Atelier de formation 225

Institut thématiques Inserm
Institut national de la santé et de la recherche médicale

Avancées technologiques en cytométrie :
applications et perspectives en recherche fondamentale et clinique
Advances in cytometry: new applications and insights in basic and clinical research
1-3 octobre 2013 ◘ 1 st -3 rd October 2013
Bordeaux, France

HYPERSPECTRAL CYTOMETRY AT THE SINGLE-CELL LEVEL USING A 32-CHANNEL PHOTODETECTOR

Gérald Grégori 1, Bartek Rajwa 2, Valery Patsekin 2,
Kathy Ragheb 2, Cheryl Holdman 2, James Jones 3,
& Paul Robinson 2

1 : Meditrranean Institute of Oceanology, AMU, Marseille
2 : Purdue University Cytometry Laboratories , West Lafayette, IN
3 : Weldon School of Biomedical Engineering, West Lafayette, IN

PUCL cytometry laboratories

mio Mediterranean Institute of Oceanography

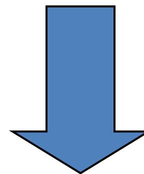
Need to understand particles (cells) heterogeneity and basic cell functions in samples

- Accumulation of information on key individual cells (lymphocyte, mammalian cells, microorganisms)

➔ good understanding of the whole community (sociology?)

- Sample diversity mustn't remain a "black box"

➔ Need to understand the cell heterogeneity (diversity, physiological state, cell activity) on the basis of single cell level.



Flow cytometry

Why is flow cytometry so popular?
(…even among marine microbiologists)

- Non invasive technology (cells remain as they are)
- **Fast analyses** (up to several thousands cells s^{-1})
  - ➔ Huge amount of cells analyzed per sample; many samples
  - ➔ Statistical results representative of the population
- **Multiparametric analyses at the single cell level** (2 scatters and several fluorescences).
- Quantitative data (can thus be correlated to other data measured)
- **Real time** measurements
- **Size class distribution and cell abundance**
- **Unique identification markers** :
  - **natural** (chlorophyll, other pigments) → autofluorescence
  - **induced** (staining) → fluorochromes (fluorescent antibodies, dyes)
- **Cell sorting (post-analyses, cultures)**

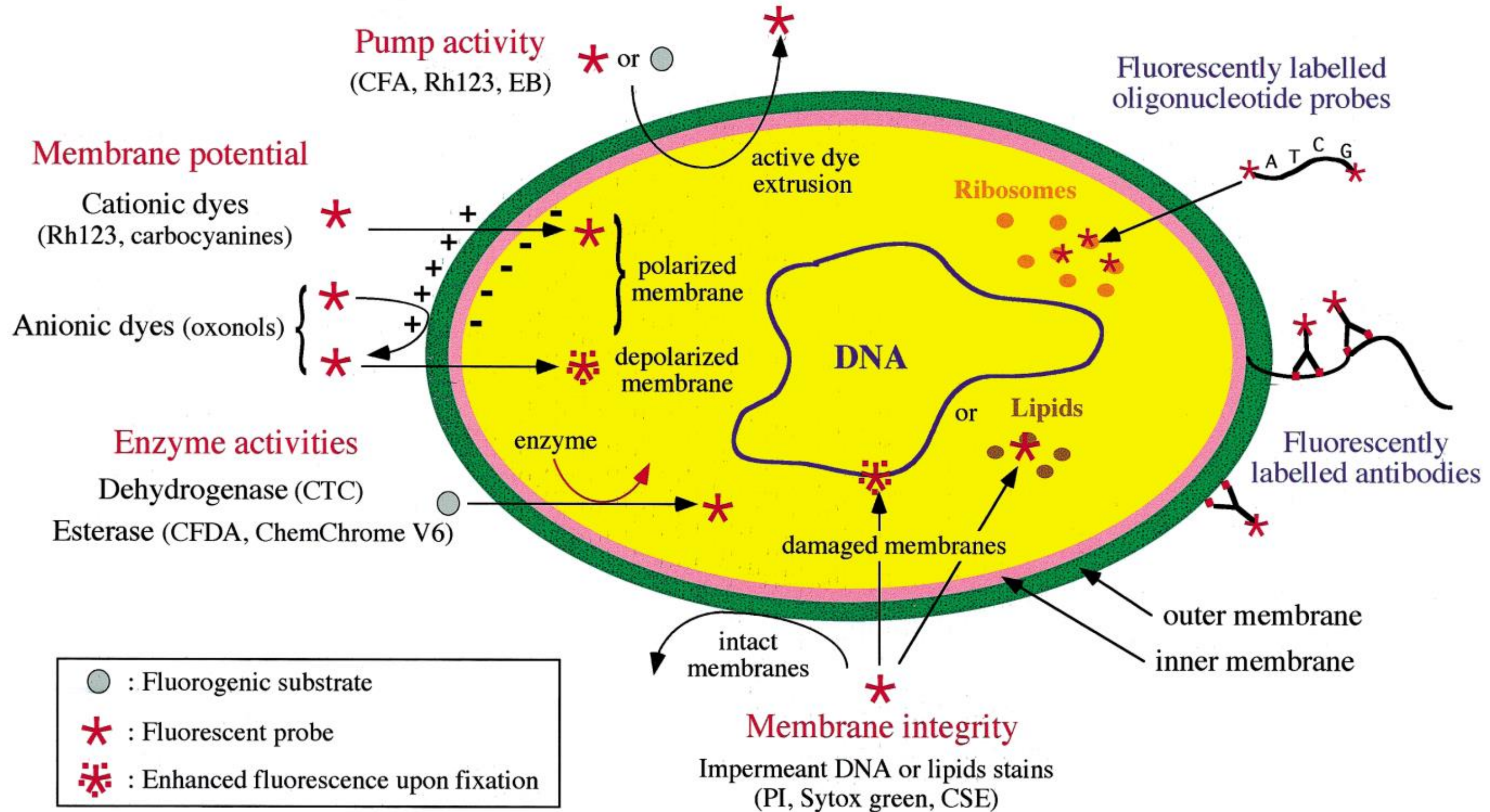
Informations needed on cells

- **Cell identification**
  - Design specific probes to target the cells of interest (Antibodies, FISH)
  - Track these cells in the samples (follow abundances, cell properties)
  - Evaluate their dynamics (natural trends, influence of a stress)

- **Cell viability**
  - Only live cells are responsible for activities observed *in vivo (in situ)*
  - Better characterize the factors controling the viability/mortality

- **Cell function**
  - Metabolic probes used to assess cell activity (metabolic pathways, respiration, enzyme activity,etc.)
  - Detect capacity of cells to divide
  - Characterize growth rate (various steps of the mitosis)

Fluorescent probes (fluorochromes)



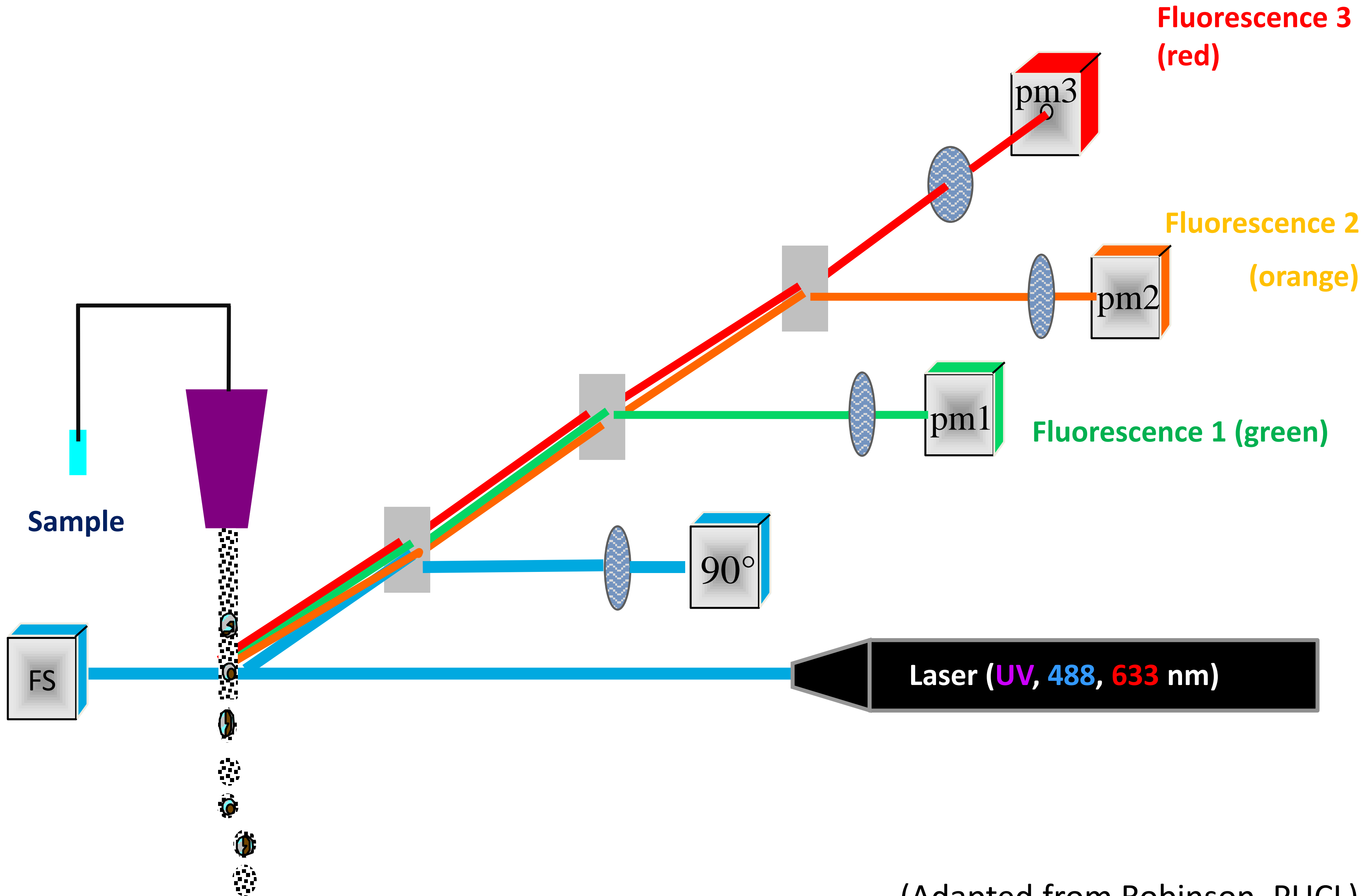
Joux *et al.*. Microbes and Infection, 2, 2000, 1523–1535

Examples of conventional flow cytometers

Analyseurs de paillasse

Sorters

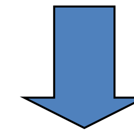
General Principle of Flow Cytometry



(Adapted from Robinson, PUCL)

Configurations of dichroic mirrors and filters

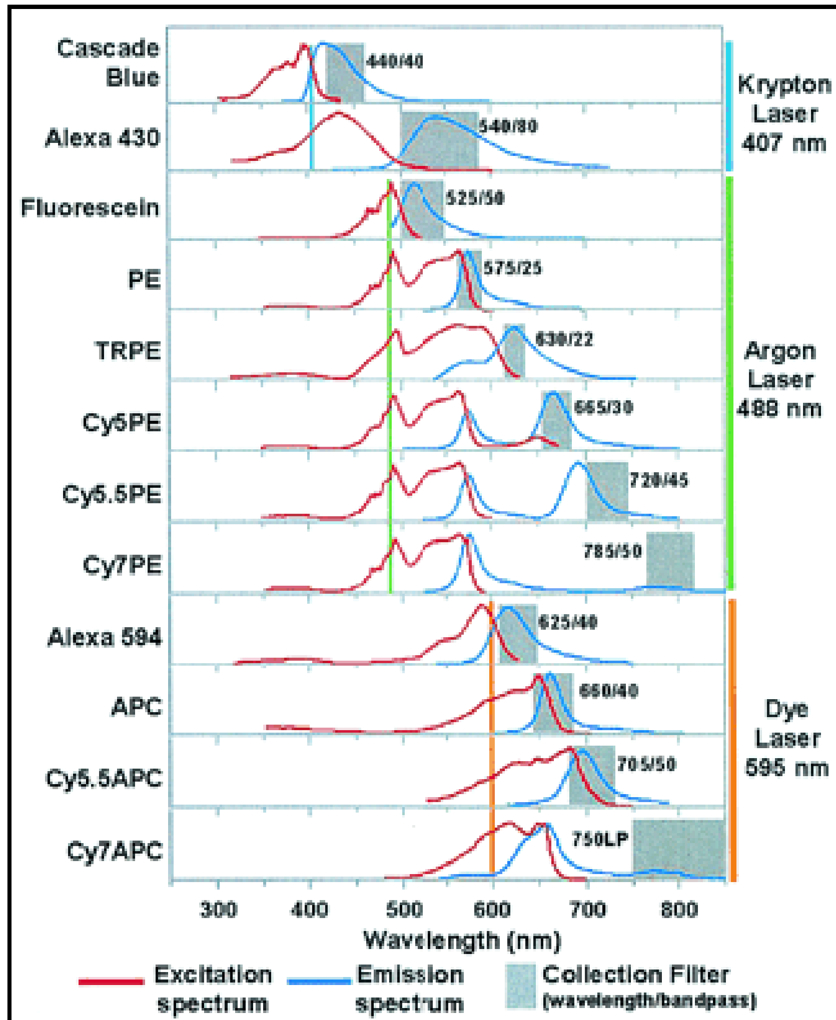
- **Light loss for dichroics ranges up to 10-15%**
- **Light reflected from dichroic represents a loss of about 1%**



Problem for dim fluorescent particles

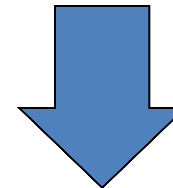
HINT: Collect long wavelengths first because they have less energy (Red → UV)

A limitation : Number of fluorescences



The excitation and emission spectral bands of dyes, lines of lasers, and types of various bandpass filters necessary to perform an 11-signal analysis (from Roederer *et al.*).

- **Large range of organic fluorochromes**
  - **→ Large emission spectra**
- **Number of photodetectors (PMTs)**
  - **→ Up to >16 (immunology)**
  - **→ Typically 3 to 5**
- **Spectral overlapping of fluorochromes**
  - **→ Compensation calculations**
  - **→ Loss of signal**



Hyperspectral Cytometry

Hyperspectral Cytometry Overview

- **1979 Wade et al. record the fluorescence spectrum of particles in a flow system**
  - **Only collection of integrated spectra from many particles (not from an individual particle)**

- **1986 Steen and Stokke measured averaged fluorescence spectra of rat thymocytes using a custom built cytometer and grating monochromator**

- **1990 Buican used a Fourier-transform interferometer to obtain single-cell spectra**
  - **Limited performance by the fact that cells needed to stay in the laser beam for a relatively long time to be scanned by his system**

- **1996 Gauci et al. used a flint-glass prism and an intensified photodiode array.**
  - **The data rate of the instrument was too slow to be of practical use. Additionally, the sensitivity of photodiodes was (and still is) below the power offered by PMT technology.**

- **1996 Asbury et al. measured spectra of cells and chromosomes using a monochromator changing the wavelength during the course of an instrument run.**
  - **The technique allowed measurement of just a single band from any individual particle.**

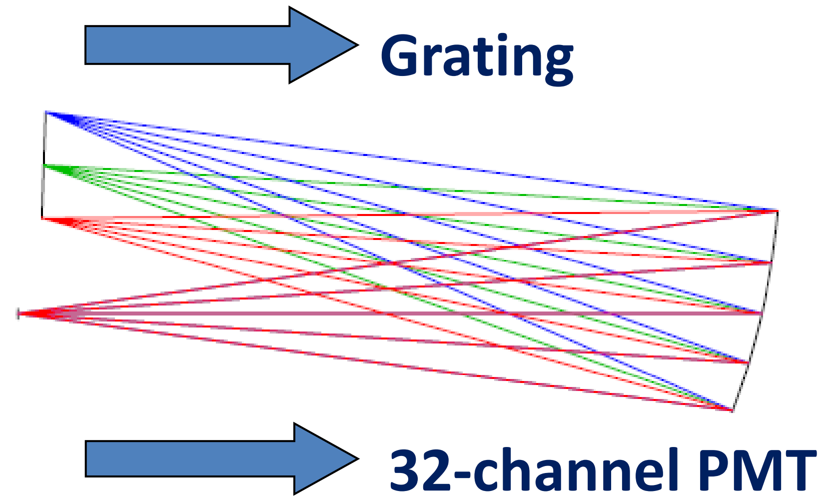
- **2001 SoftRay Inc. and group of researchers from Wyoming and Utah Universities recently pursued another prism-based concept**

2004 Robinson JP (2004) Multispectral cytometry: the next generation. Biophotonics Int 36–40

2012 SONY and the first commercial spectral flow cytometer

Toward a new generation of flow cytometer

- **No dichroic mirrors** → **Grating**



- **No filter**
- **32 fluorescences collected** (range 500-800 nm) → **32-channel PMT**

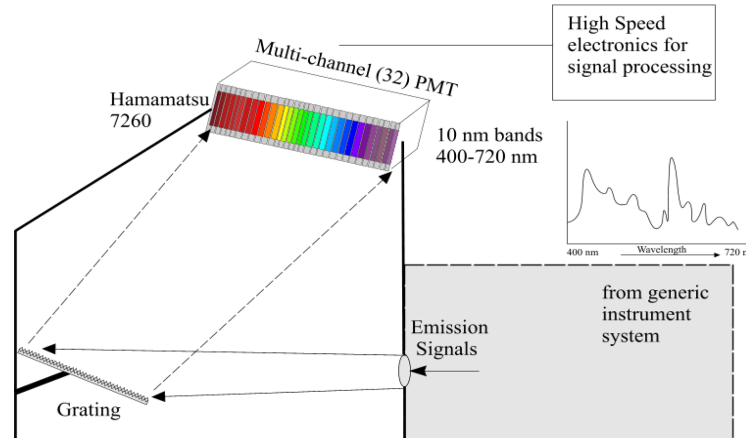


figure 4

Robinson J.P., Rajwa B., Grégori G., Patsekine V., James Jones (2007) Multi-spectral detector and analysis system. US Patent n° 7280204 B.

The very first prototype

A) Modified EPICS Elite (Beckman Coulter)

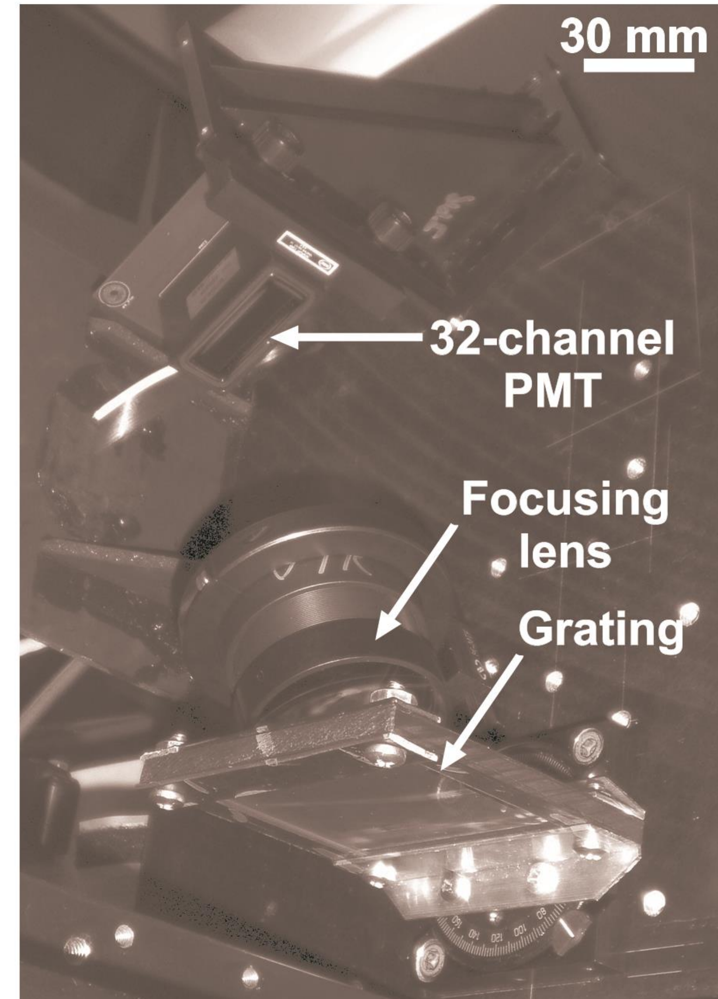
6 PMT detection unit

32 channel PMT

PMT1
PMT3
PMT5
PMT2
PMT4
PMT6
Focusing lens
Side scatter PMT
Grating
488 nm BP filter
Optical bentch
Laser beam
Epics Elite Flow cell
Jet
Mirror
Empty slots
50/50 beam splitter
10/90 beam splitter for alignment or 488nm LP filter for analysis

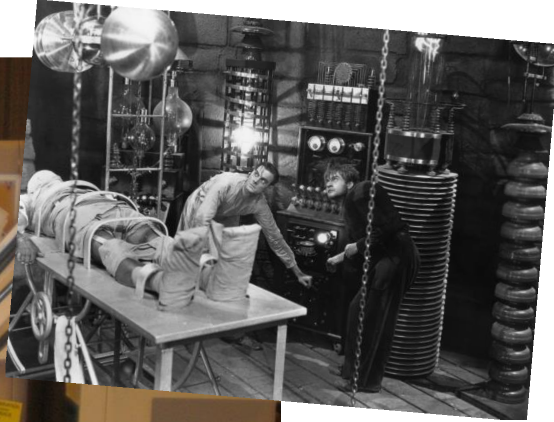
44 variables (parameters) recorded !!!

B) Optical design



Grégori G., Patsekin V., Rajwa B., Jones J., Ragheb K., Holdman C., Robinson J.P. (2012). Hyperspectral cytometry at the single-cell level using a 32-channel photodetector. Cytometry Volume 81A, Issue 1, pages 35–44.

Welcome Frankein-Flow!!!!

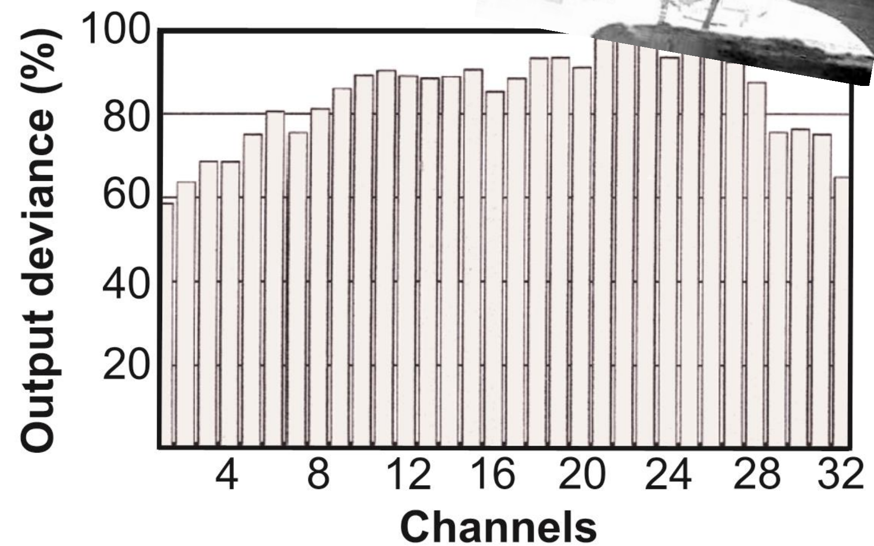


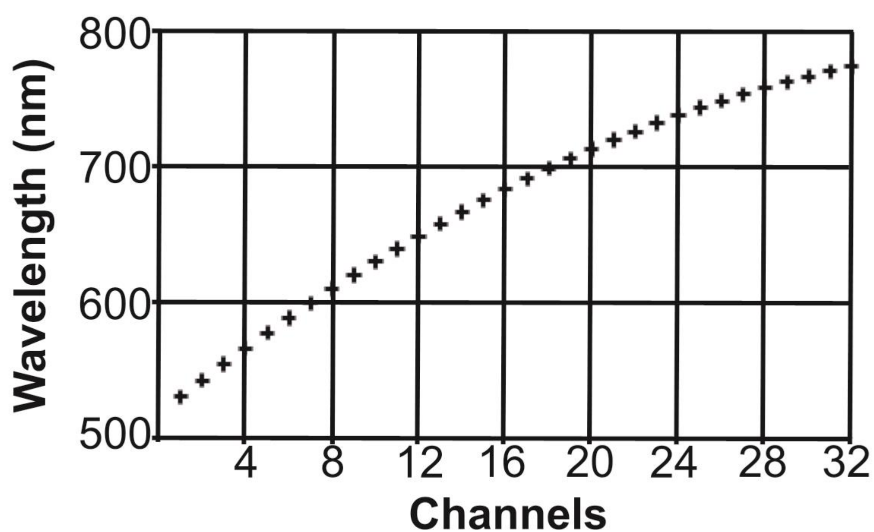
4500
4000
3500
3000
2500
2000
1500
1000
500
0

450 470 490 510 530 550 570 590 610 630 650 670 690 710 730 750 770 790

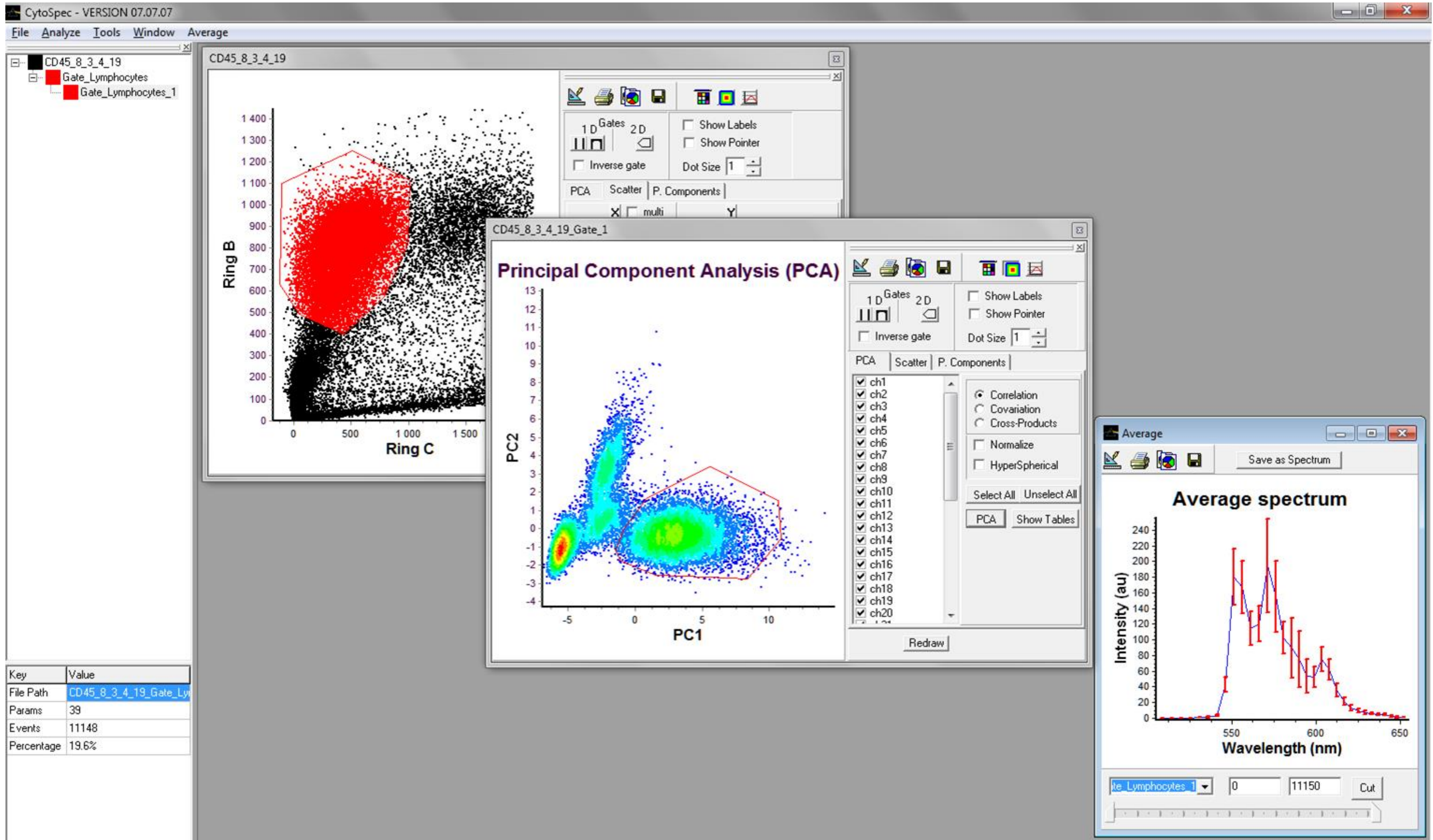
32-PMT

Calibration Lamp



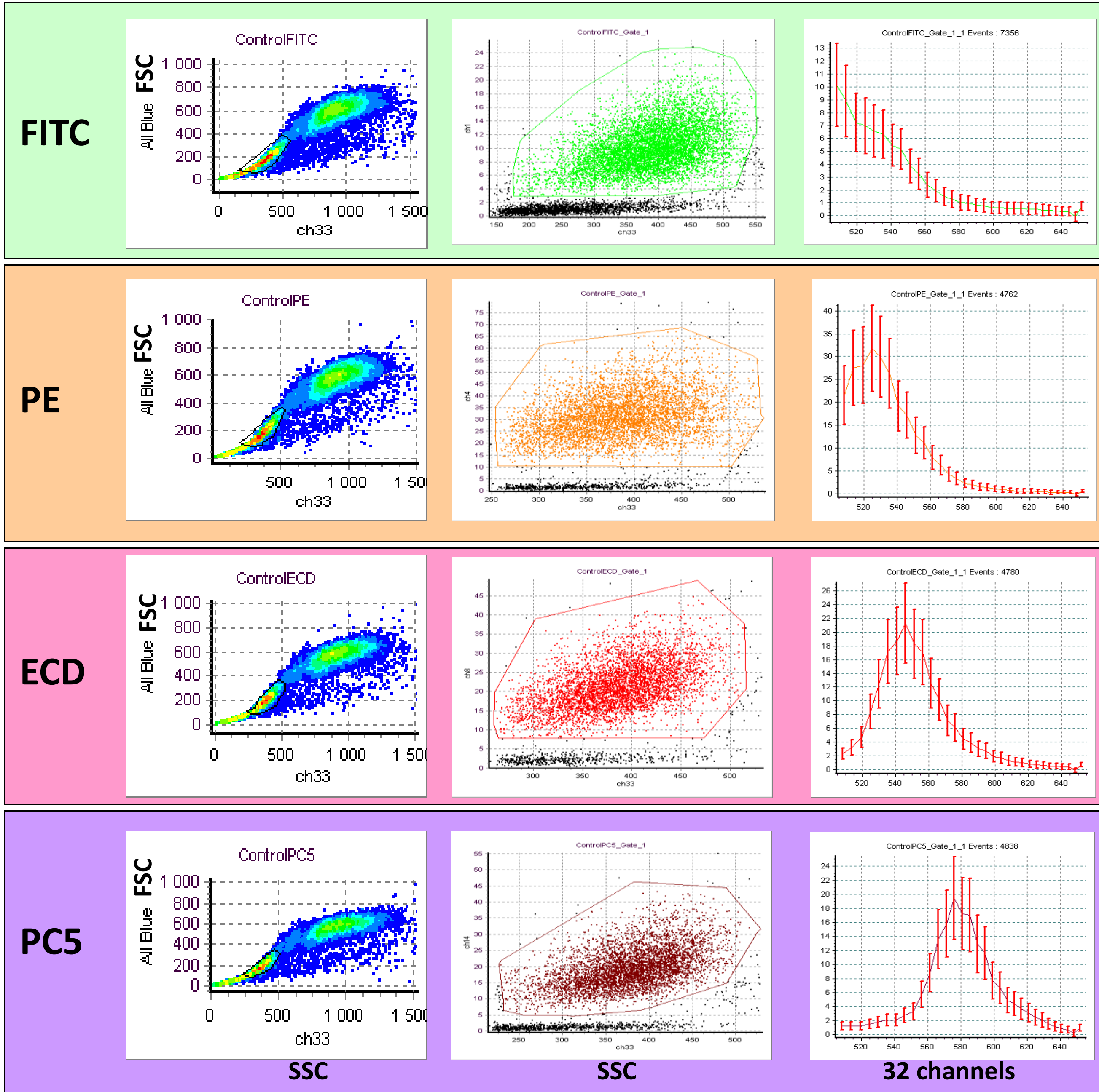


Cytospec software package : acquisition and processing of hyperspectral cytometry data



The package was developed at PUCL by Valery Patsekin and is freely available at : http://www.cyto.purdue.edu/Purdue_software

Blood with either FITC, PE, ECD, or PC5 Ab (controls)



- 1st column : Gating of lymphocytes only on FS vs SSC cytogram for each control (control = 1 dye only)

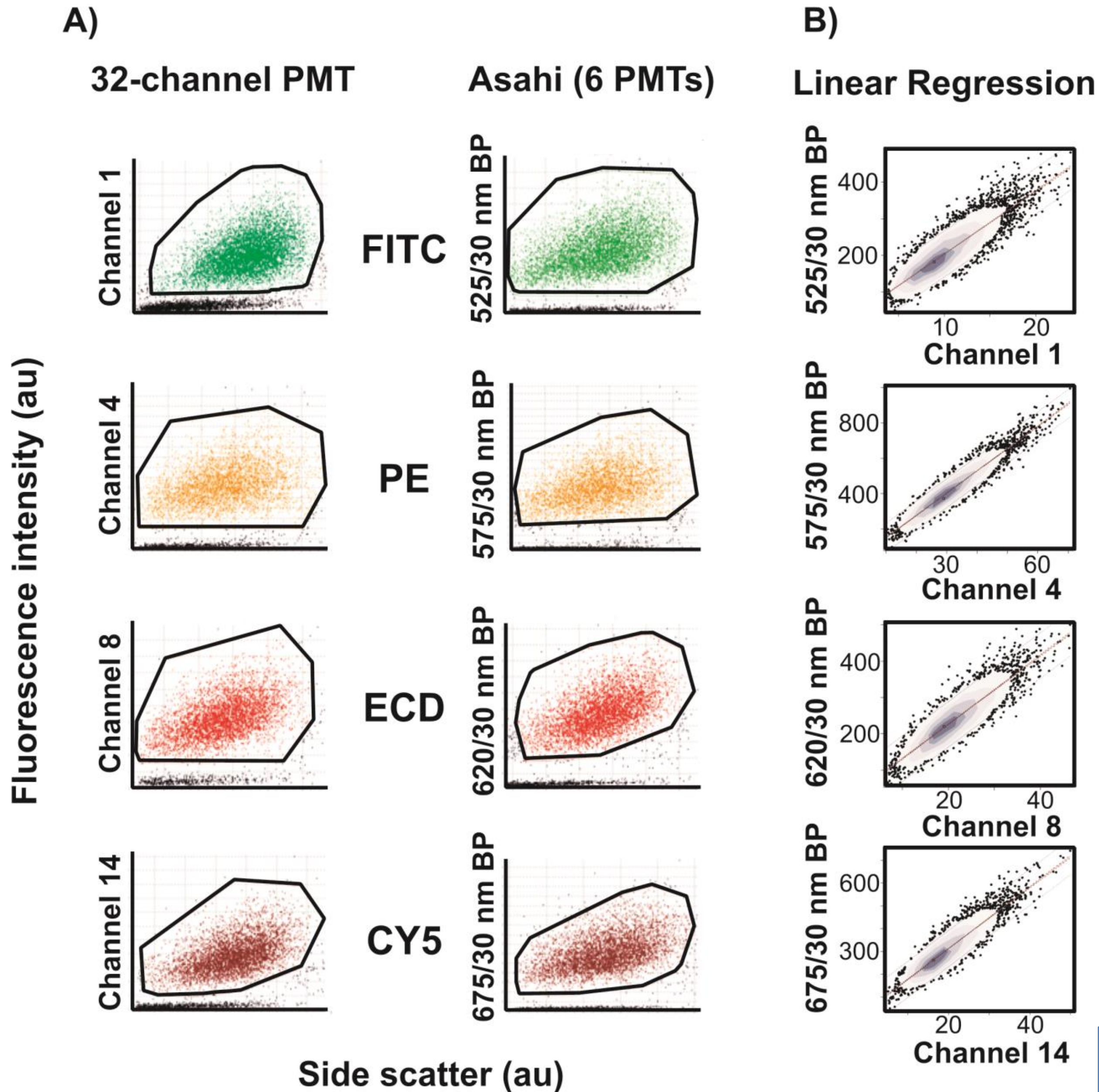
- 2nd column : Gating of stained lymphocytes on each control. This remove the non fluorescent noise from SSCvsFS

- 3rd column : Average spectra for each control (with Sd)

Event # 7

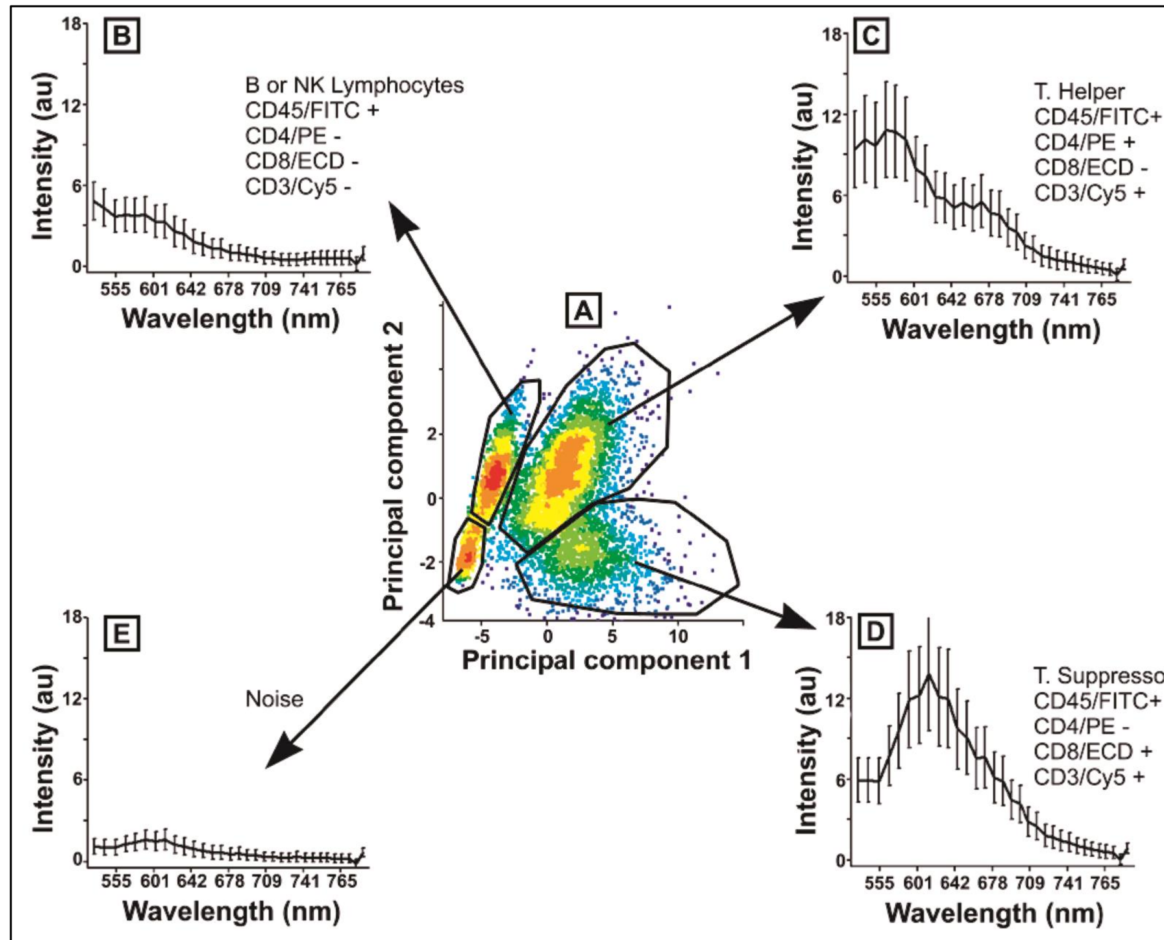
Cytospec Software

Validation of the data collected



Cytospec Software

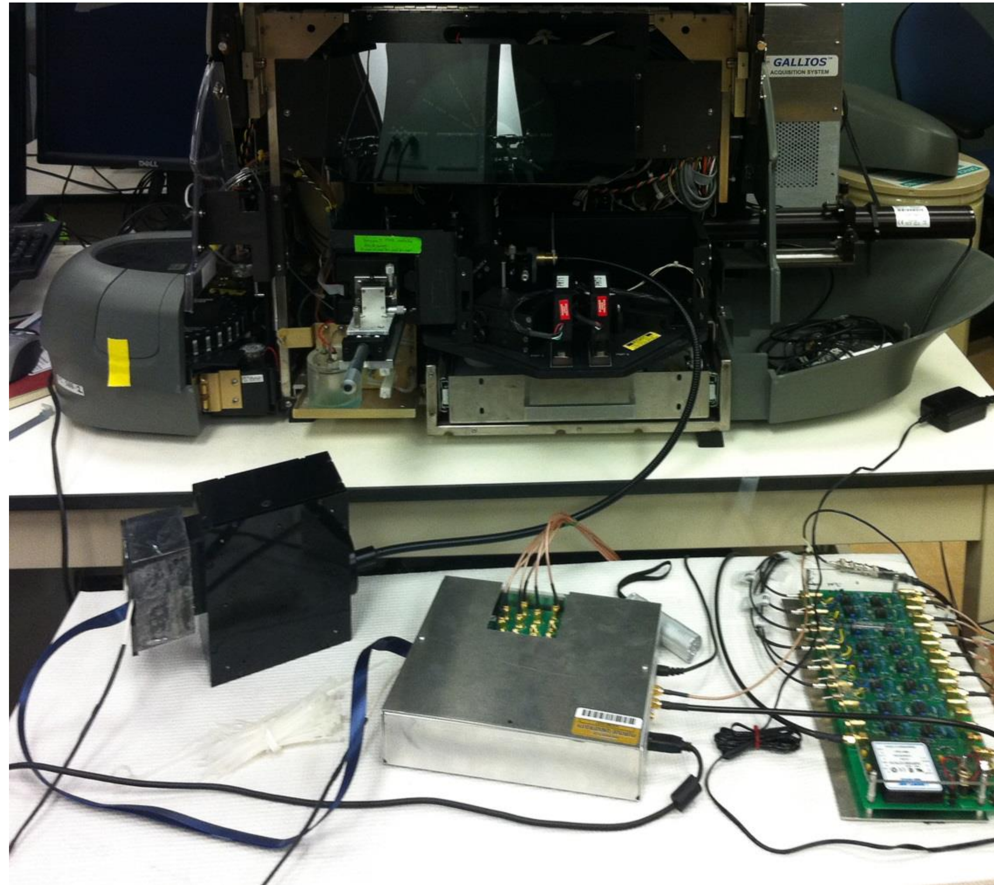
Average spectra for the 4 clusters identified by PCA (>7000 lymphocytes)



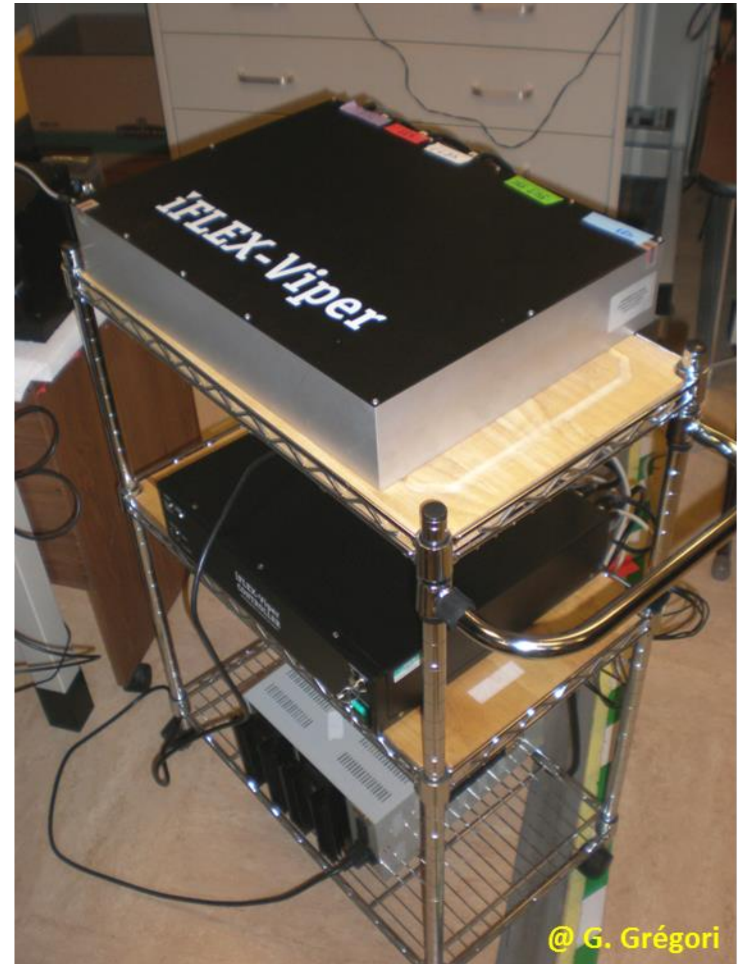
Cytospec Software

Grégori G., Patsekin V., Rajwa B., Jones J., Ragheb K., Holdman C., Robinson J.P. (2012). Cytometry Volume 81A, Issue 1, pages 35–44.

A new generation of hyperspectral detector installed on a conventional flow cytometer

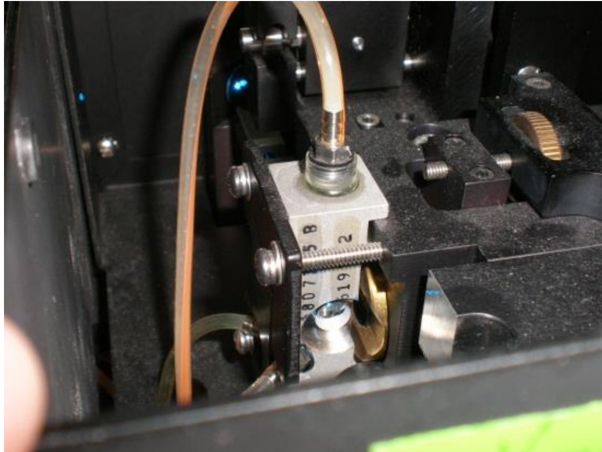


iFlex-Viper (Qioptiq) mounted on a FC500 flow cytometer

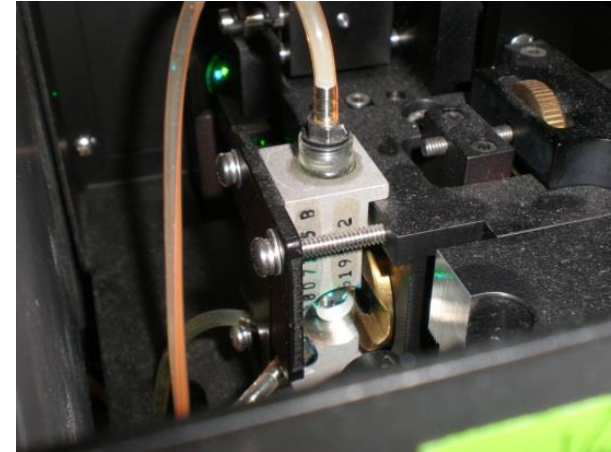


Pictures of the 5 laser beams from the iFlex-Viper and the flow cell of the FC500

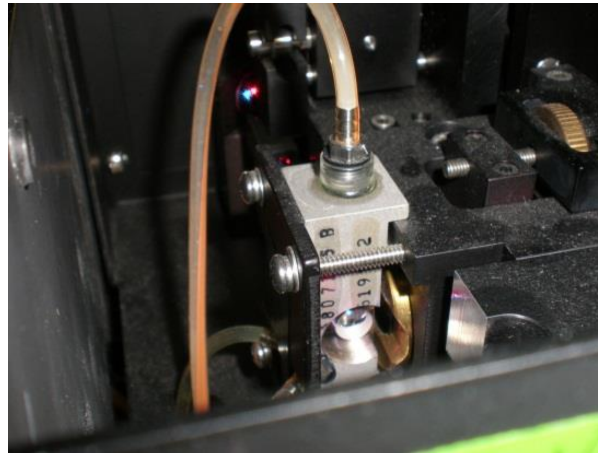
445nm = 21.1mW



561nm = 16.7mW

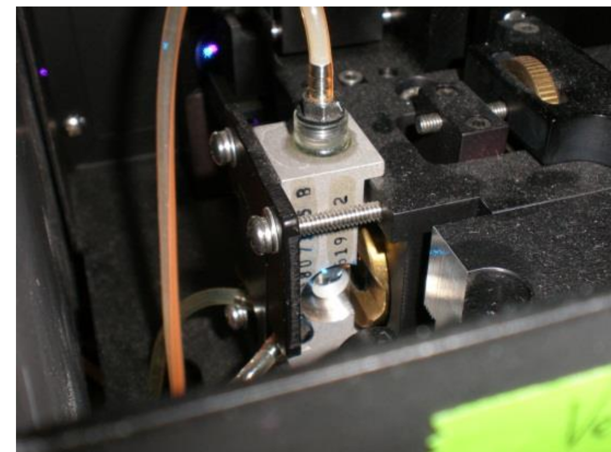


640nm = 14.4mW

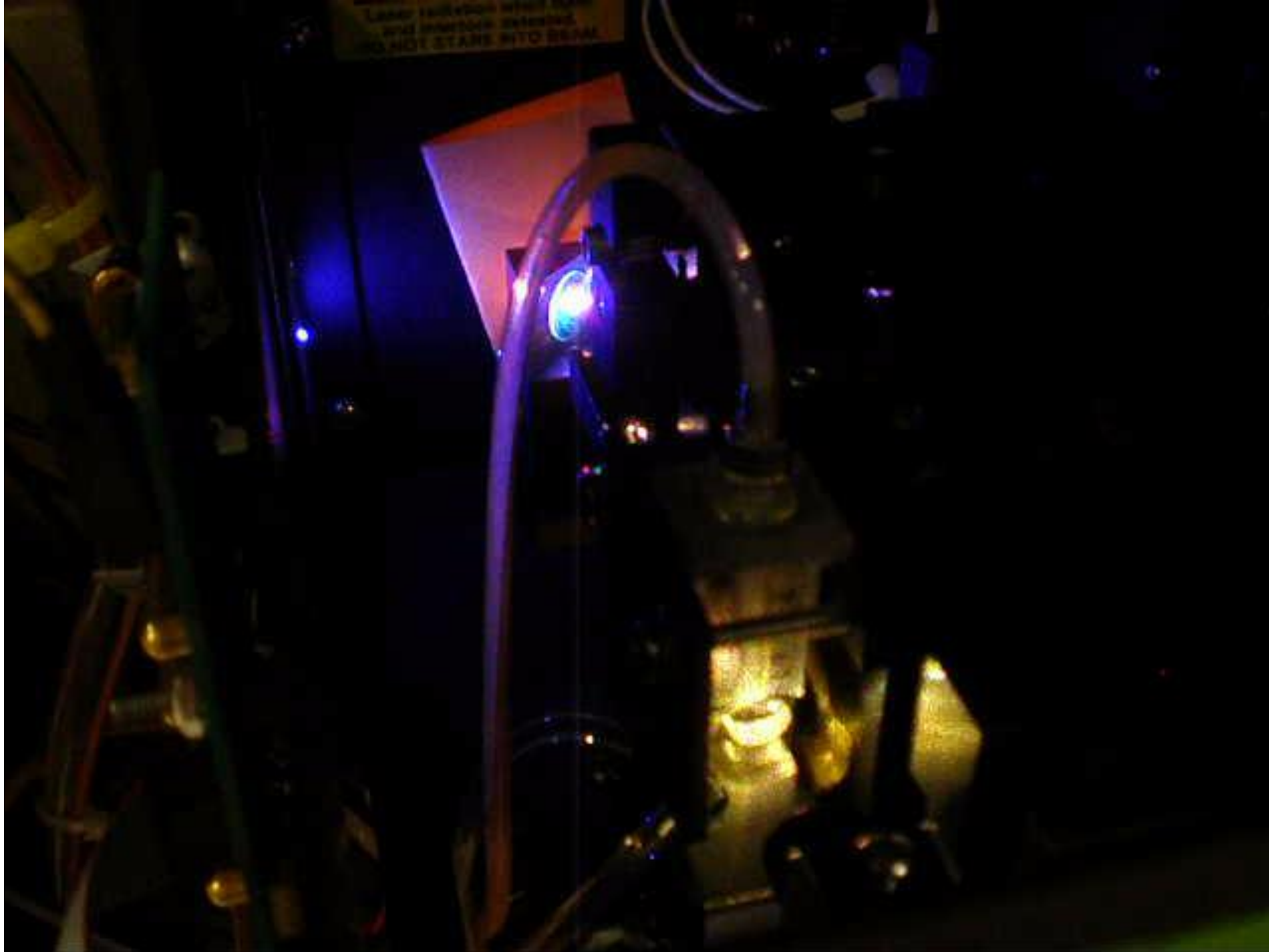


488nm = 19.3mW

405nm = 13.0mW



All lasers together



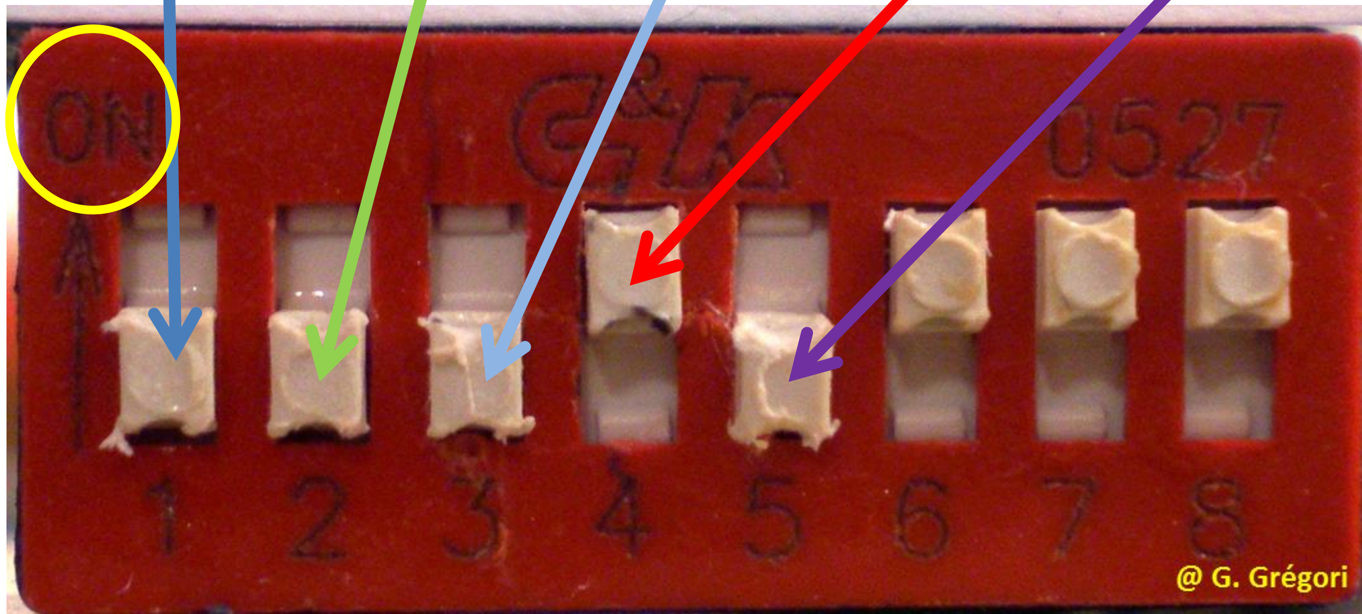
Do you see the « white » light?

« Advanced » Switches to control each single laser independently

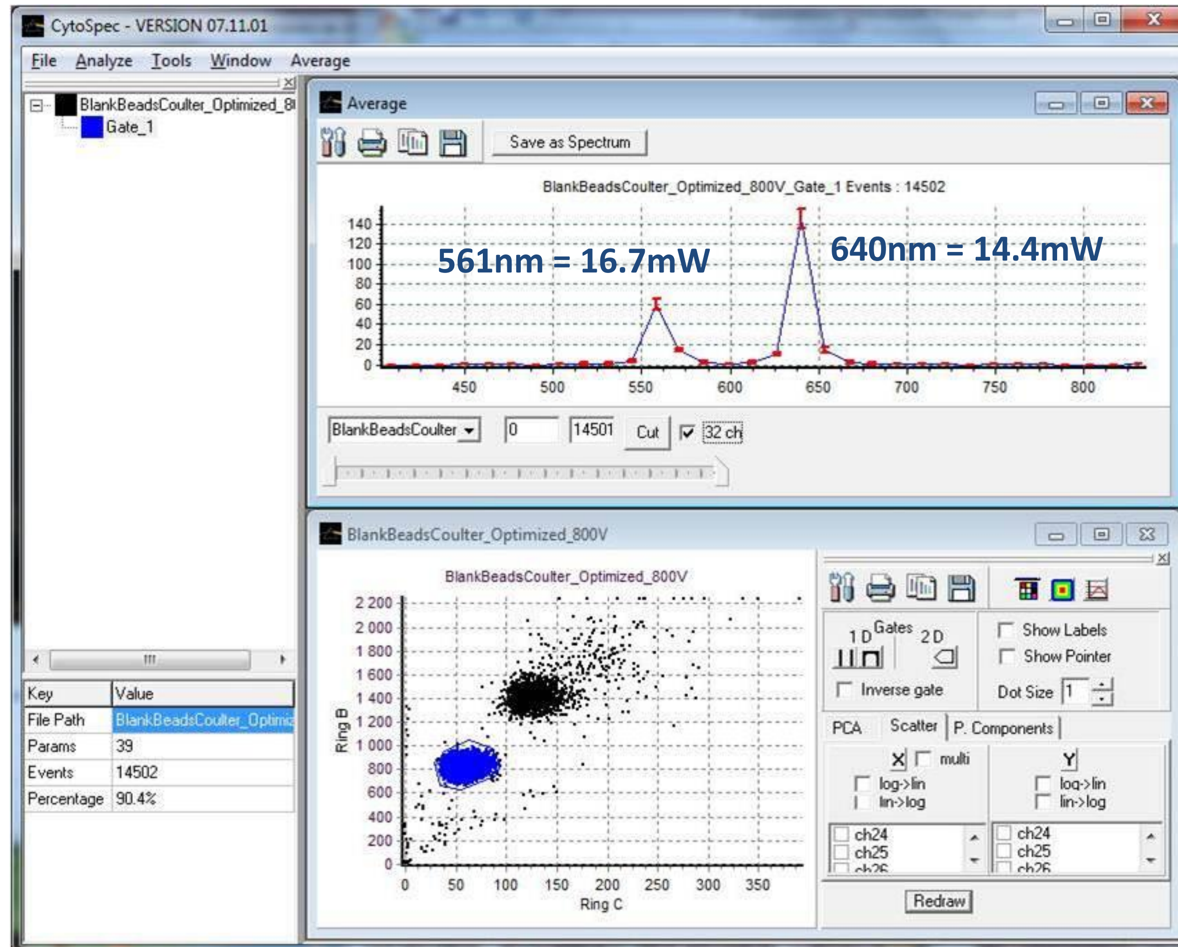
488nm = 19.3mW 561nm = 16.7mW 445nm = 21.1mW 640nm = 14.4mW 405nm = 13.0mW

ON

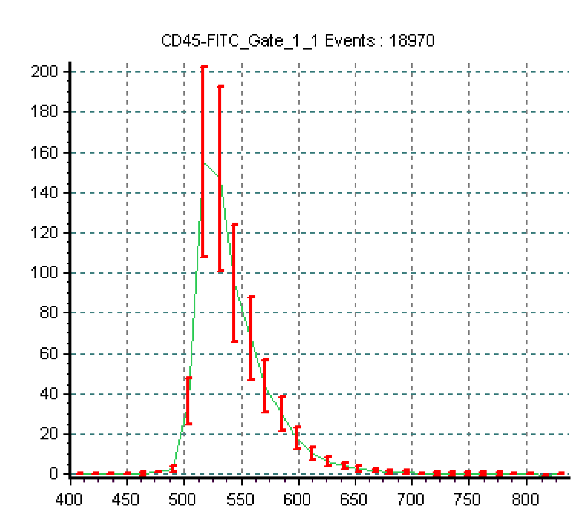
OFF

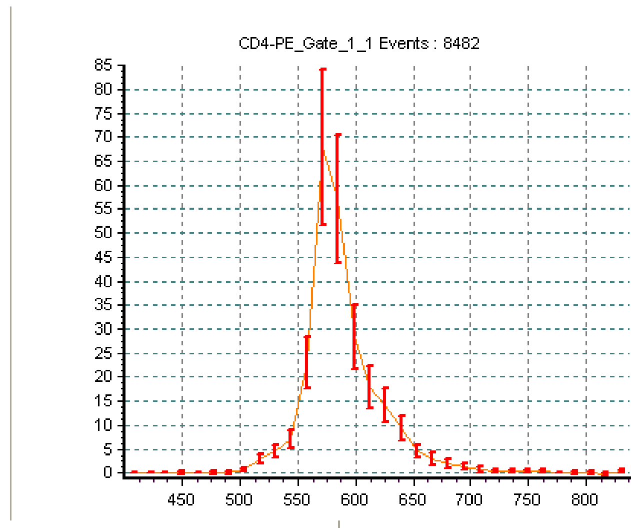


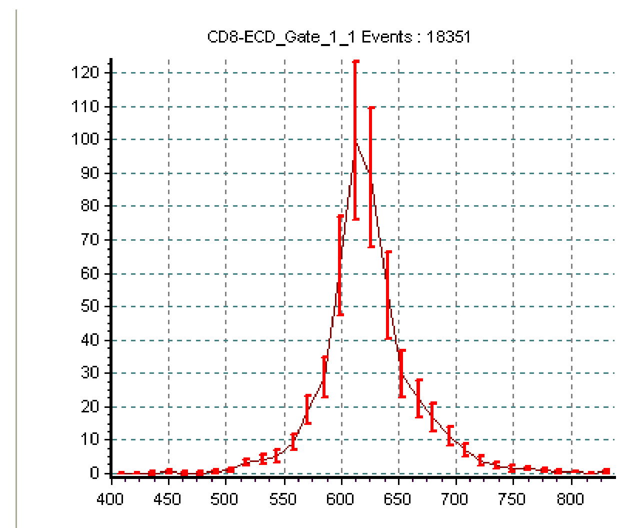
90° Light scatter collected for blank beads from 2 laser beams

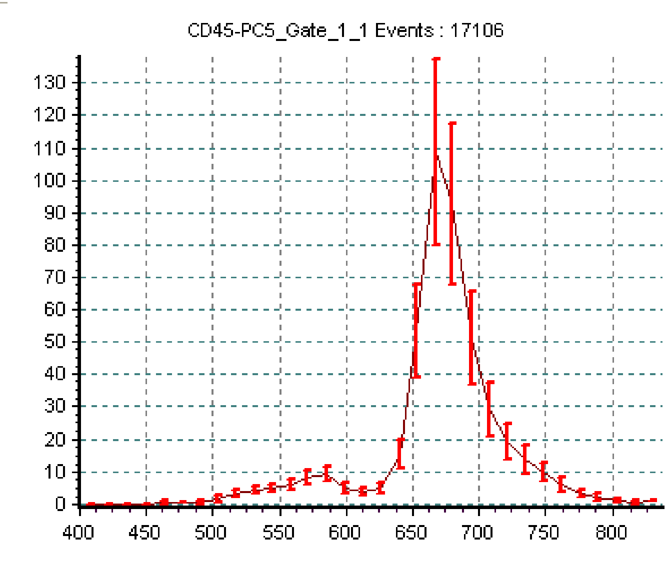


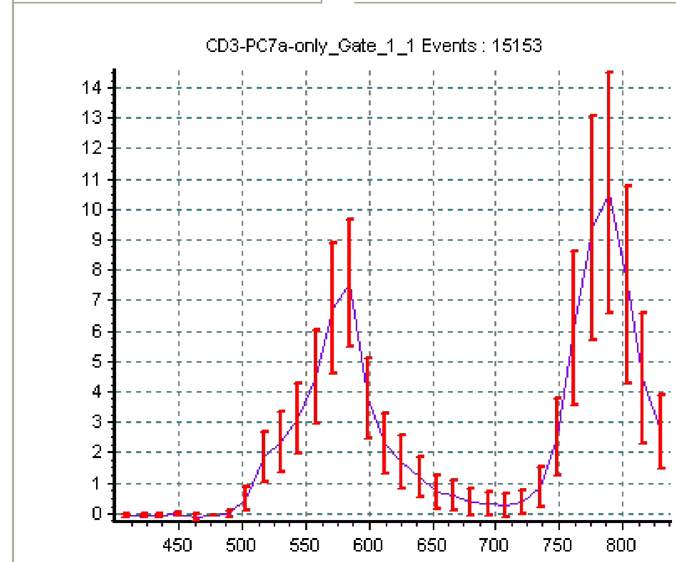
Blood sample analysis :
5 controls (CDs 45 FITC, 4 PE, 8 ECD, 45 CY5, 3 PC7)





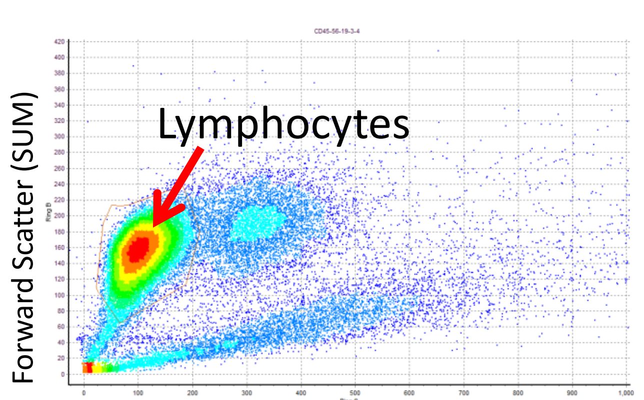




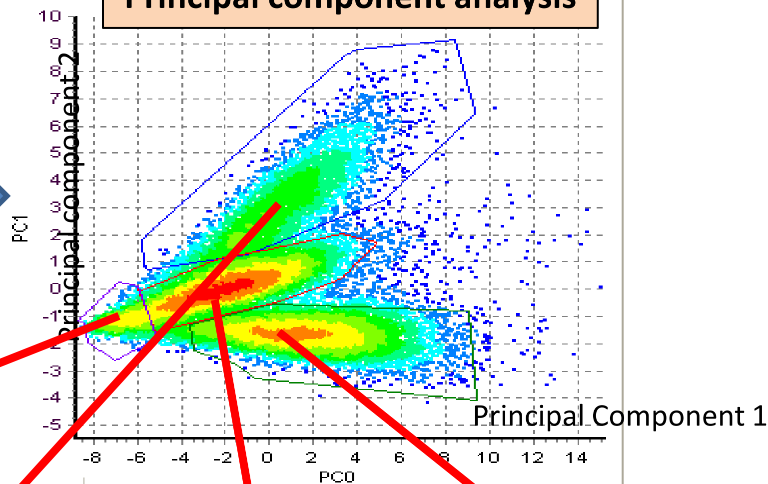


Blood sample :
5 colors (CDs 45 FITC, 4 PE, 8 ECD, 45 CY5, 3 PC7)

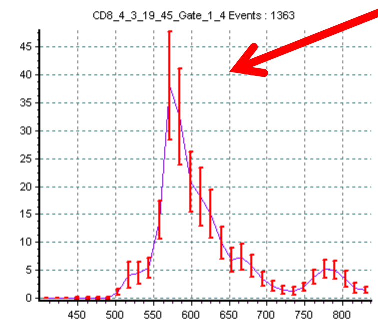
Gating of the lymphocytes

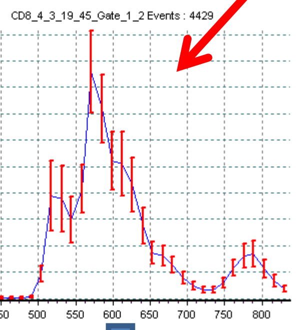


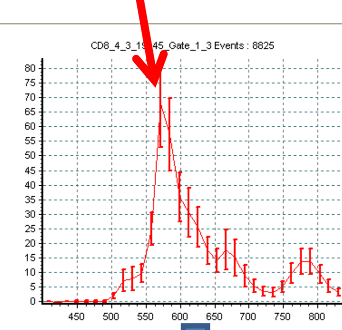
Principal component analysis

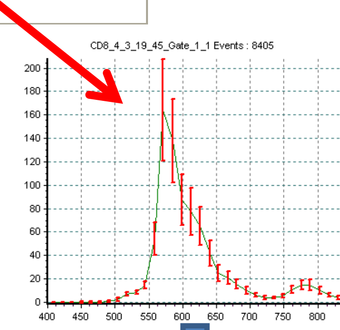


Average spectrum for each cluster (with Standard deviation)





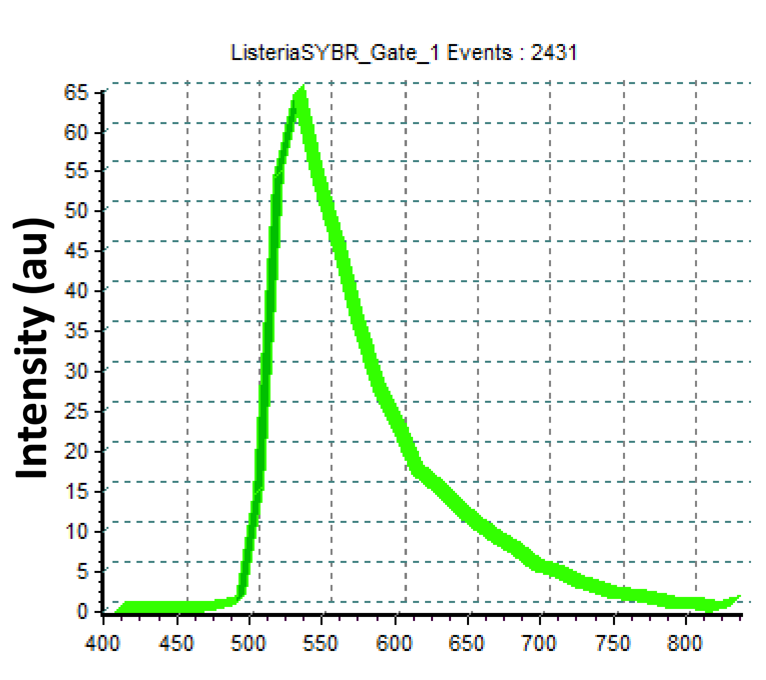


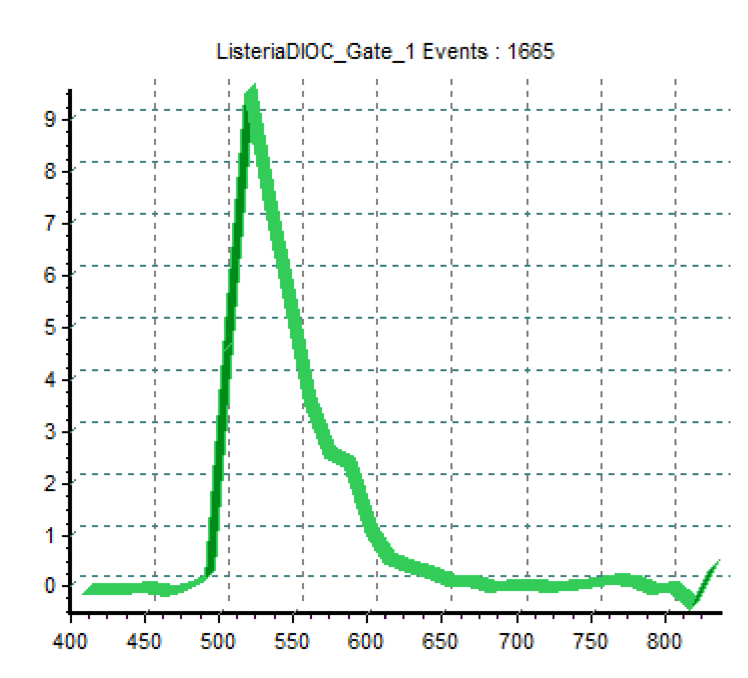


Decomposition of the sources

B, Helper T, Suppressor T-Lympho

First measurements on bacteria: Listeria





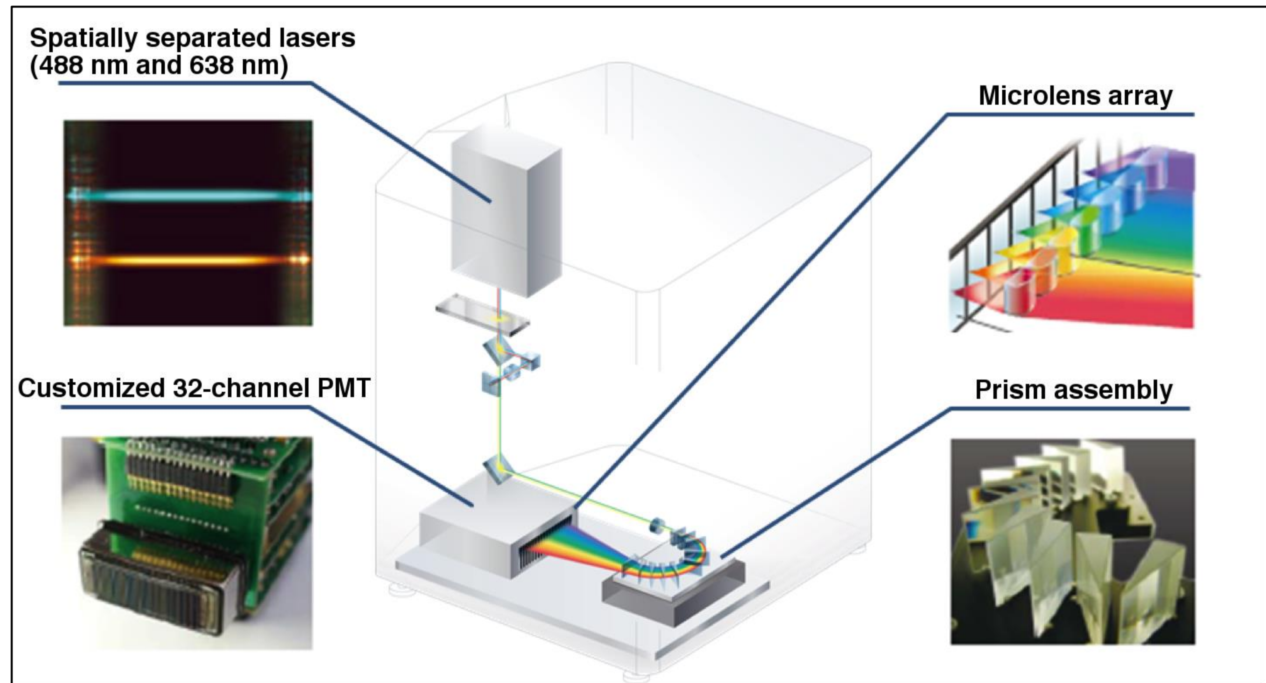
Wavelength (nm)

SYBRGreen I

DiOC6(3)

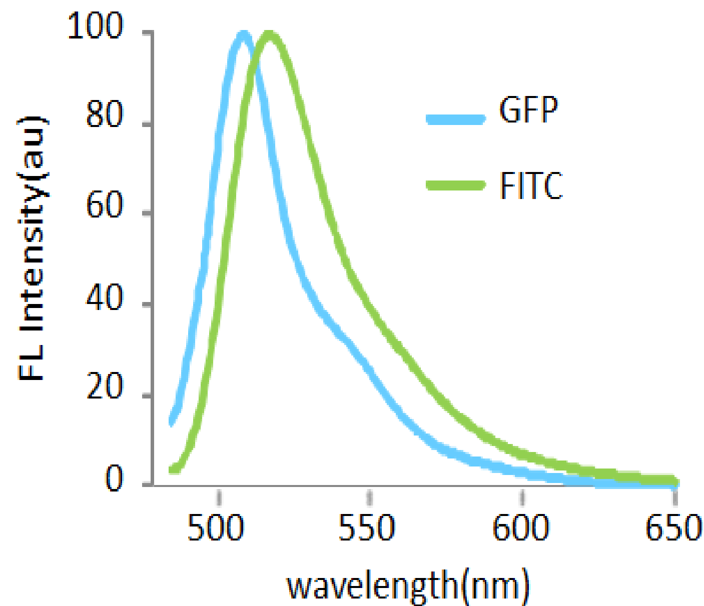
« J'en ai rêvé … SONY l'a fait »

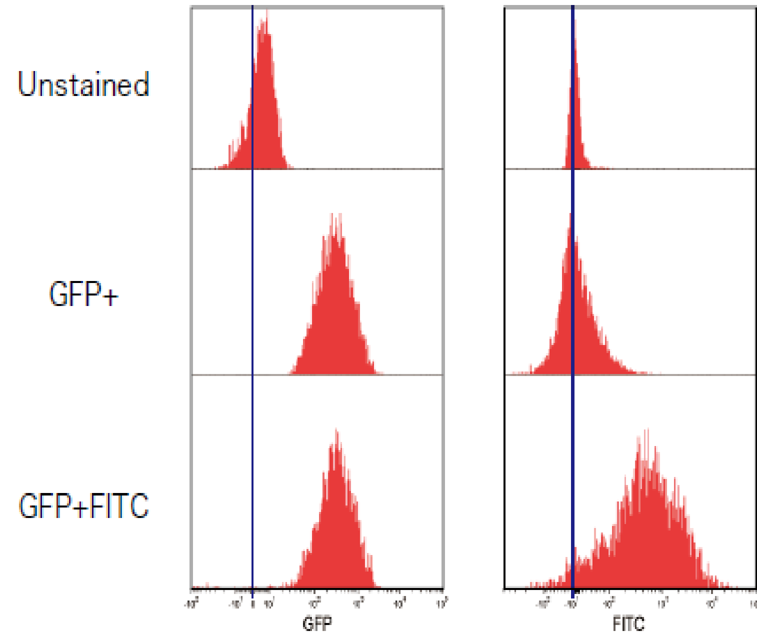
Sony SP6800 spectral cell analyzer



- Spatially separated lasers (488 nm and 638 nm)
- The sample's fluorescence spectra ranging from 500 nm to 800 nm are collected by a 32-channel linear array PMT detector equipped with a multi-prism monochromator

Separation of GFP and FITC signals using Sony spectral system





Data courtesy of W.C. Hyun (UCSF)

SP6800 is capable of combing the channels in the PMT to form "virtual filters"

- ➔ Function as a regular polychromatic FC
- ➔ Capability of fine-tuning the detection to adjust for particular staining strategies as well as problems with label intensities or markers abundances.

New approach to the problem of unmixing in multispectral FC.

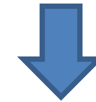
(A) OLS–based unmixing

(B) NNLS–based unmixing

(C) MAPE–based unmixing

(D) Poisson–based unmixing

Extension of the well-established concept of unmixing as used in other fields such as **remote sensing, spectral imaging, and chemometrics** and modification for use with cytometry systems



Unmixing using Poisson regression

Novo D., Grégori G., Rajwa B. (2013). Generalized unmixing model for multispectral flow cytometry utilizing non-square compensation matrices. Cytometry Part A 83A : 508-520

Conclusion and Prospective

PUCL Prototypes have demonstrated :

- Collection of a fluorescence "spectrum" at the single cell level (range 500-800 nm)
- Analysis at a flow rate up to 1,000 events.s^{-1} (as on regular FC)
- System sensitive enough to detect dim signals (bacteria)

****

SONY has optimized the system

- Bring its technology (Blue Ray and DVD laser control technology; PMT more sensitive; extension of the wavelength range →800 nm)
- Develop the Software :
  - → To optimize data display and analysis;
  - → Deconvolution of the emissions of several dyes used together

➔Commercialization of the SONY SP6800 spectral cell analyzer

****

Still to do :

- Work on data analysis (automated deconvolution and cell clustering)
- Improve particle discrimination in a sample thanks to current & emerging bio-labeling methodologies (new dyes; nanocrystals)

Merci

Gracias

Speacial thanks to :

- Kathy
- Cheryl
- Jennie
- Valery
- Bartek
- Gretchen
- Jim

& Paul

Grazie

Danke

Obrigado