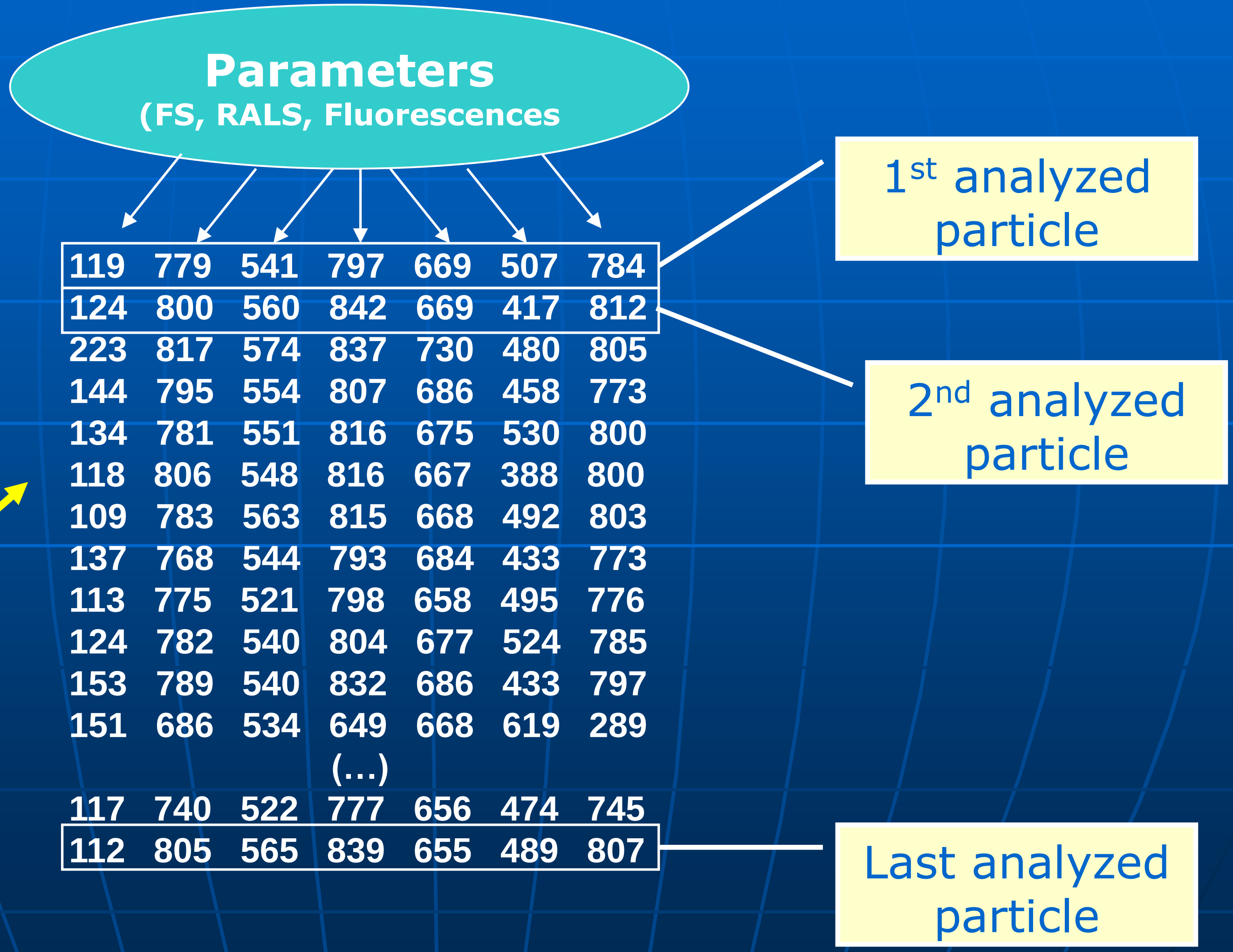
Text

```
$P1N:        FS   Peak
$P1S:        FS   Peak
$P1R:        1024
$P1B:        16
$P1V:        550
$P1GAIN:     15.000000
$P1PGAIN:    3.000000
@P1ADDRESS:10
$P1E:        0,0
@P1X:          0.0,   0.0
@P1U:
@P1C:        ARITHMETIC
@P1Z:        ON
$P1Q:        FS   Peak
$P2N:        PMT2   Log
$P2S:        PMT2   Log
$P2R:        1024
$P2B:        16
$P2V:        880
$P2GAIN:     5.000000
$P2PGAIN:    5.000000
@P2ADDRESS:   15
$P2E:          4.0,0.1024
@P2U:
@P2C:        GEOMETRIC
@P2Z:        ON
$P2Q:        PMT2   Log
```

```
$P3N:        PMT3   Log
$P3S:        PMT3   Log
$P3R:        1024
$P3B:        16
$P3V:        740
$P3GAIN:     5.000000
$P3PGAIN:    5.000000
@P3ADDRESS:19
$P3E:          4.0,0.1024
@P3U:
@P3C:        GEOMETRIC
@P3Z:        ON
$P3Q:        PMT3   Log
$P4N:        PMT4   Log
$P4S:        PMT4   Log
$P4R:        1024
$P4B:        16
$P4V:        796
$P4GAIN:     5.000000
$P4PGAIN:    5.000000
@P4ADDRESS:   23
$P4E:          4.0,0.1024
@P4U:
@P4C:        GEOMETRIC
@P4Z:        ON
$P4Q:        PMT4   Log
$P5N:        FS   Log
$P5S:        FS   Log
$P5R:        1024
```

```
$DATATYPE: I
$EXP:
$PROJ:
$INST:       Purdue   University   Cytometry
Labs
$INSTADDRESS:
$LOCATION:
$RUNNUMBER:              964
@FILEGUID: E53F8C1E65D8D7119D9D0004
$OP:         kathy
$CYT:        Beckman   Coulter   EPICS   Altra
$SMNO:       964
$SRC:
$CELLS:
$BTIM:       11:37:14
$ETIM:       11:38:15
$DATE:       27-Aug-03
@Y2KDATE:  20030827
@BASELINEOFFSET:         OFF
$DFC2TO1:  0.000
(…)
$DFC5TO6:  0.000
@SAMPLEID1:              Euglena
@SAMPLEID2:
@SAMPLEID3:
@SAMPLEID4:
@COMPENSATIONMODE:  Advanced
@ABSCALFACTOR:           NOT   SET
TESTNAME:  euglenaSort
TESTFILE:    euglenaSort
@CYTOMETERID:
$FIL:        Euglena   00000964   002.LMD
```

Example of FCS file (next)



Parameters

(FS, RALS, Fluorescences

119	779	541	797	669	507	784
124	800	560	842	669	417	812
223	817	574	837	730	480	805
144	795	554	807	686	458	773
134	781	551	816	675	530	800
118	806	548	816	667	388	800
109	783	563	815	668	492	803
137	768	544	793	684	433	773
113	775	521	798	658	495	776
124	782	540	804	677	524	785
153	789	540	832	686	433	797
151	686	534	649	668	619	289
			(…)			
117	740	522	777	656	474	745
112	805	565	839	655	489	807

1st analyzed particle

2nd analyzed particle

Last analyzed particle

Data

3 formats:
- List mode
- Correlated
- Uncorrrelated

Software Sources

- Flow cytometer manufacturers

- Commercial software sources

De Novo Software → FCS Express
http://www.denovosoftware.com

Management Sciences Associates → MacLAS & WinLAS
http://www.msa.com

Phoenix Flow Systems → MultiCycle AV, Win-FCM, MultiTime , etc.
http://www.phnxflow.com

Ray Hicks → FCSPress (Macintosh)
http://www.fcspress.com

Tree Star, Inc. → FloJo
http://www.flowjo.com

Verity Software House → WinList, ModFit, IsoContour
http://www.vsh.com

Non Commercial Software Sources

- Autoklus (T. Bakker Schut)
  - http://flowcyt.cyto.purdue.edu/flowcyt/software.htm
- Cylchred (T. Hoy)
  - http://www.uwcm.ac.uk/study/medicine/haematology/cytonetuk/documents/software.htm
- CYTOWIN (D. Vaulot)
  - http://www.sb-roscoff.fr/Phyto/cyto.html#cytowin
- Flow Explorer 4.0 (R. Hoebe)
  - http://wwwmc.bio.uva.nl/~hoebe/Welcome.htm
- IDLK (R. Habbersett)
  - robb@beatrice.lanl.govj
- MFI (E. Martz)
  - http://www.umass.edu/microbio/mfi/
- RFlowCyt (T. Rossini)
  - http://software.biostat.washington.edu/wikis/front/RFlowCyt
- Soft Flow Hungary, Ltd.
  - http://www.visi.com/~soft-flow/
- WinMDI (J. Trotter)
  - http://facs.scripps.edu/software.html

See Tutorial on your free CD-ROM

Flow Cytometry Software? What for?

- **Display flow cytometry data** (1D, 2D, and 3D displays)

- **Identification of cells of interest**

- Define a cluster ➔ *Region*
- Mixed populations and noise ➔ *Gating*

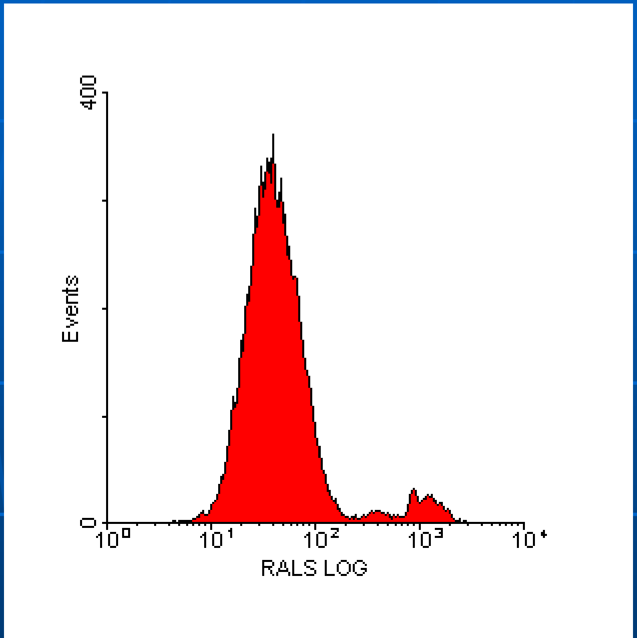
- **Characterization of cells of interest**

- Intrinsic parameters (mean/median scatter and fluorescence intensities ; positive/negative cells)
- Cell counts (abundance)
- Kinetics (evolution of a cell parameter with time)
- Cell cycle analysis

Classical Data Analysis: Various types of data displays

- Frequency distribution
- Dot plot
- Density plot
- Contour plot

Frequency distribution



Histograms display the distributions of the Events for one parameter.

➔ Simplicity of the plot

➔ No correlation with the other parameters

➔ Problem for cluster identification

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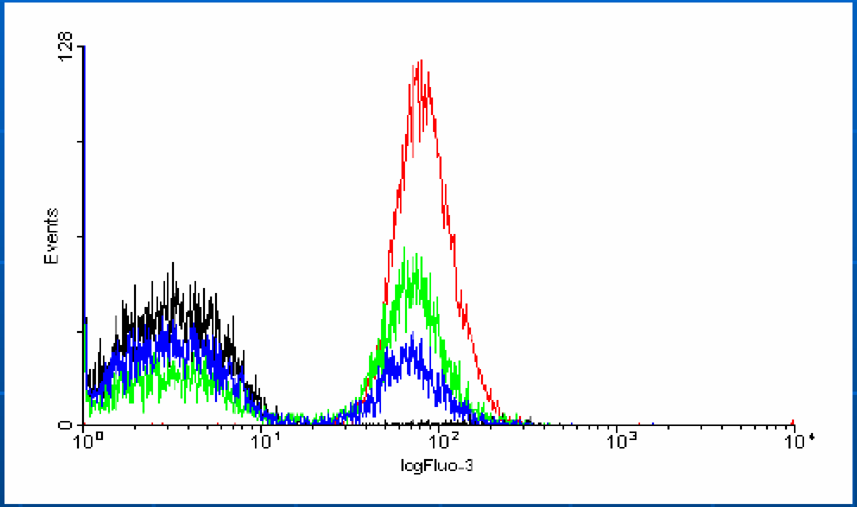
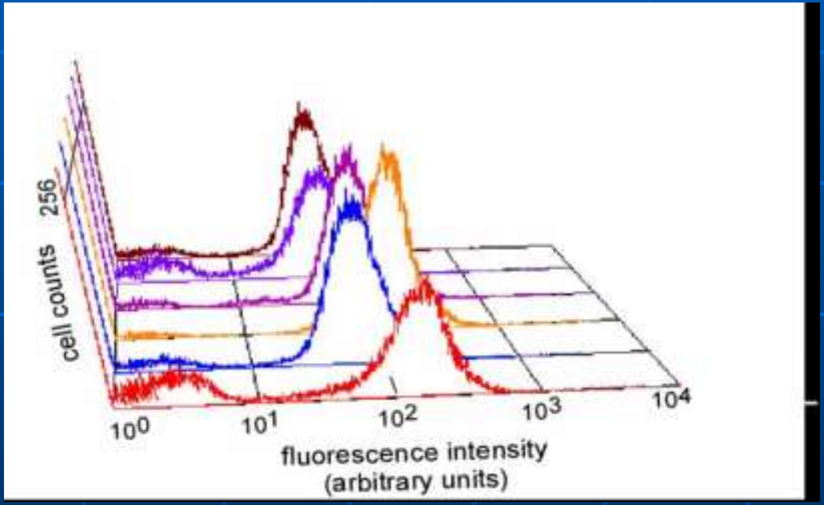
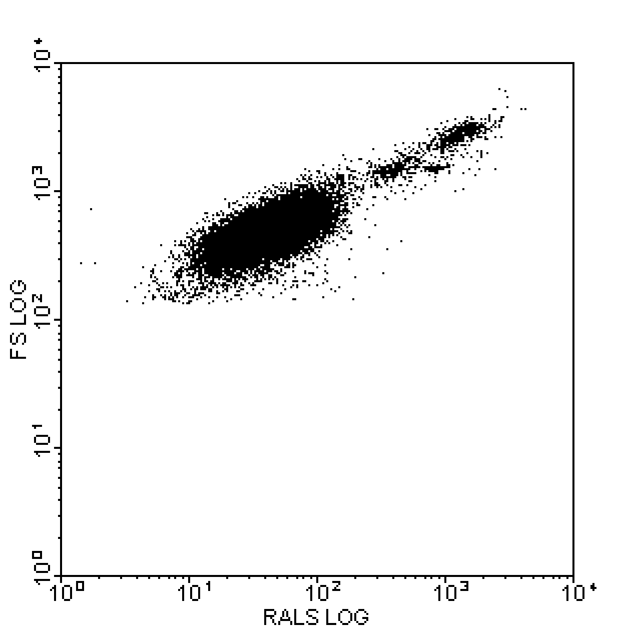
Histogram overlay





Superimpose the data from several data files

Dot plot



- Displays correlated data from any two parameters.

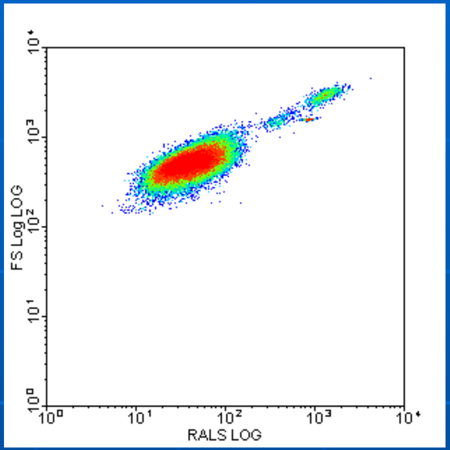
- Each dot corresponds to a particle (event) analyzed by the flow cytometer.

- Several events can occupy the same dot if they have the same parameter intensities.

➔ No indication of the relative density of the events

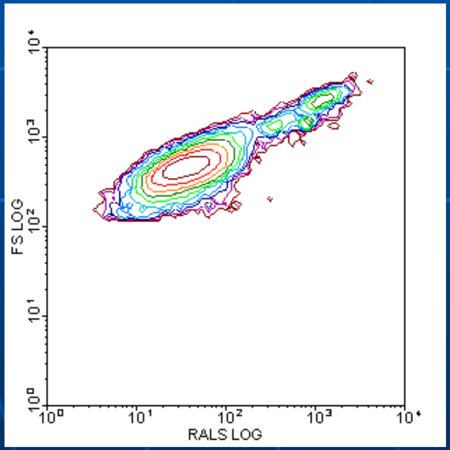
→ Problem with large data files

Density and Contour plot



Density plot:

- Displays two parameters as a frequency distribution.
- Color is used to code the different frequencies of events.



Contour plot:

- Displays correlated data from any two parameters, with contour lines joining points of equal elevation (frequency distribution).

➔ Simulation of a 3D display with a " *third* " parameter being the number of events.

→ Can clarify clusters

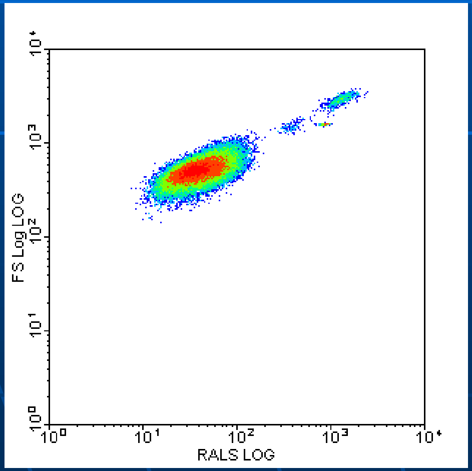
Danger!!!

With Density plots and Contour plots some options like

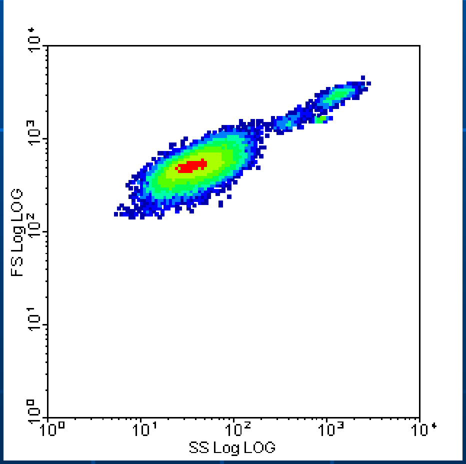
- -Resolution
- -Smoothing

can emphasize or hide clusters of cells.

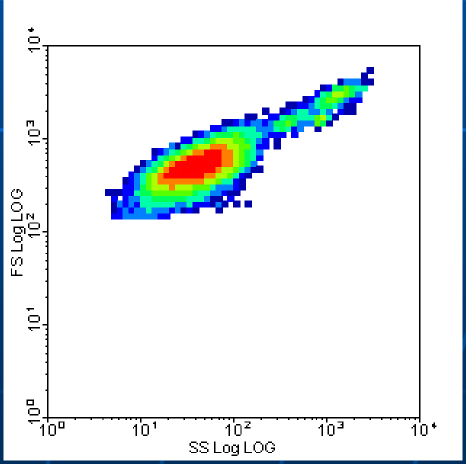
Example : Changing Resolution



256x256

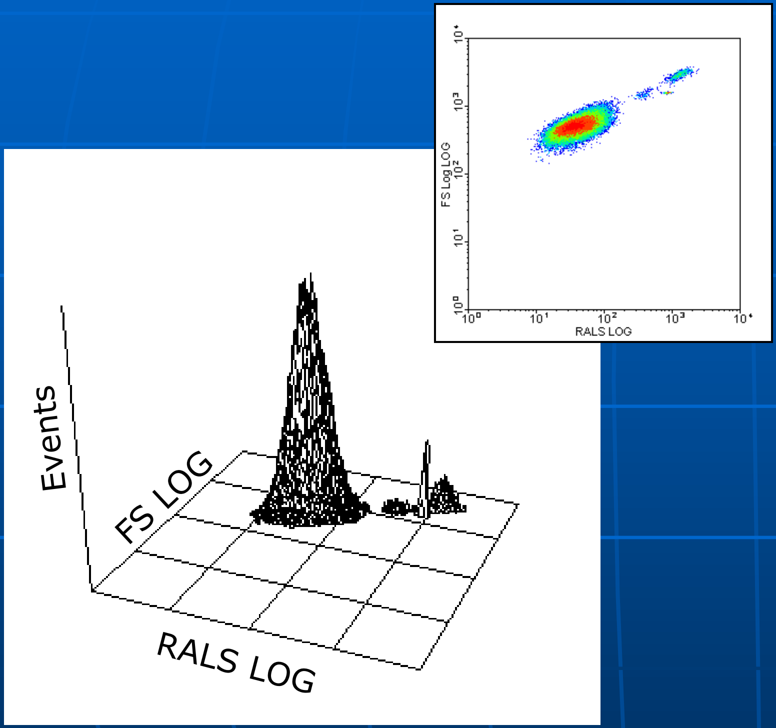


128x128

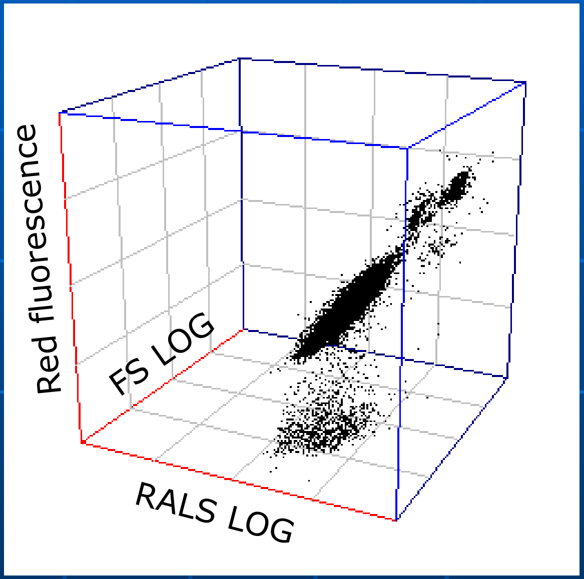


64x64

3D Displays



2 parameters *versus* density

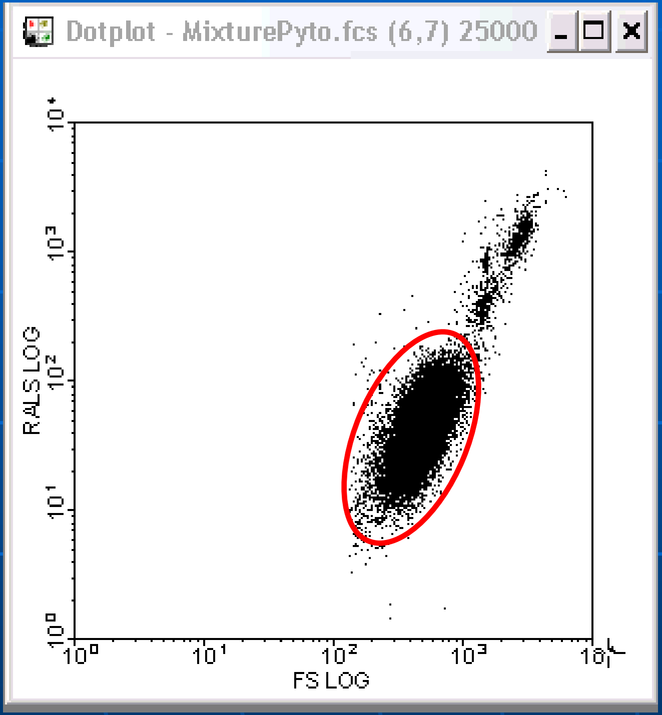


3 parameters displayed together

Particle (cell) Discrimination

- Problem :
  - Very often, samples are heterogeneous
    →there are events which are not of interest
    (other cells, debris, electronic noise).
  - Several clusters of interest mixed together
- Solution :
  - Discriminate the cells of interest.
  - Need to exclude the unwanted events from the analysis.

What is a Region?

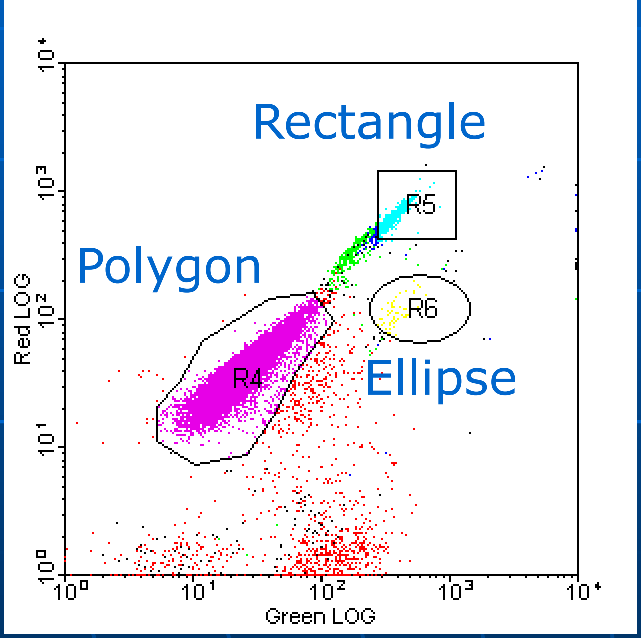


A **region** can be defined as set of points carefully selected by the user that determine an area on a graph.

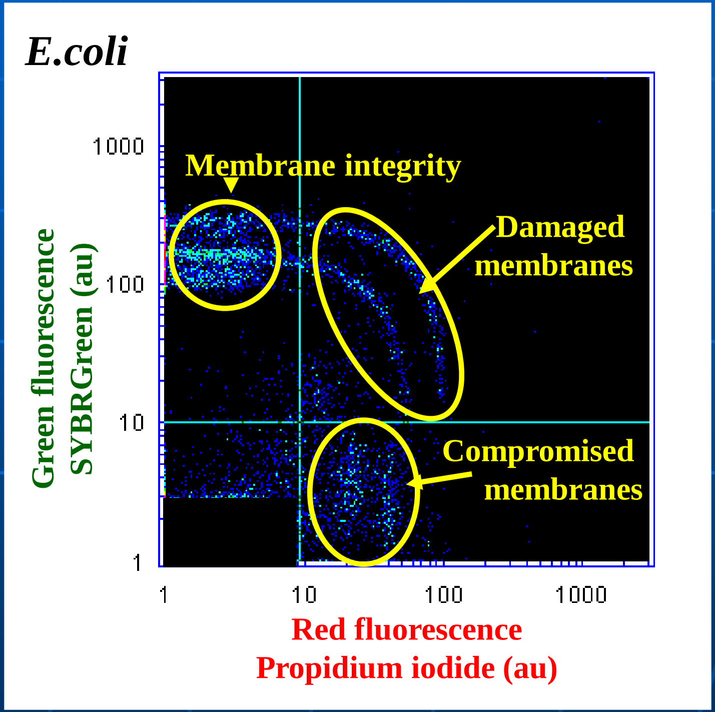
Several regions can be defined on the same graph.

➔ Isolate the cluster(s) of interest
➔ Better discrimination of the cluster(s) using color

Different styles of regions

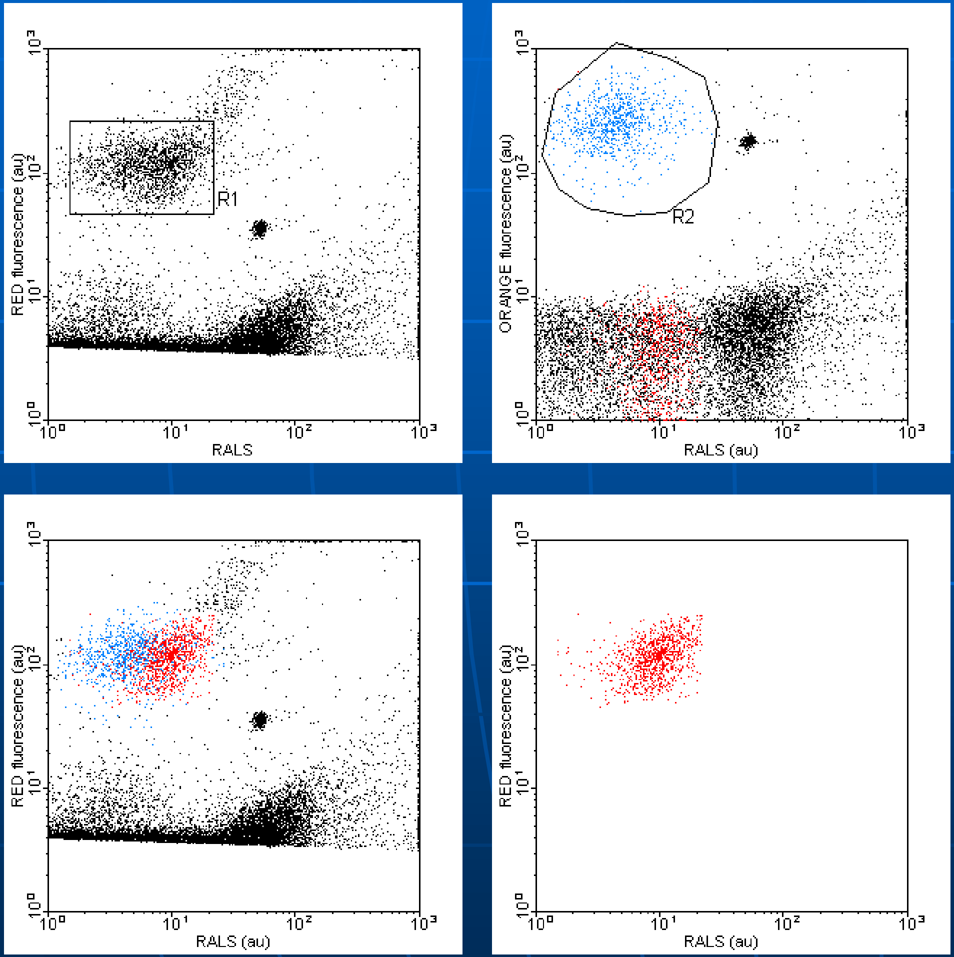


Cluster discrimination



Positive/Negative cell identification

What is a Gate?



A **gate** can be defined as one or more regions combined using Boolean (logic) operators (AND, NOT, OR)

Defines a subset of the data to be displayed.

- Used to compute **statistics** and characterize the subset of events selected
- Get rid of noise and save space on disks

Statistics

Prior the statistical analysis of the clusters, consider these two factors :

1. **Sample size**:

The precision of the statistical analysis depends on the number of cells analyzed (Poisson Law → Std Deviation = √(n))
When the number of events increases the coefficient of variation of the estimate decreases.

2. **Incorrect choice of statistics** impacts the relevance of the results.

The mean(s)

The **mean** = one of the most widely used statistics in flow cytometry.
Gives the **average intensity** of a parameter in a population.

⬇

Two types :

➔ the **arithmetic** mean

➔ the **geometric** mean.

⬇

Choosing the wrong one can impact the results.

Some definitions

- Arithmetic Mean (“average”)
  - Sum of the “n” individual values of a group divided by n

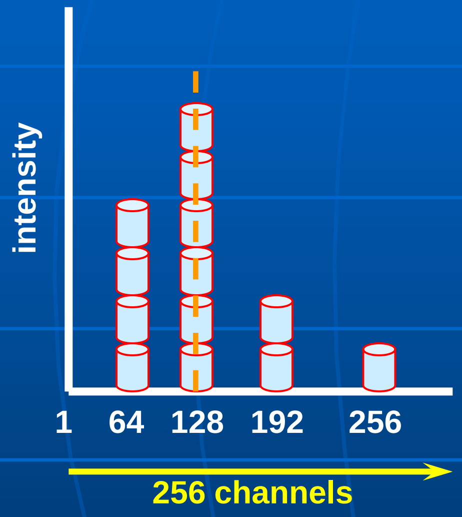
$$\text{Arithmetic mean} =(V_1 + V_2 + V_3 \ldots +V_n)/n$$

- Geometric Mean
  - Multiply the “n” individual values of a cluster together and get the nth root of this product.

$$\text{Geometric mean} = \sqrt[n]{(V_1 \text{ x } V_2 \text{ x } V_3 \ldots \text{ x}V_n)}$$

What does it mean?

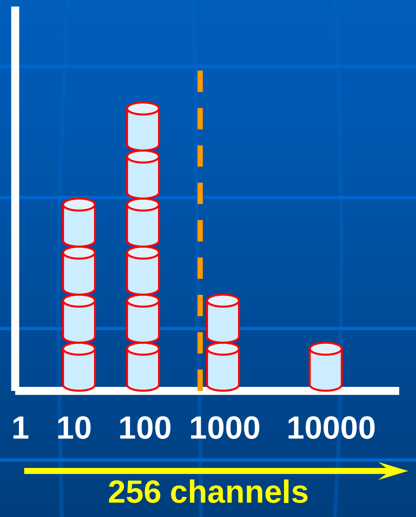
Linear scale



Arithmetic mean:

$$\frac{4x64 + 6x128 + 2x192 + 256x1}{13} = 128$$

Logarithmic scale

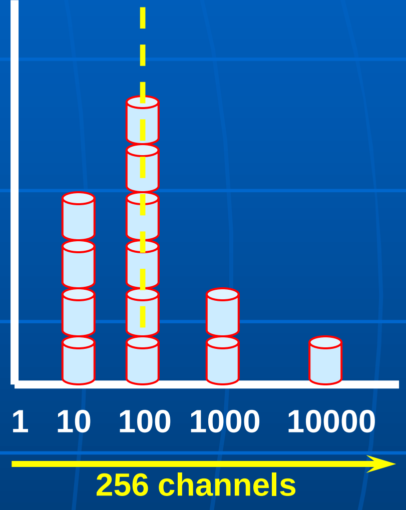


Arithmetic mean:

$$\frac{4x10 + 6x100 + 2x1000 + 10000x1}{13} = 972.30$$

→ **NOT display resolution dependent**

Sensitive to small numbers of events in the higher decades



Geometric mean:

$$\sqrt[13]{10^4 x100^6 \text{ x } 1000^2 \text{ x } 10000^1} = 100$$

→ **Display resolution dependent**

The median

- Frequently used to describe flow cytometry data.

- Refers to the point at which 50% of the events are on either side of a particular channel. *Example : the 2501st cell in a population of 5001.*

- If population normally distributed : Median = Mean = Mode

- Median shifted to a higher intensity value than the mode if the population distribution is skewed to the right and shifted to a lower intensity if skewed to the left.

If data pile up in the last channel, how far off scale are they ?

→ Impossible to get a true mean value

→Median gives a better information about the central tendency of the population

→ If more than half the population is off-scale, then median and mean cannot give the central tendency of the population.

Other Statistics

Standard Deviation (Sd)

Measures the spread of a distribution
= the dispersion of the values from each event around the mean of a population.

Coefficient of Variation

Defined as the (Standard Deviation /mean) X100.
➔ CVs are always a percentage
➔ Measure of the peak width.

Mode

The mode is the most frequently occurring value in a data range.
If symmetrical distribution, then mode = mean = median
If the distribution is skewed, then these three values are different.

Skewness

Characterizes the asymmetry of a distribution ➔ So it is related to the mean value of the population.
If Value < 0 ➔ asymmetrical distribution ➔ tail towards the left ➔ lower values with respect to the mean.
If Value > 0 ➔ tail towards the right ➔ higher values with respect to the mean.

Kurtosis

Kurtosis refers to the relative “flatness” of a distribution and is also related to the mean of the distribution.
A Value<0 ➔ relatively flat distribution,
A Value>0 ➔ a relatively peaked distribution
} compared to the normal distribution

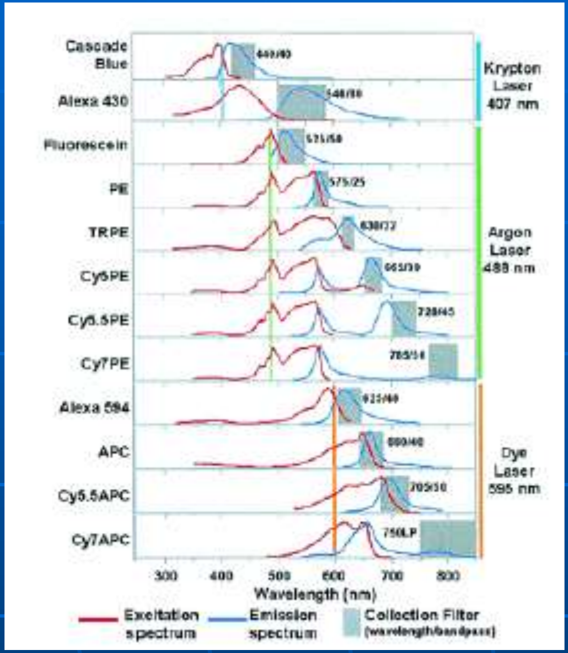
Flow Cytometry : next generation?

- **New technologies** available for Flow Cytometry:
  - light sources (LEDs ; solid state lasers);
  - photodetectors (multichannel PMTs ; avalanche photodiodes);
  - Fast electronic;
  - Compact size;
  - Cheaper
  - New fluorescent compounds (organic dyes; nanocrystals)
- **New computer** (faster; more memory)

- More data collected per particle (cell) → more Multiparametric than ever
- New data types (spectra; volume; etc.)

Some examples…

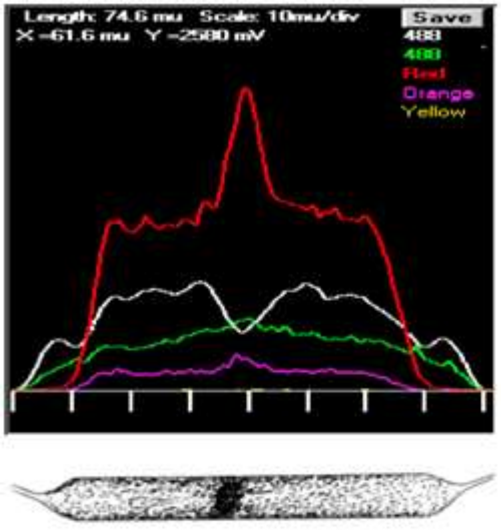
Eleven Colors



Excitation and emission spectral bands of dyes, lines of lasers, and types of various bandpass filters necessary to perform an 11-signal analysis.

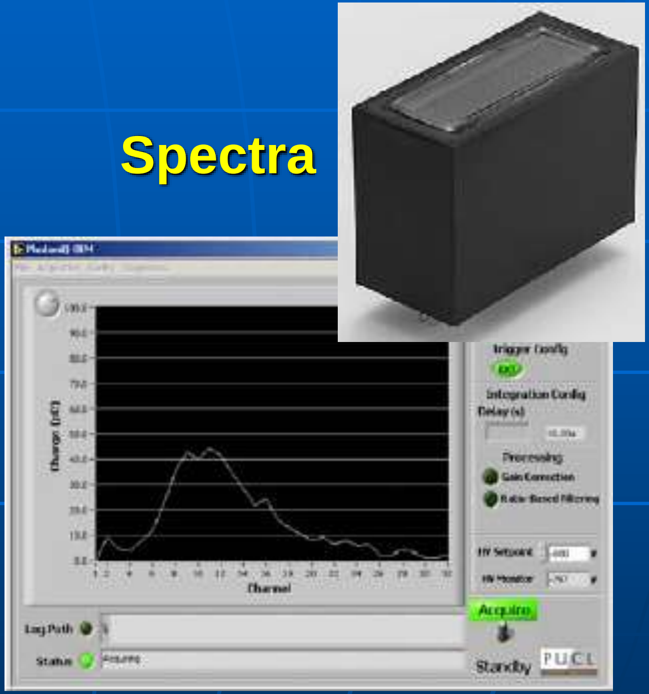
Figure from **De Rosa,S.C. & Roederer,M.** Eleven-color flow cytometry. A powerful tool for elucidation of the complex immune system. *Clin. Lab Med.* **21**, 697-712, vii (2001).

Profiles



CytoBuoy raw pulse data
From George Dubelaar
http://www.cytobuoy.com/

Spectra



32 fluorescence channels
Collected for each
single particle

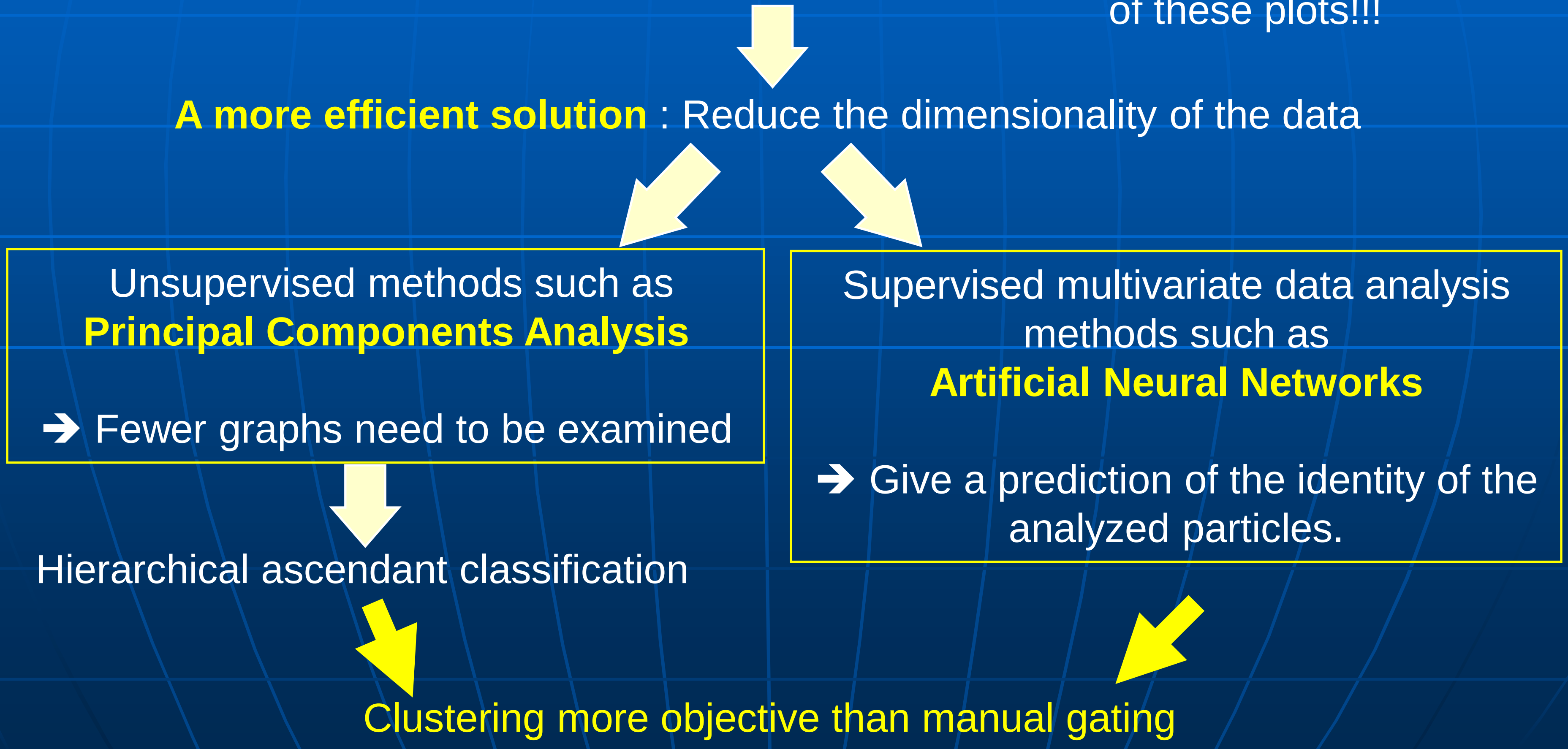
Purdue University Cytometry Laboratories

(Lafayette, Indiana USA)

Multivariate Methods for multiparametric data analysis

Traditionally, single and dual-parameter plots are used to visualize FCM data.

Problem : For a data set defined by 7 parameters ➔ one should examine 21 of these plots!!!



A more efficient solution : Reduce the dimensionality of the data

Unsupervised methods such as **Principal Components Analysis**

➔ Fewer graphs need to be examined

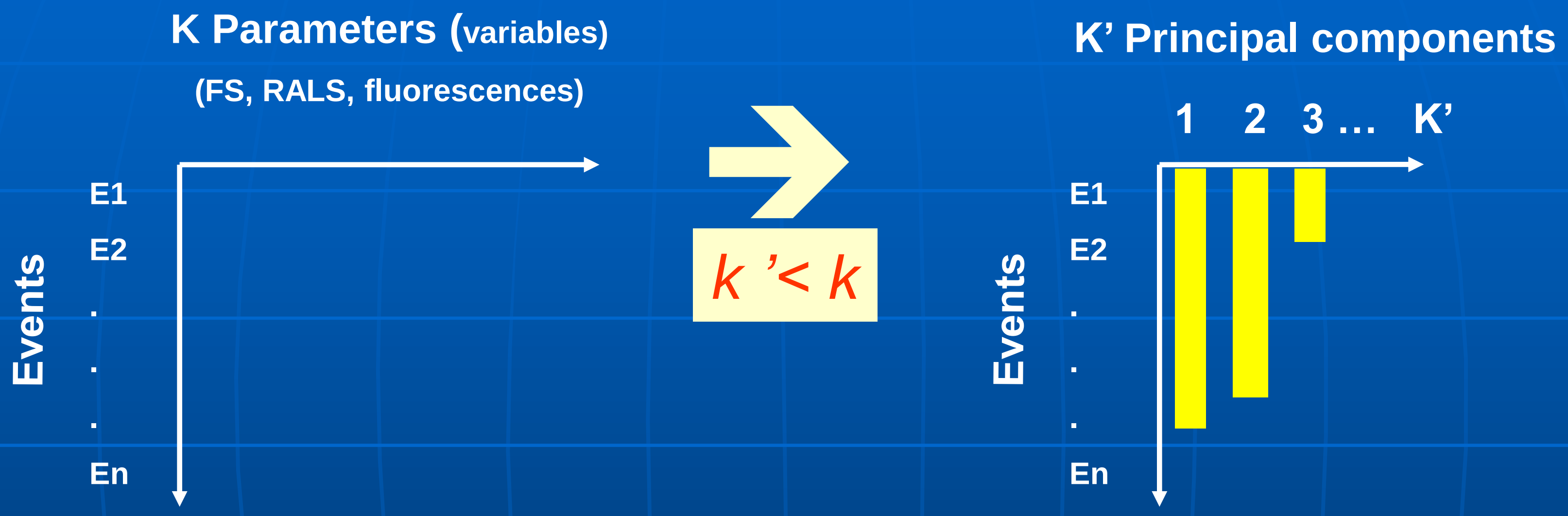
Hierarchical ascendant classification

Supervised multivariate data analysis methods such as **Artificial Neural Networks**

➔ Give a prediction of the identity of the analyzed particles.

Clustering more objective than manual gating

Principal Component Analysis



Principal Components Analysis :

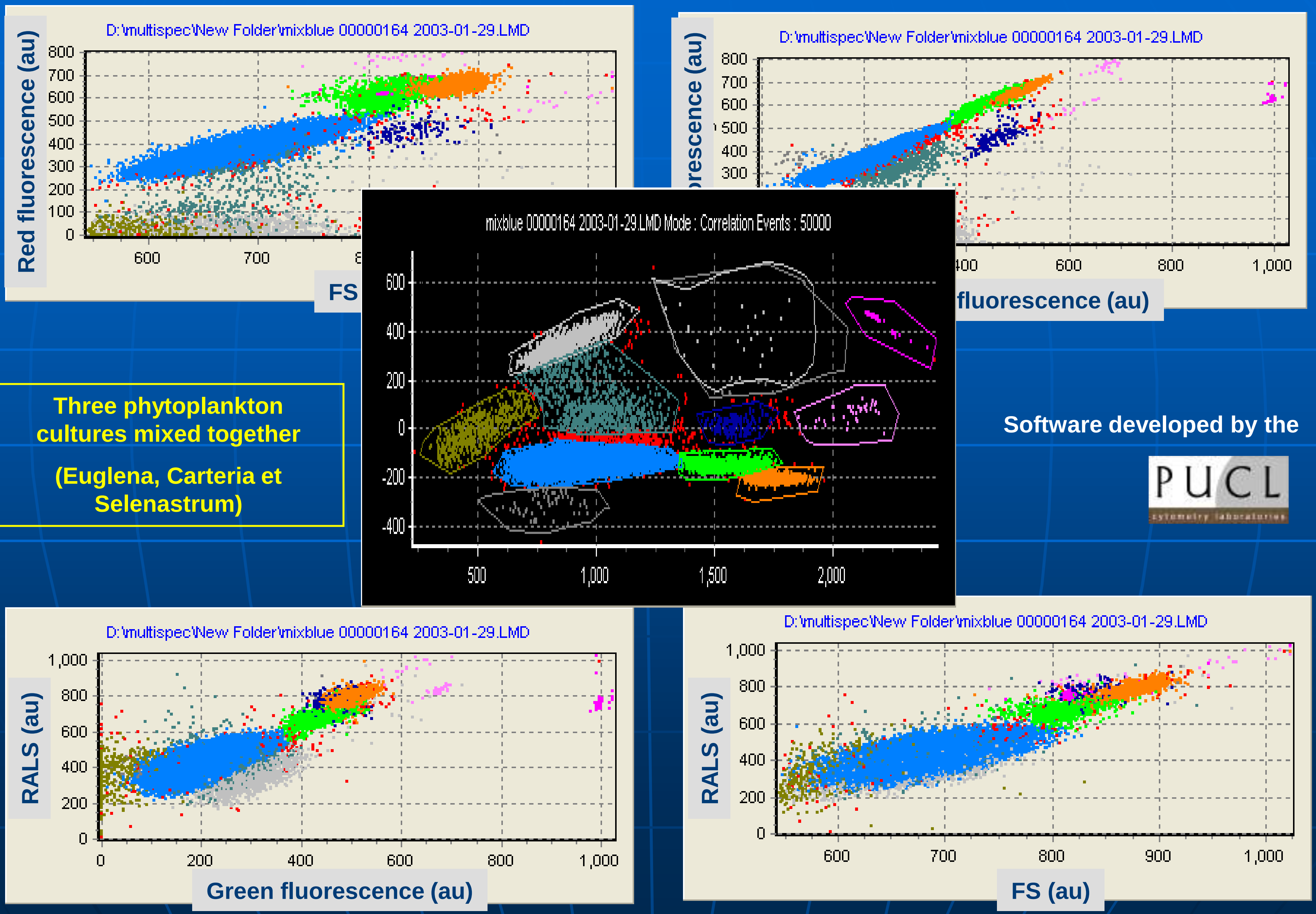
- Computation of **new variables** = **Linear combination** of the old ones (parameters)

  → The 1st new variable accounts for most of the variation (variance) in the data

  → The 2nd new variable accounts for the next most, and so on.

= Translation and rotation of the coordinate axes
(axes remain orthogonal to each other)

Example of PCA



Three phytoplankton cultures mixed together

(Euglena, Carteria et Selenastrum)

Software developed by the

PUCL
cytometry laboratories

Artificial Neural Network: Kohonen Self Organizing Map (SOMs)

- SOMs are "unsupervised classifier systems“

- SOMs provide a straightforward mapping of points from a “n” dimensional space (input) into a 2-dimensional space (output)

  → Output = regular array of nodes (neurones)

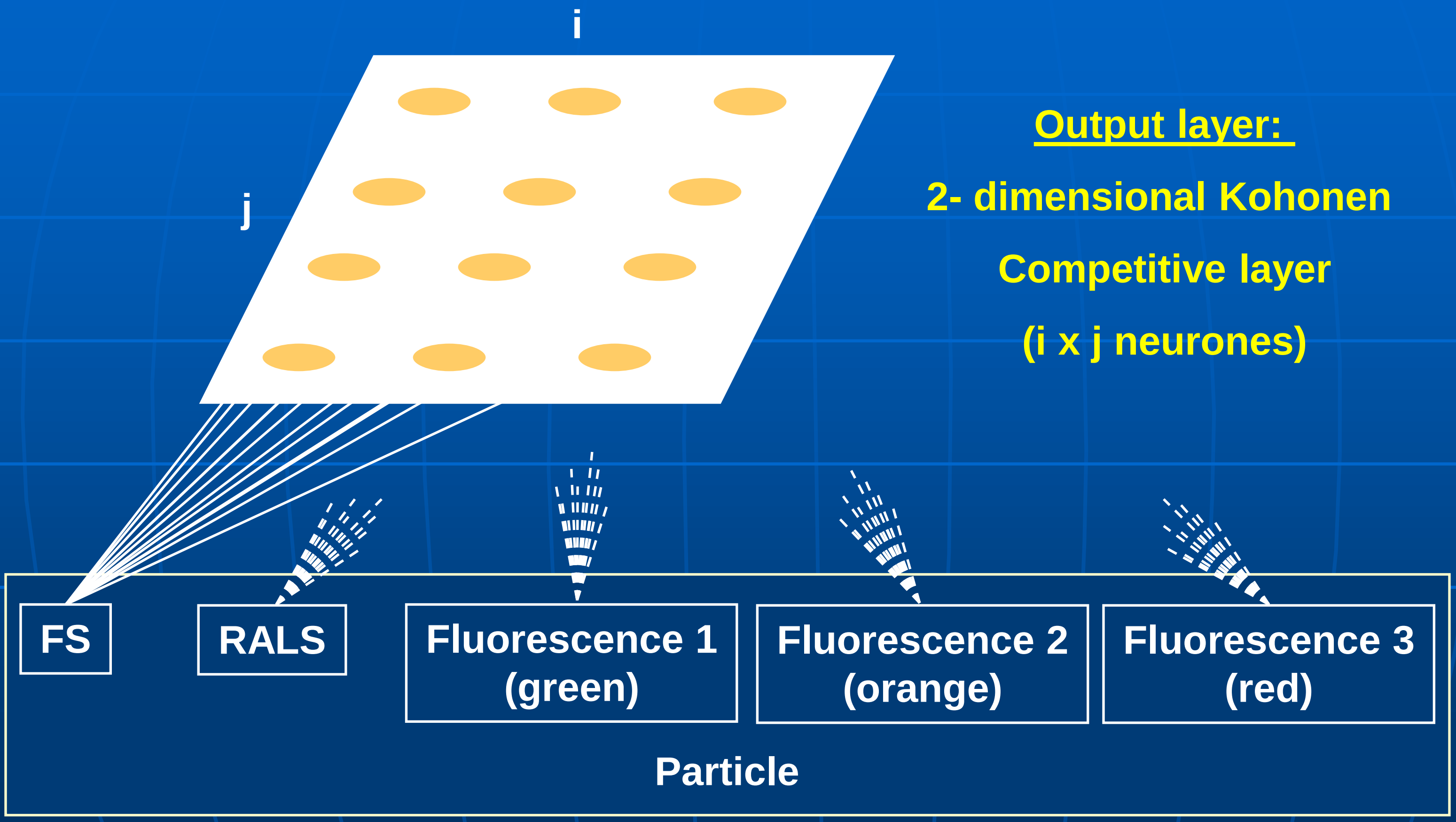
- Preservation of the same spatial relationships among points in the 2 spaces (topology conservation)

- Input space = flow cytometric variables (parameters)

- Output nodes (neurones) = the classes potentially available for the observed events (particles).

The original SOMPAK suite of programs can be downloaded for free at : http://www.cis.hut.fi/nnrc/som_pak/).

SOMs in brief…



Output layer:

2- dimensional Kohonen

Competitive layer

(i x j neurones)

input layer:

FCM parameters

SOMs principle

- A weight matrix connecting locations in the input and output spaces is calculated in a preliminary phase called “Learning phase”.
  - a large number of points is considered in the input space and the best mapping of those points is done in the output space (this step is repeated thousands of times)

- Once this phase is completed, any new observation (particle) in the input space is directed to a specific location (**classification**) in the output map by means of the weight matrix

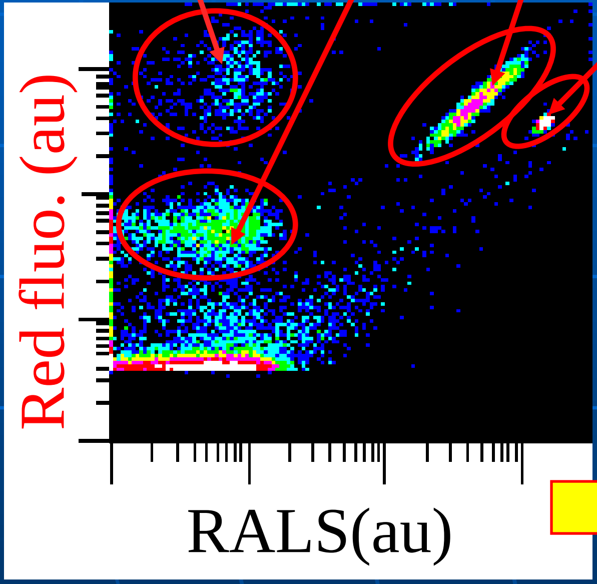
Some results

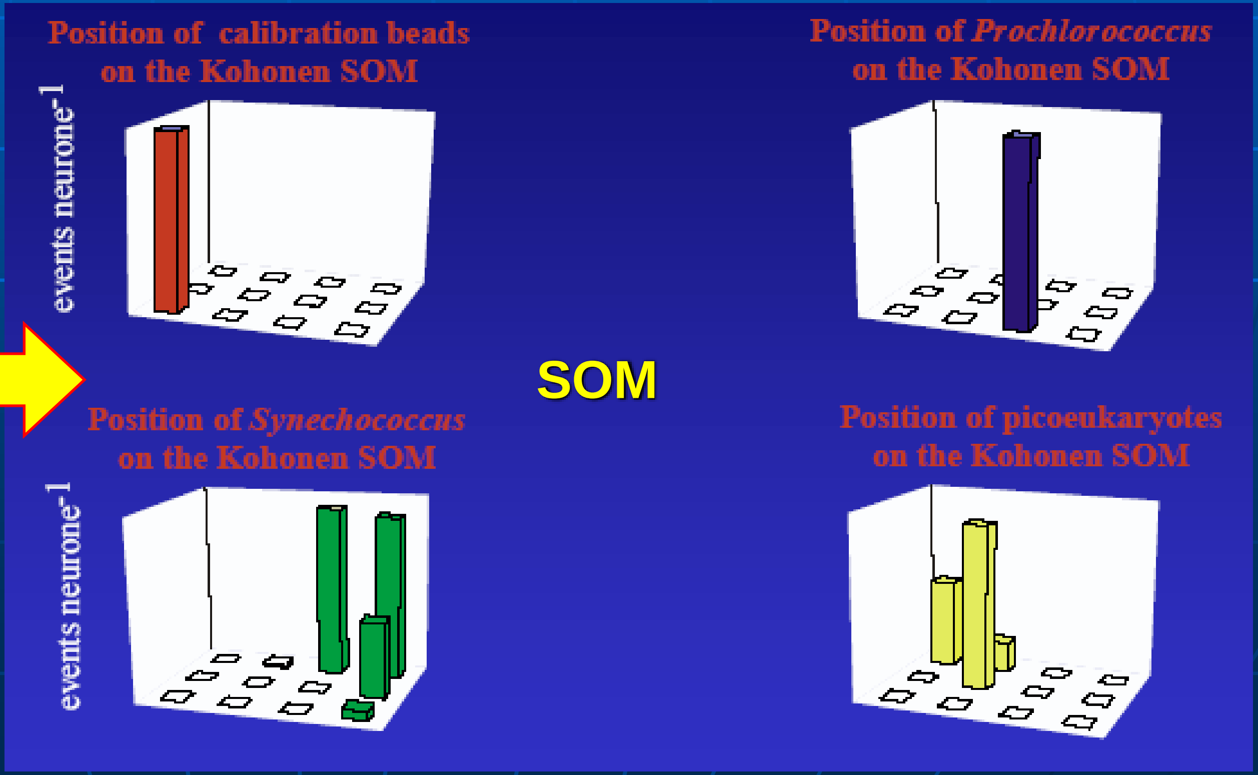
picoeukaryotes

Synechococcus

Fluorescent beads (1 µm)

Prochlorococcus




Position of calibration beads on the Kohonen SOM

Position of *Prochlorococcus* on the Kohonen SOM

SOM

Position of *Synechococcus* on the Kohonen SOM

Position of picoeukaryotes on the Kohonen SOM

Conclusion

Shapiro's Seventh Law of Flow Cytometry:

“No data analysis technique can make good data out of bad data”

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