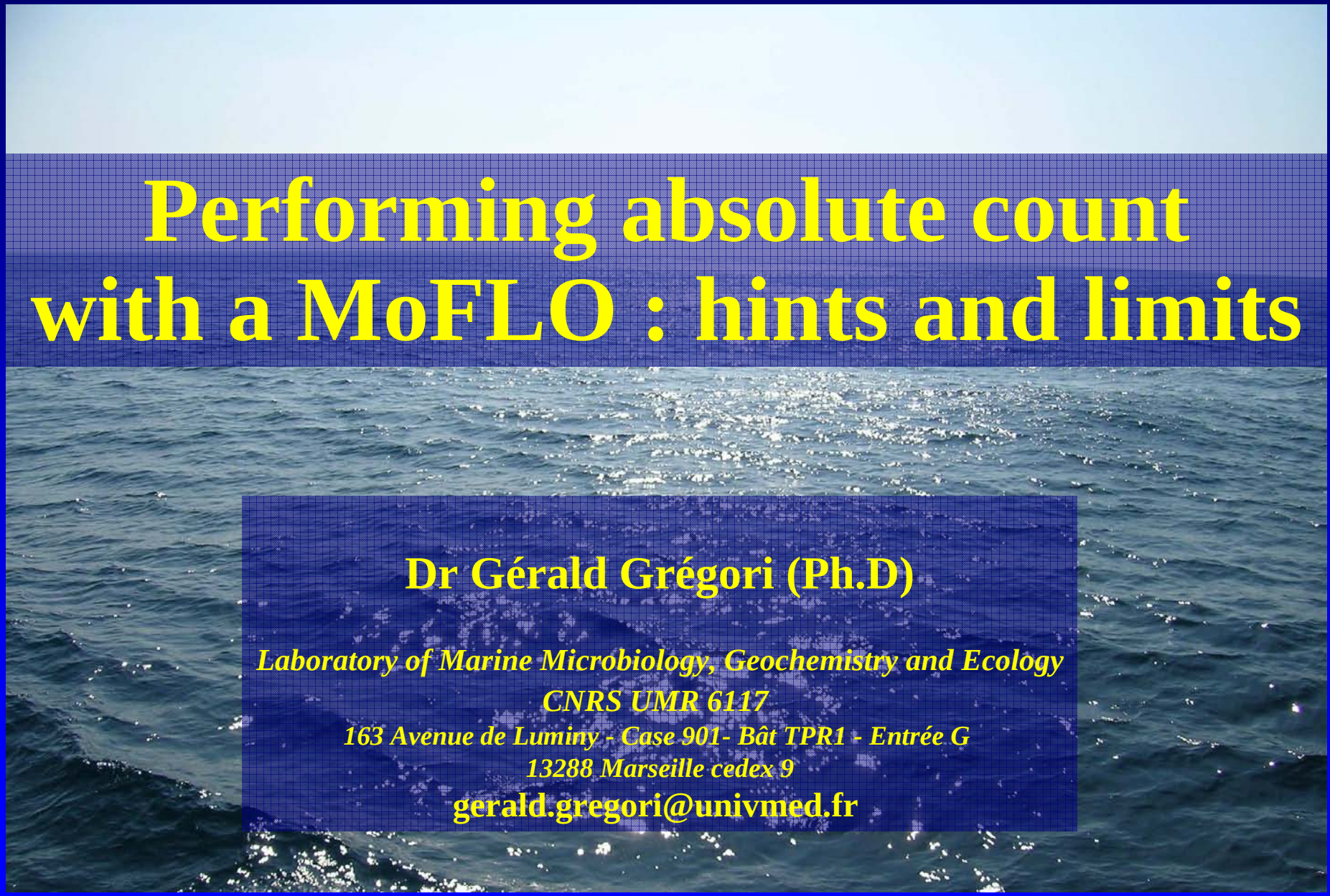
European Flow Cytometry User Group Meeting
Edinburgh 15/16th May 2007

Performing absolute count with a MoFLO : hints and limits



Dr Gérald Grégori (Ph.D)

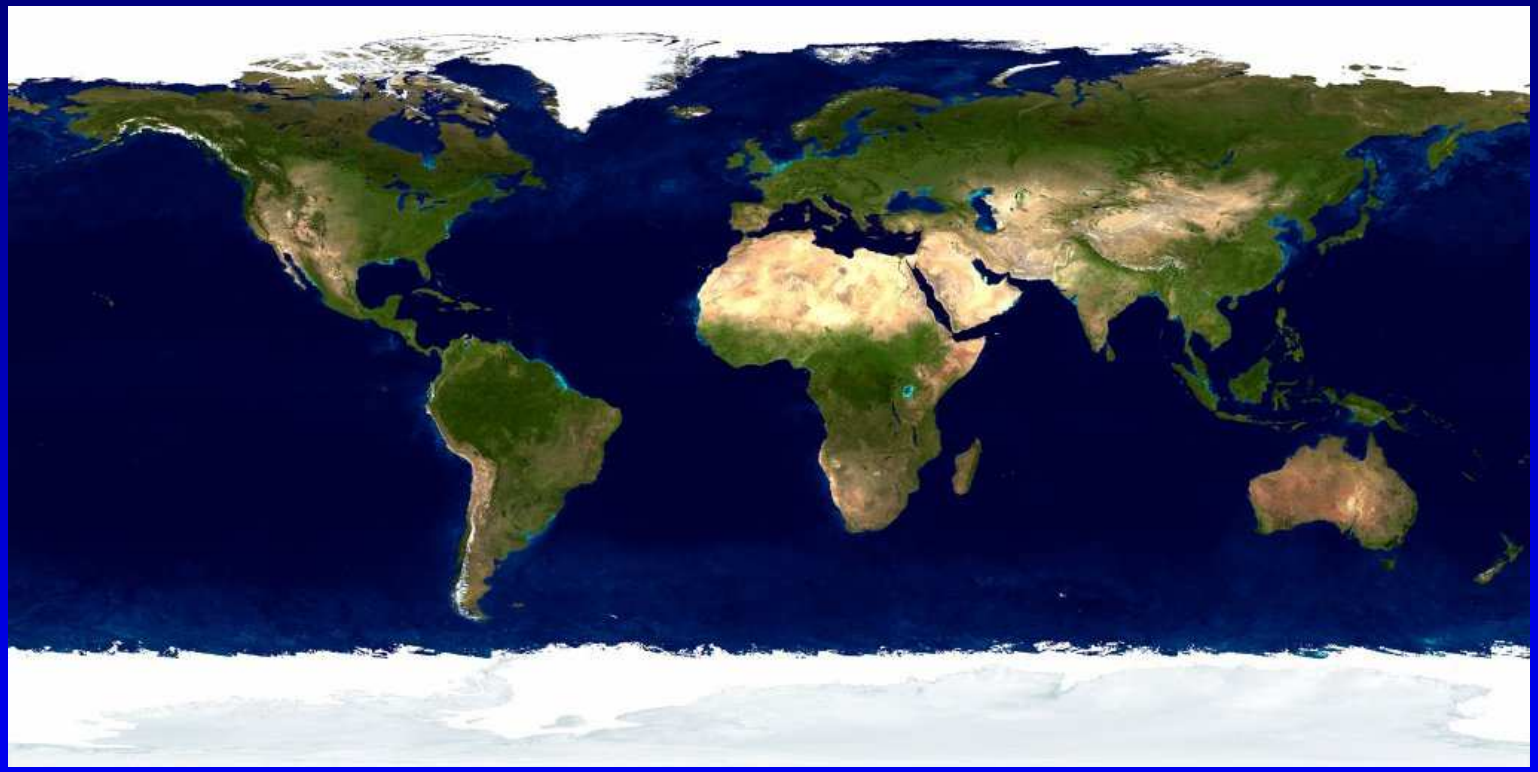
Laboratory of Marine Microbiology, Geochemistry and Ecology
CNRS UMR 6117
163 Avenue de Luminy - Case 901- Bât TPR1 - Entrée G
13288 Marseille cedex 9
gerald.gregori@univmed.fr

I. Importance of Marine Microorganisms

“Life on Earth is microscopic!”

Sean Nee, 2004

Importance of the hydrosphere

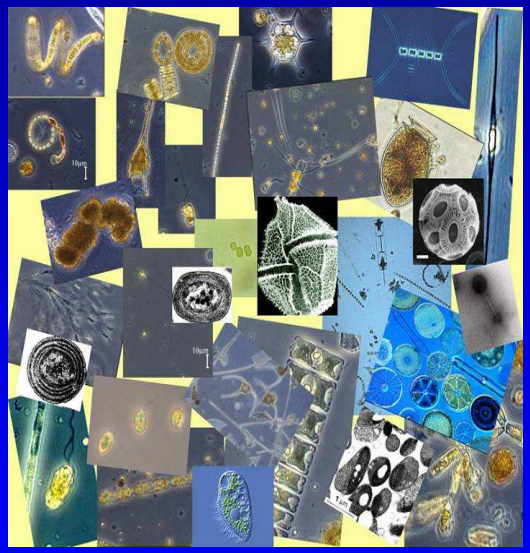


SEAWIF (NASA)

Hydrosphere ➔ >70% of the Earth

Do you know?

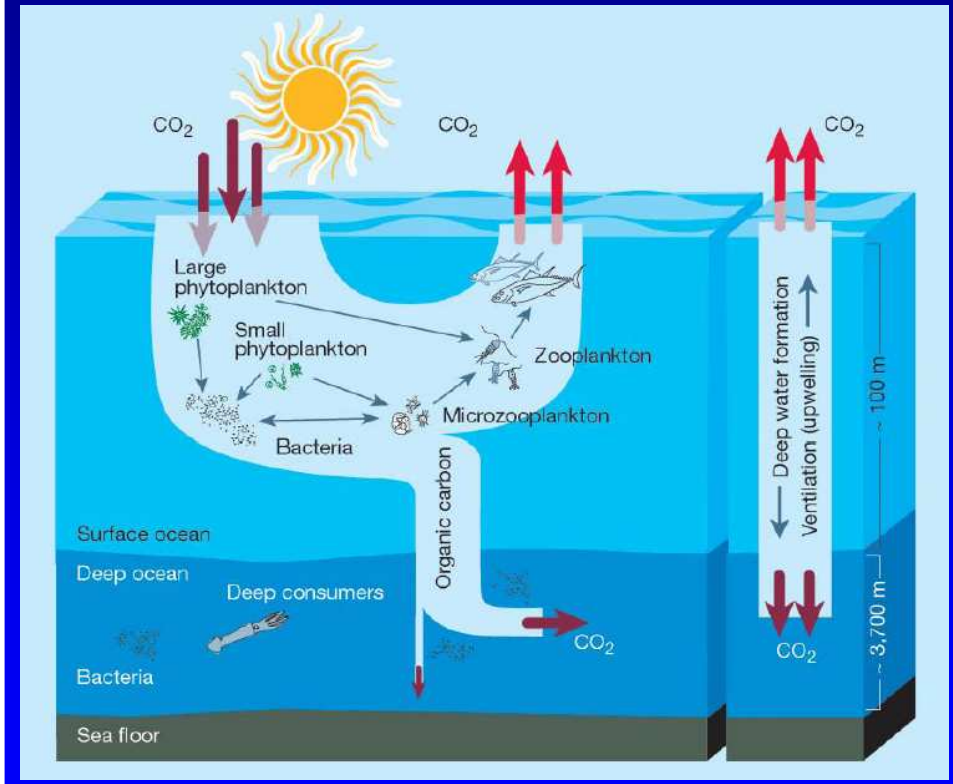
Aquatic unicellular microorganisms from 0.2 -100 µm
➔ 50% of the total biomasse of the planet



	Pg Carbon (10^{15}g)
Phytoplankton (<20µm)	3 – 4
Bacteria (0.5-2 µm)	2.8 - 13.7
Virus (0.2 µm)	0.027 – 0.27
Whales	0.0041 – 0.012
Human beings	0.03

"Life on Earth is microscopic!" (*Sean Nee, 2004*)

Role of microorganisms in the ecosystem



Chisholm, 2000, Nature 407: 685-687

- Crucial roles in the **functioning of the Earth's biosphere**

- Dominate the marine ecosystem (**biomass, high rate of turnover**)

Responsible for :

- (i) The **production of organic matter** (about half of our Planet's annual primary production) → CO_2 uptake

- (ii) Oceanic **mineralization** (water column) → CO_2 release

- (iii) Playing an essential role in **regulating the climate** (contribution to the atmospheric CO_2 sequestration in the deep ocean ; producing chemically-active biogases

- (iv) Toxicity (ecosystem and sanitary risks)

How to characterize the microbial community in natural samples?

1. **Identification** (clusters, taxa, species) ➔ biodiversity

2. A**bundances** and estimation of the **biomass**
   ➔ Spatio-temporal variability of populations (natural or induced)

3. **Physiological state** ➔ Heterogeneity
   ➔Viability (active, inactive, and dead cells)

4. Qualify and quantify **metabolic** or **enzymatic** activities

Why is flow cytometry so popular among (aquatic) microbiologists?

- **Fast analyses** (up to several thousands cells s^{-1})
  - ➔ Huge amount of cells analyzed
  - ➔ Statistical results representative of the population
- **Multiparametric analyses at the single cell level** (several scatters and fluorescences).
- Quantitative data (correlated to biochemical data)
- **Real time** measurements
- **Size class distribution and cell abundance**
- **Unique identification markers** :
  - **natural** (chlorophyll, other pigments) → autofluorescence
  - **induced** (staining) → fluorochromes (dyes)
- **Cell sorting (post-analyses, cultures)**

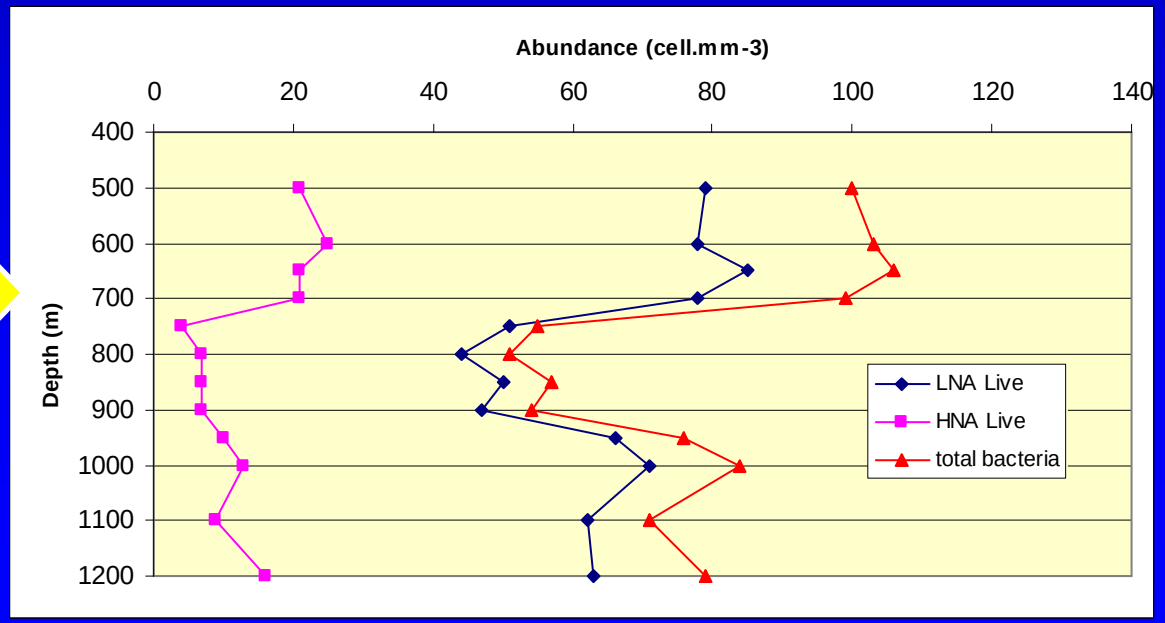
Adaptation of the MoFLO to small and dim particles

- **Forward angle light scatter detector = PMT**
- **Trigger in Log scale (signal from the oscillo)**

Change trigger signal by pressing those buttons

II. Cell abundance assessed by flow cytometry

➔ Need to know the volume analyzed



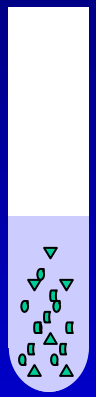
a. Direct Absolute Count

Sample

Cell concentration
(# of events / volume)

To analyze a bead solution of known concentration
➔ Control of the fluidic

b. Weigh a Sample



Sample

Weigh the sample before analysis (Weight W_0)

Analysis (Flow Cytometer)

cell number (N)

Weigh the sample after analysis (Weight W_1)

Volume analyzed $V = (W_0 - W_1)/\rho$

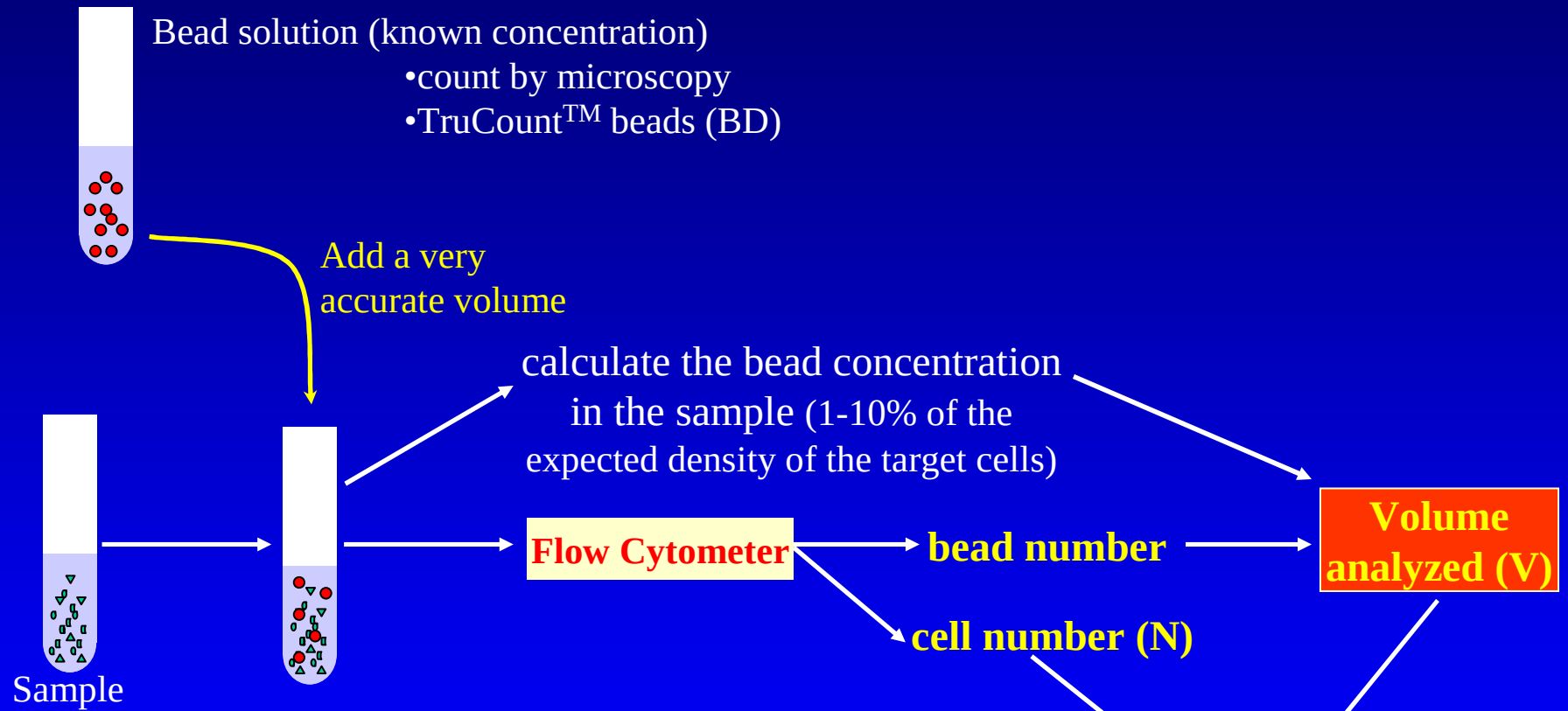
density

Cell concentration = N/V

Disadvantages

- Time consuming
- Less accurate (back flow of sheath fluid in the sample)
- Analysis in one run

c. Add Beads in the Sample



Stewart & Steinkamp, 1982, Cytometry 2 : 238-243

d. Determination of the Flow Rate

Hypothesis: flow rate (µl/s) through a flow cytometer = constant

Bergeron *et al*, 2003, Cytometry 52B:37-39

↓

Conclusion: volume (V) analyzed in a fixed time = constant

No need to add beads in the samples.

↓

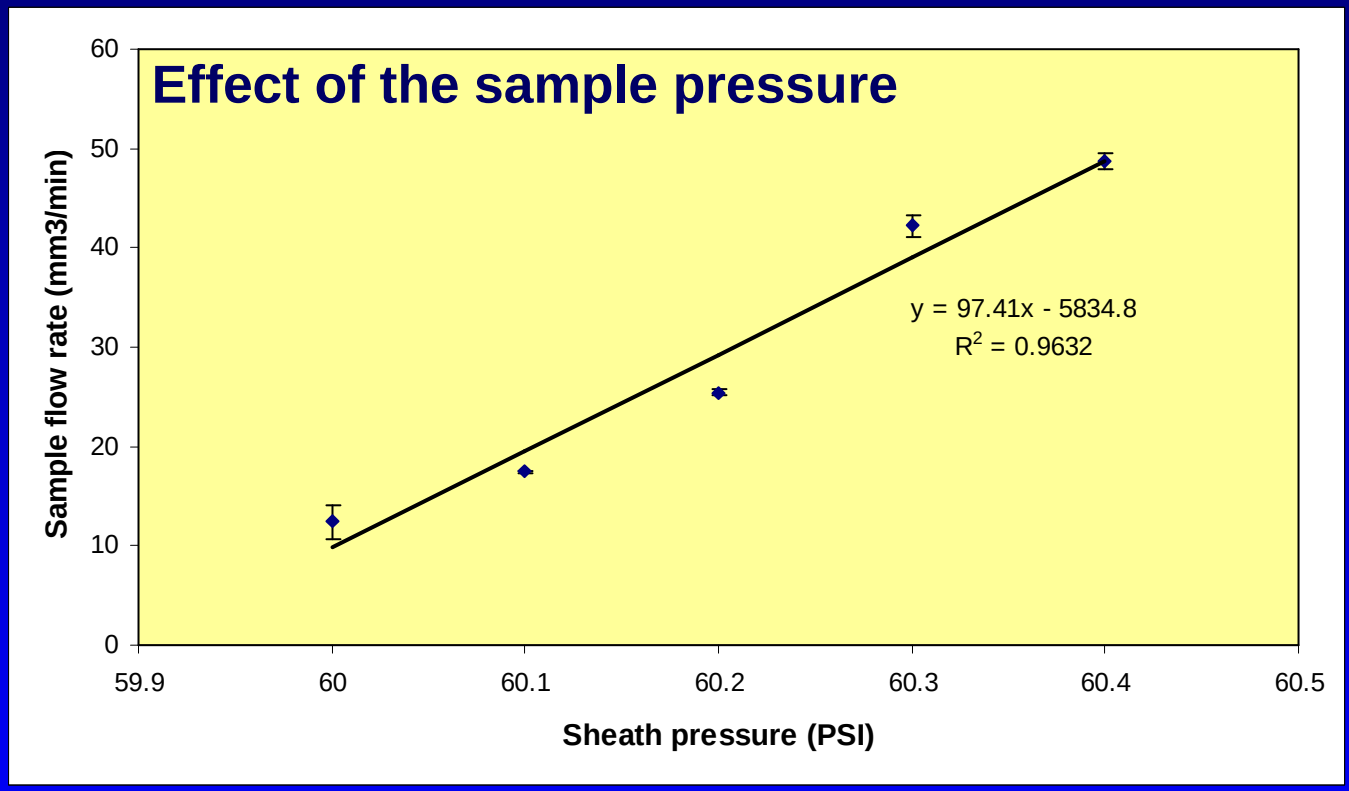
If N events are analyzed by the cytometer in the fixed time

then cell concentration = N/V (cells/µl)

Hint!

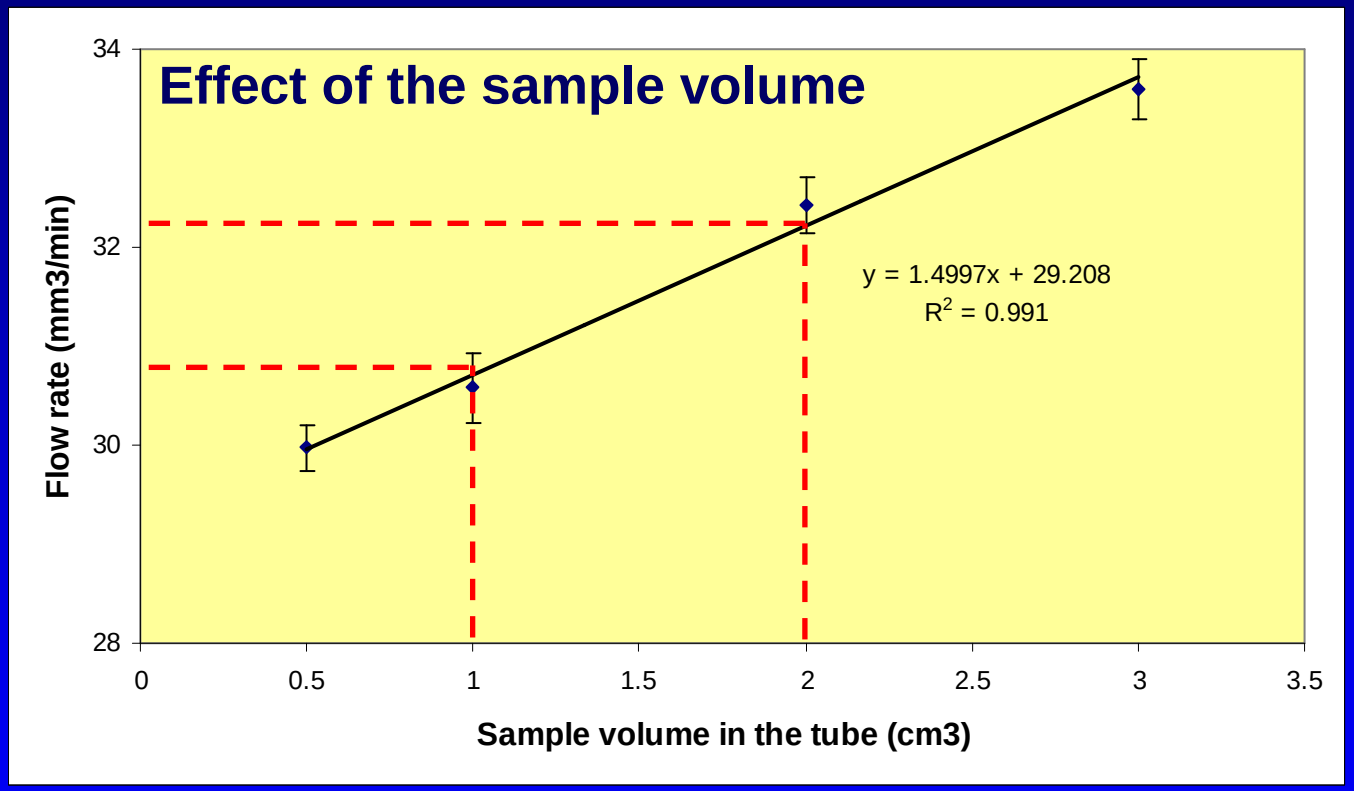
- *Analyzes must be done with the same flow rate*
- *Volume accurately determined (microscopy*, Flow Count™ beads) *and controlled*
- *Beads not necessary in the sample, but can be used as internal standard*

Testing the fluidics



Please Dako's folks could you add a second digit on the pressure controller screens?

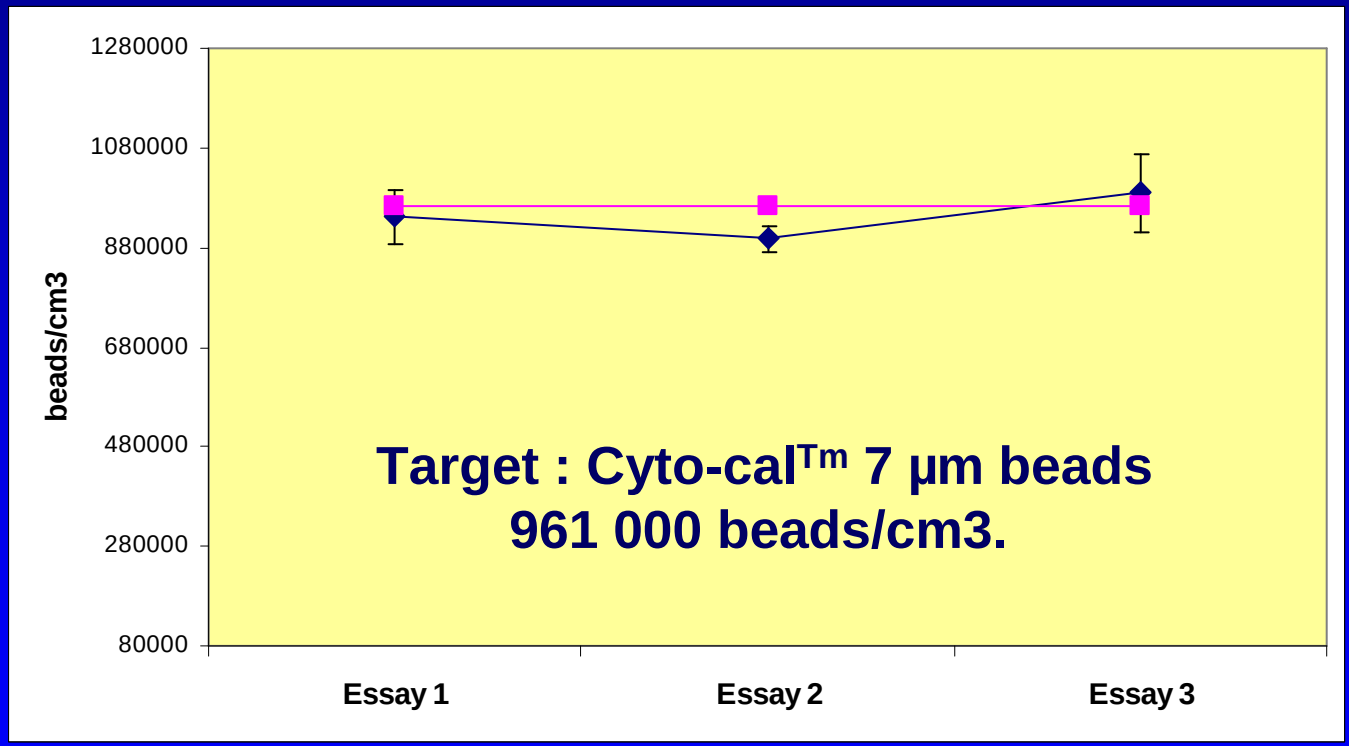
Testing the fluidics



➔Sample volume between 1 and 2 ml

➔Analysis for 3 minutes

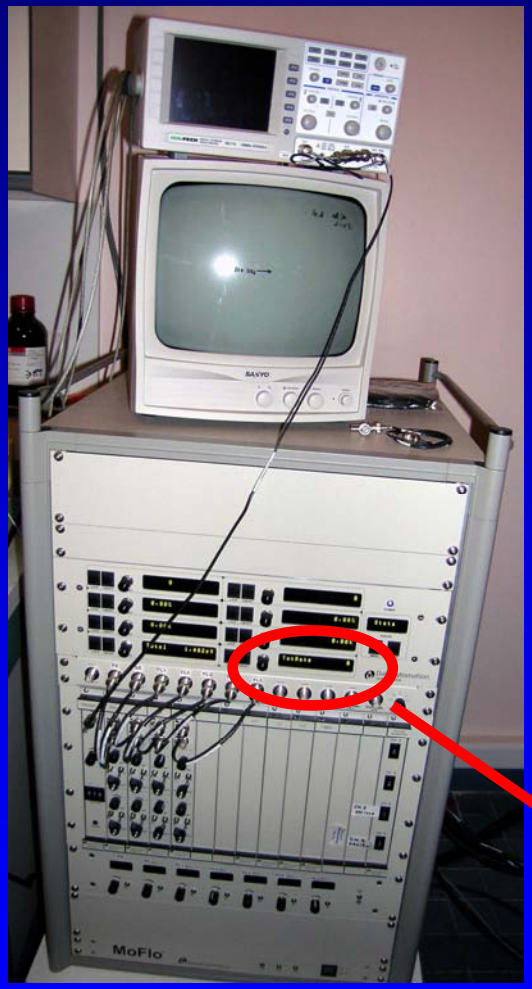
Verification with beads

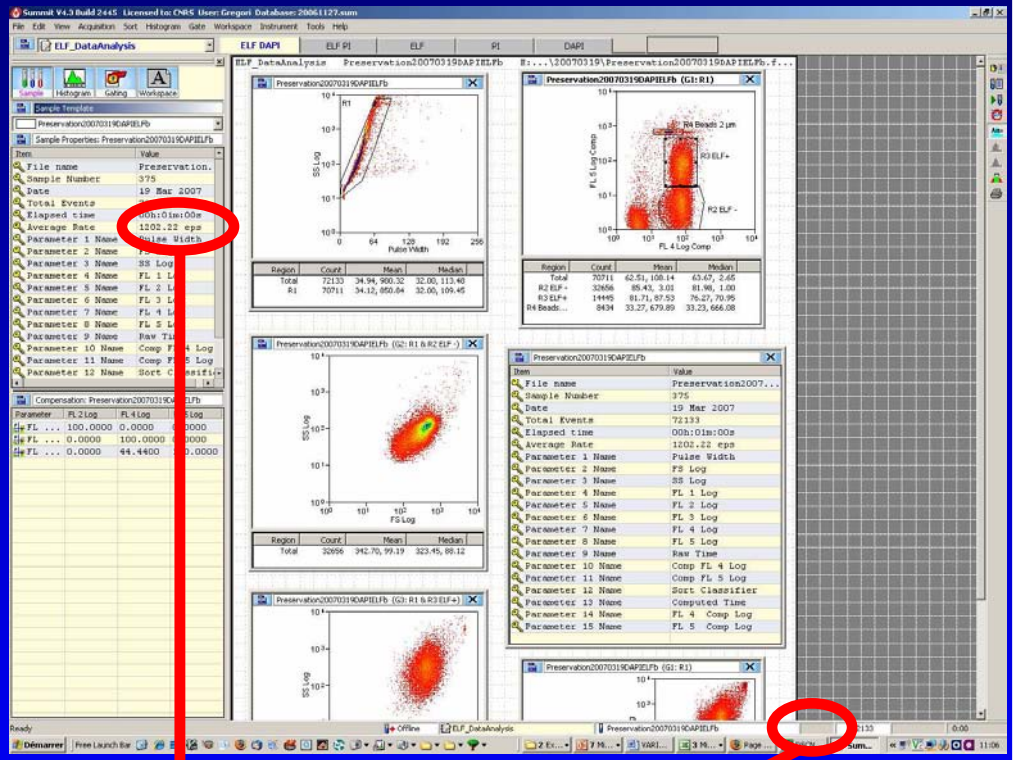


II. Cell abundance Assessed by flow cytometry

➔Need to count all the particles

Counter discrepancy



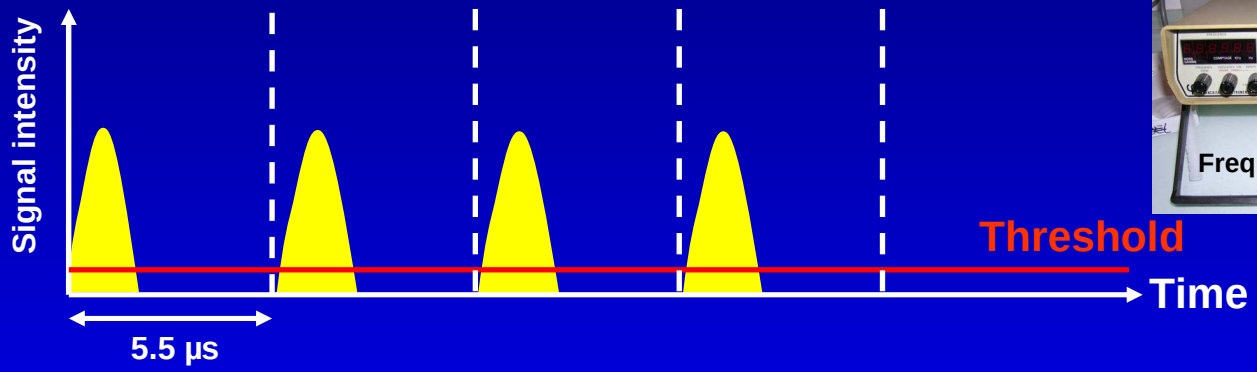


Different values

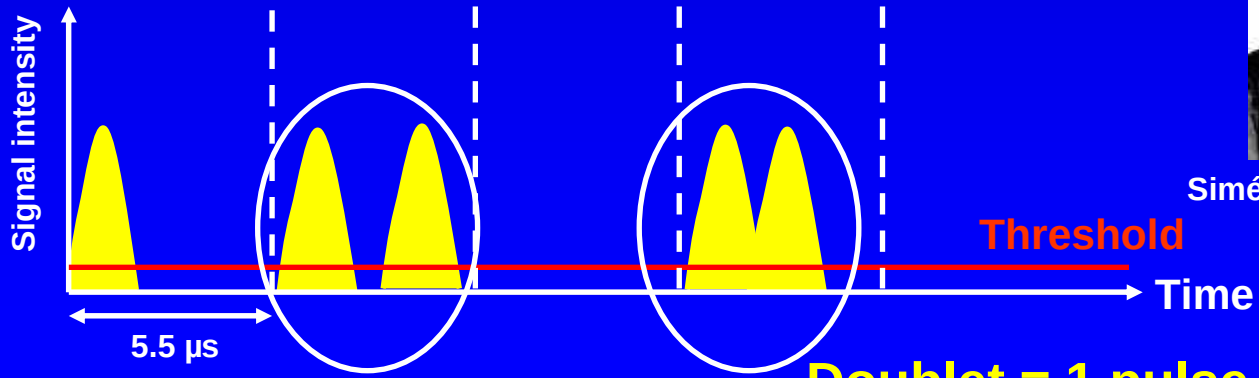
Dead time

Dead time defines the number of signals that can be processed per unit of time.

On a MoFLO, the dead time is 5.5 µs ➔ 181818 pulses/s

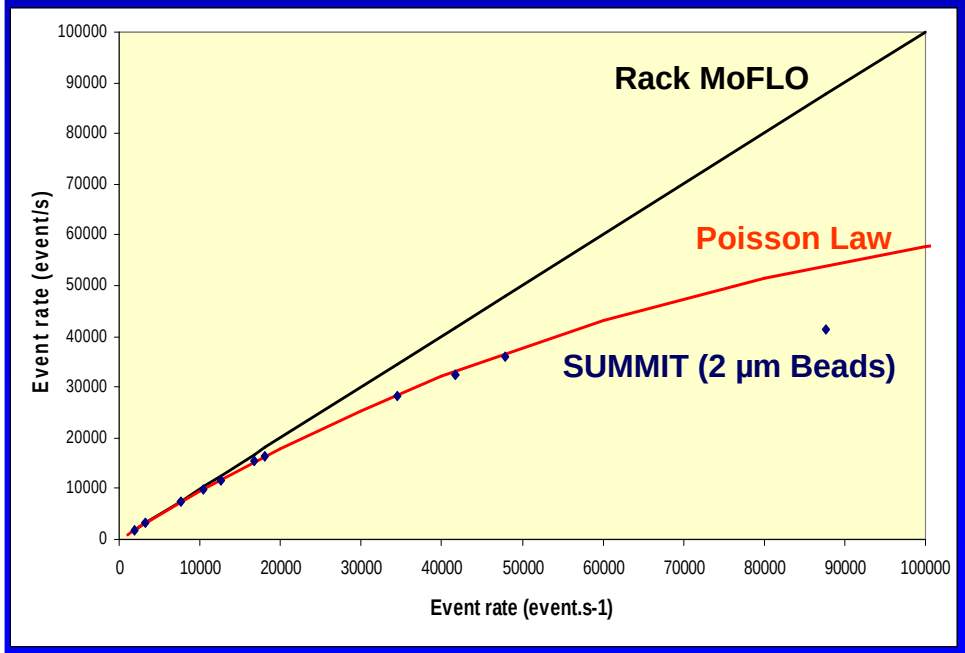


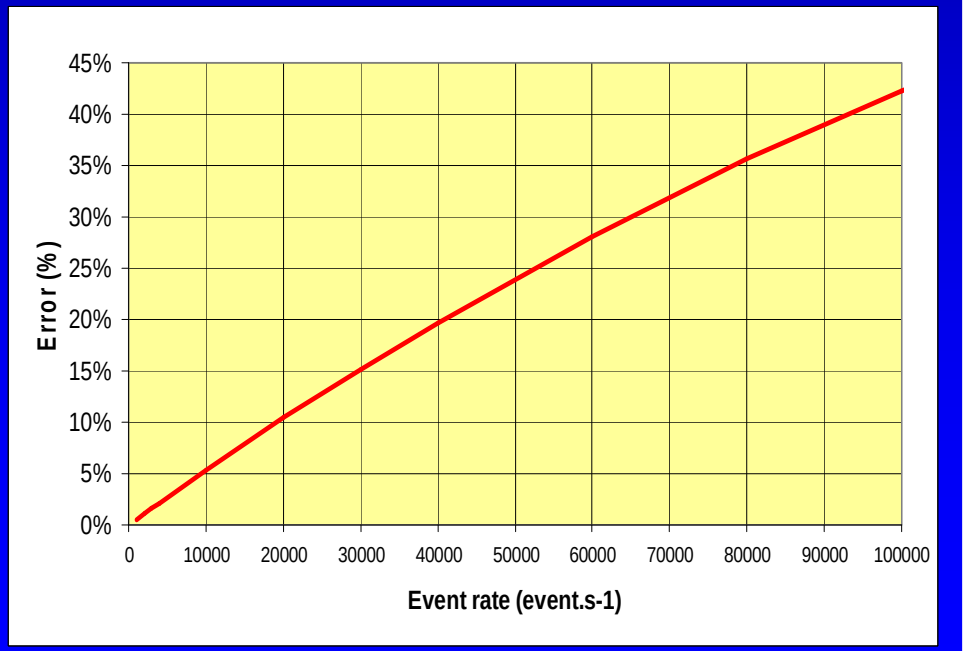
In the « real world » two pulses can occur in 5.5 µs



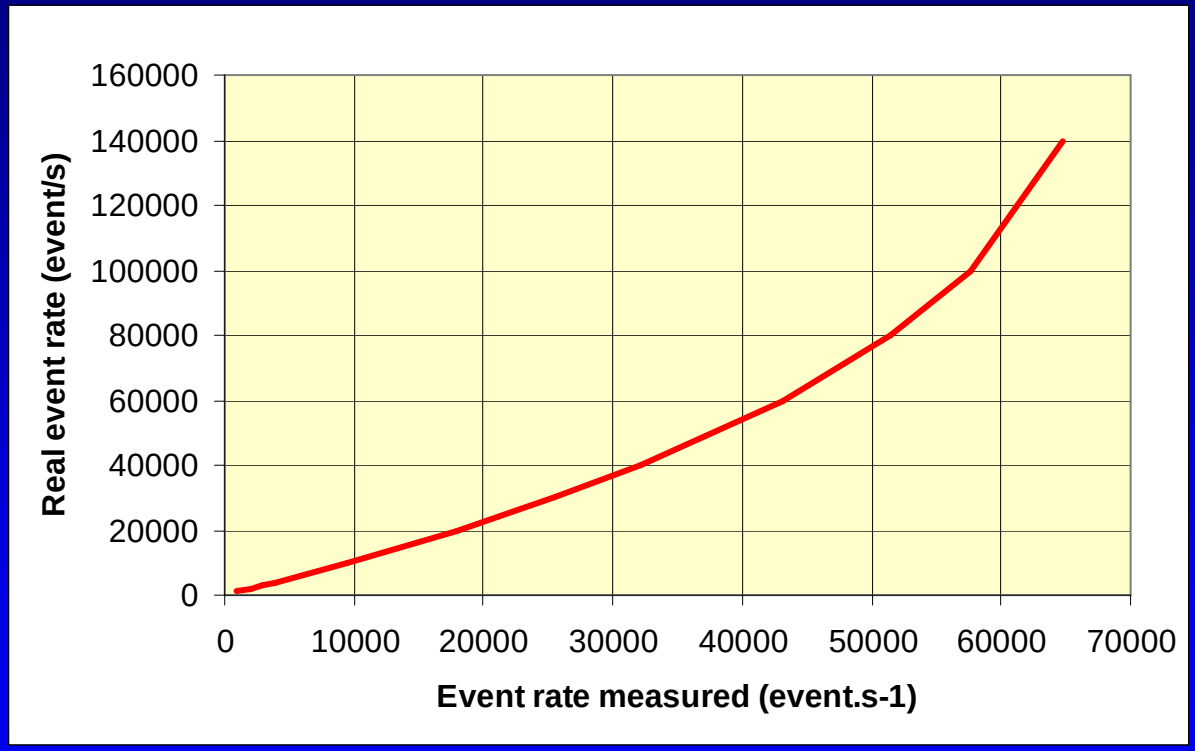
Siméon Denis Poisson
1781-1840

Use of a frequencymeter





A model to solve the problem



Conclusion

To make absolute count with a MoFLO:

- **Define optimal fluidic conditions for your analysis (sample and sheath pressure)**
- **Check the number of events/s analyzed**
- **Determine the volume analyzed (weighing a sample before and after a 3 minute analysis)**
- **Do this weighing on a half day basis (every 4 hours)**
- **Add beads in your sample as internal control**
- **Ask Dako folks to design a syringe based system, with an option in the software that would give the direct absolute count.**


Thank you for your attention ...
Merci…
Maire Island (Marseille, France)