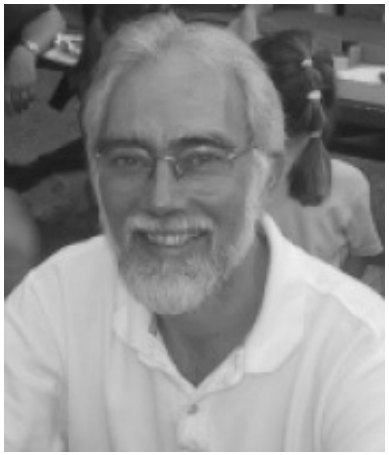
Next-Generation Cytomics: Spectral Fingerprinting

J. Paul Robinson

SVM Professor of Cytomics
Professor of Immunopharmacology
Professor of Biomedical Engineering
Director, Purdue University Cytometry Laboratories
Deputy Director, Bindley Biosciences Center



PURDUE UNIVERSITY

PUCL cytometry laboratories

Cell measurement technologies in ‘cytomics’

“…the systematic study of biological organization and behavior at the cellular level…

….has developed out of computational imaging and flow cytometry and promises to provide essential data for systems biology.”

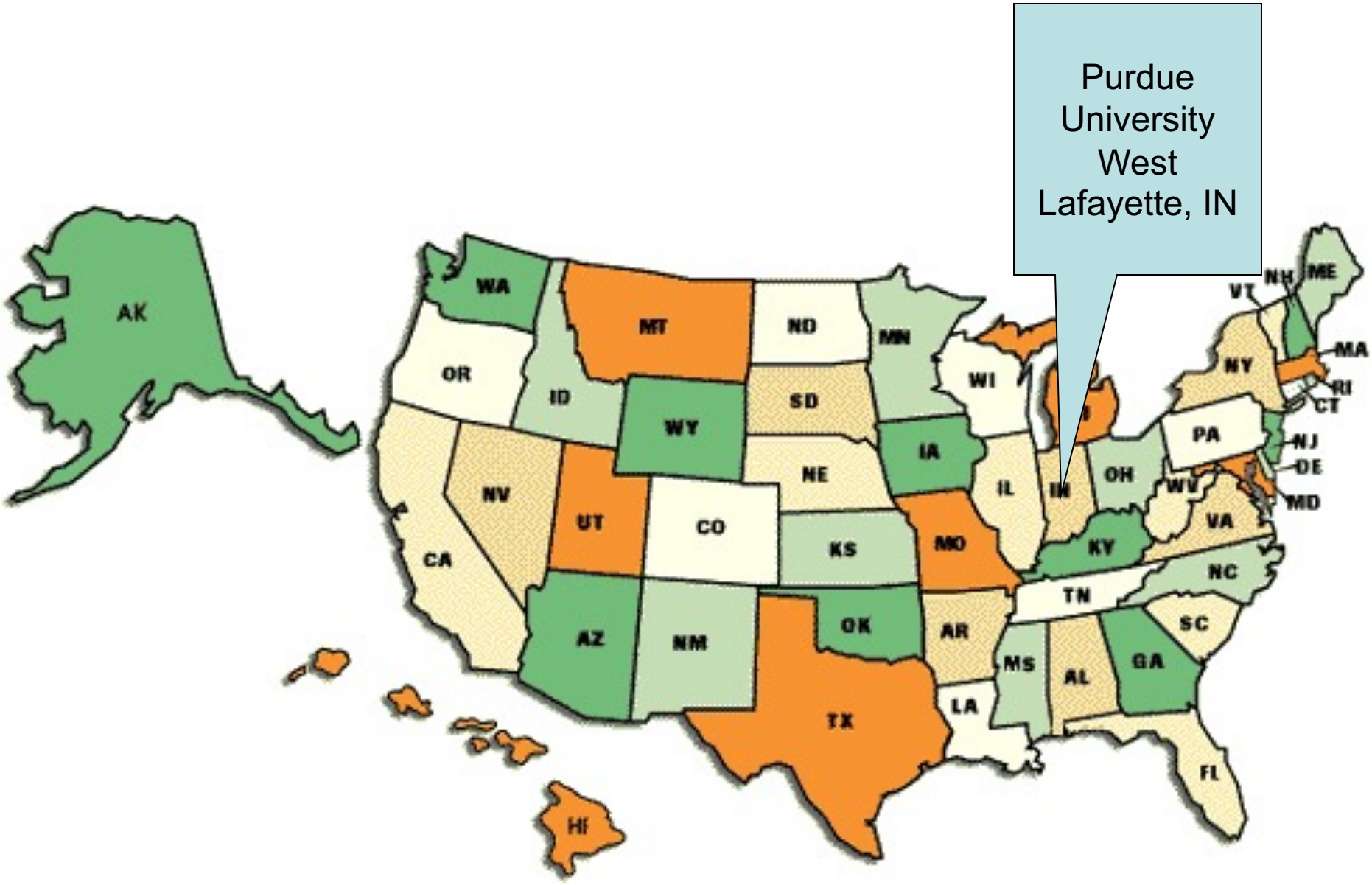
PUCL
cytometry laboratories

The National Center for Applied Cytomics

An NIH Research Resource

Sabbatical visit program funded by the Center as well as postdoctoral opportunities

PUCL
cytometry laboratories



PUCL
cytometry laboratories

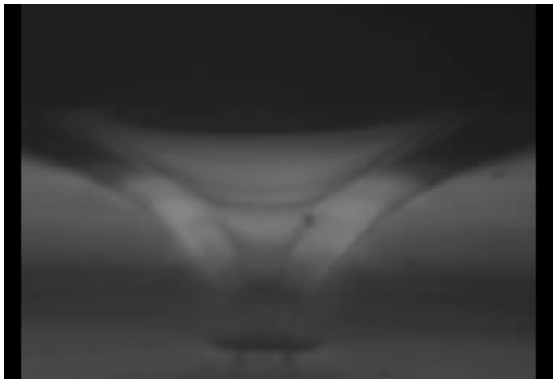
Biophotonic Projects

- High speed spectral analysis of particles
- Angle-resolved light scattering of particles
- Whole animal spectral imaging
- Bacterial classification by angular scattering fingerprints
- Spectral endoscopy using multimodal imaging
- Calibrated fluorescence opthalmoscopy
- Microdroplet delivery/manipulation systems

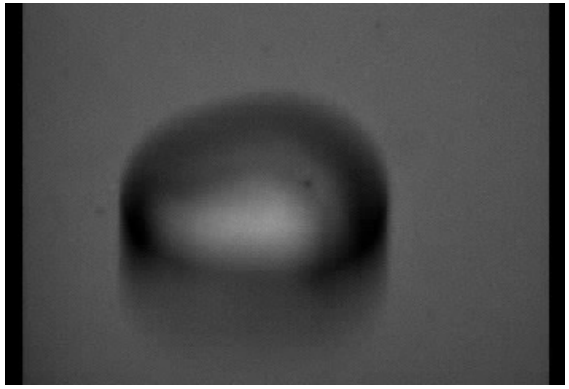
PUCL
cytometry laboratories

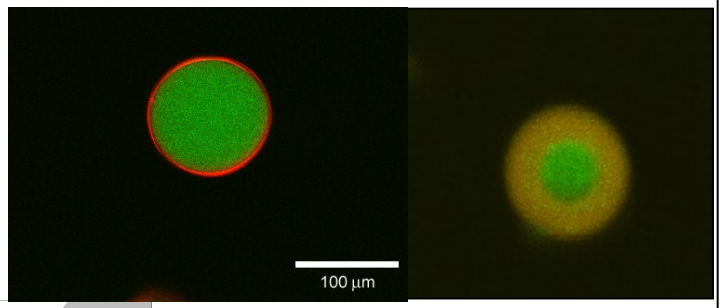
Microdroplet Generation & Manipulation for Drug Delivery

Controlled pressure regulation changes

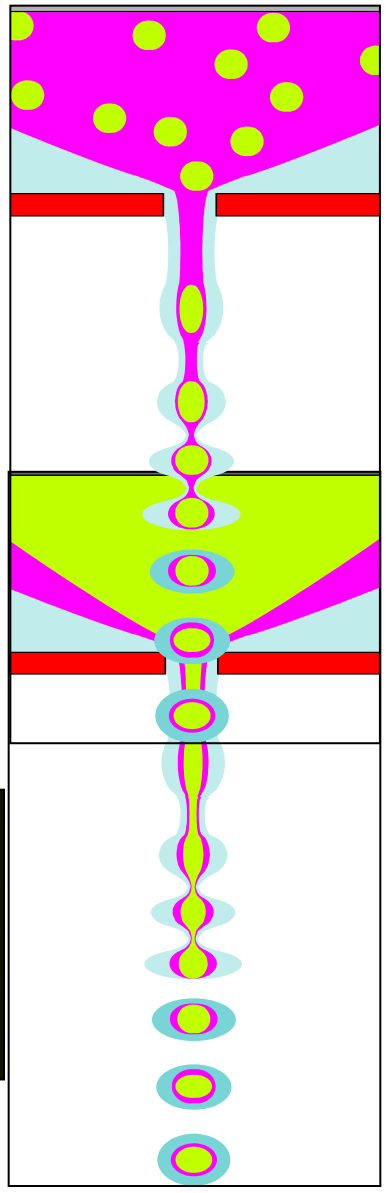


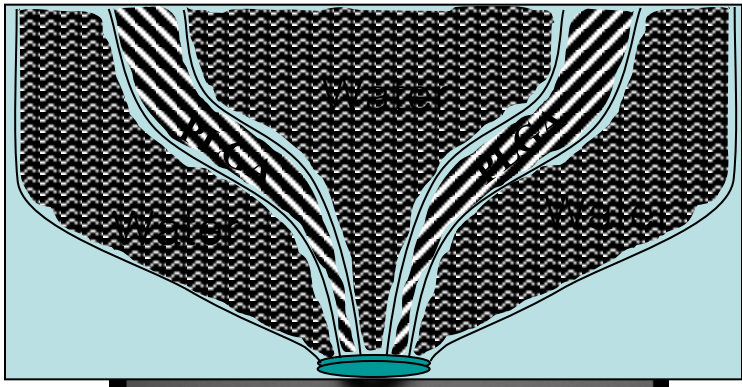
Addition of small beads

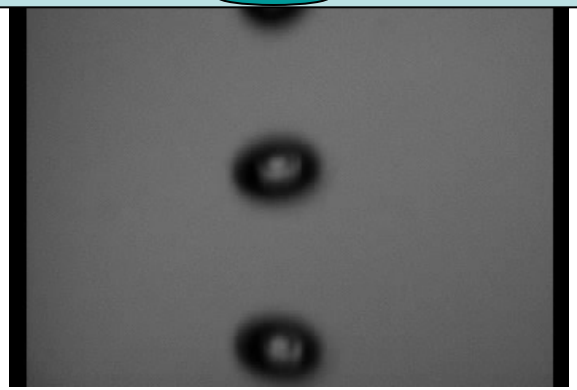


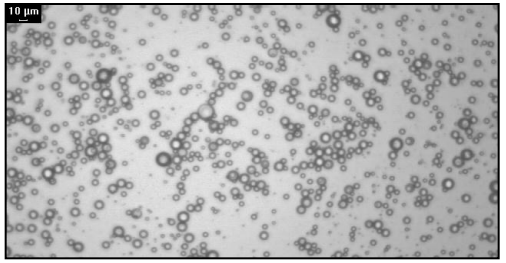


PUCL
cytometry laboratories



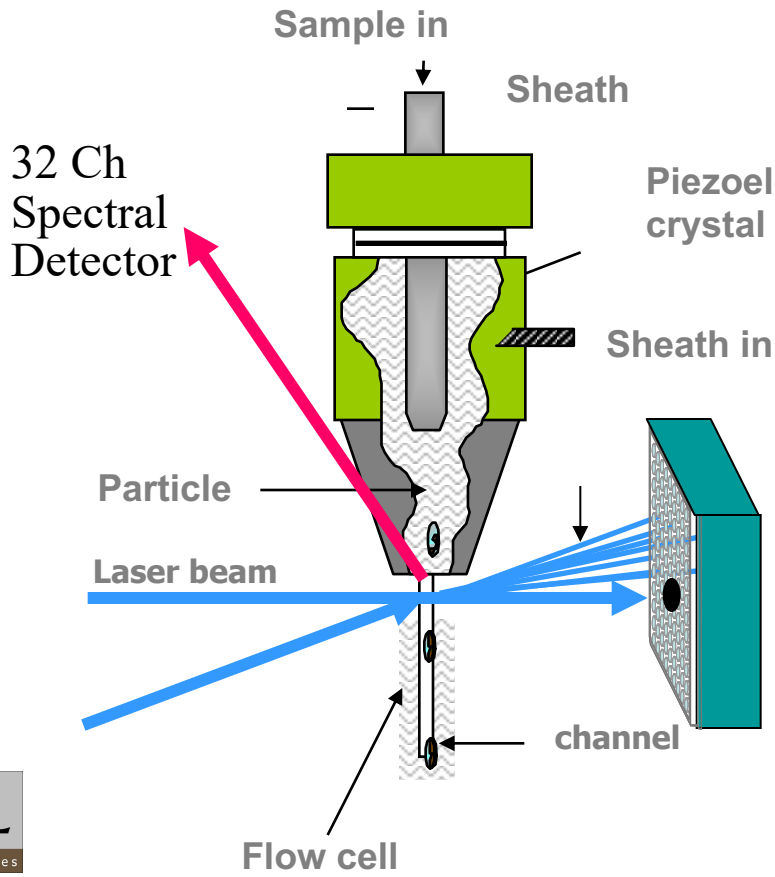






hydrogels

Focus the advanced capabilities offered by angle-resolved light scattering on cytometry,

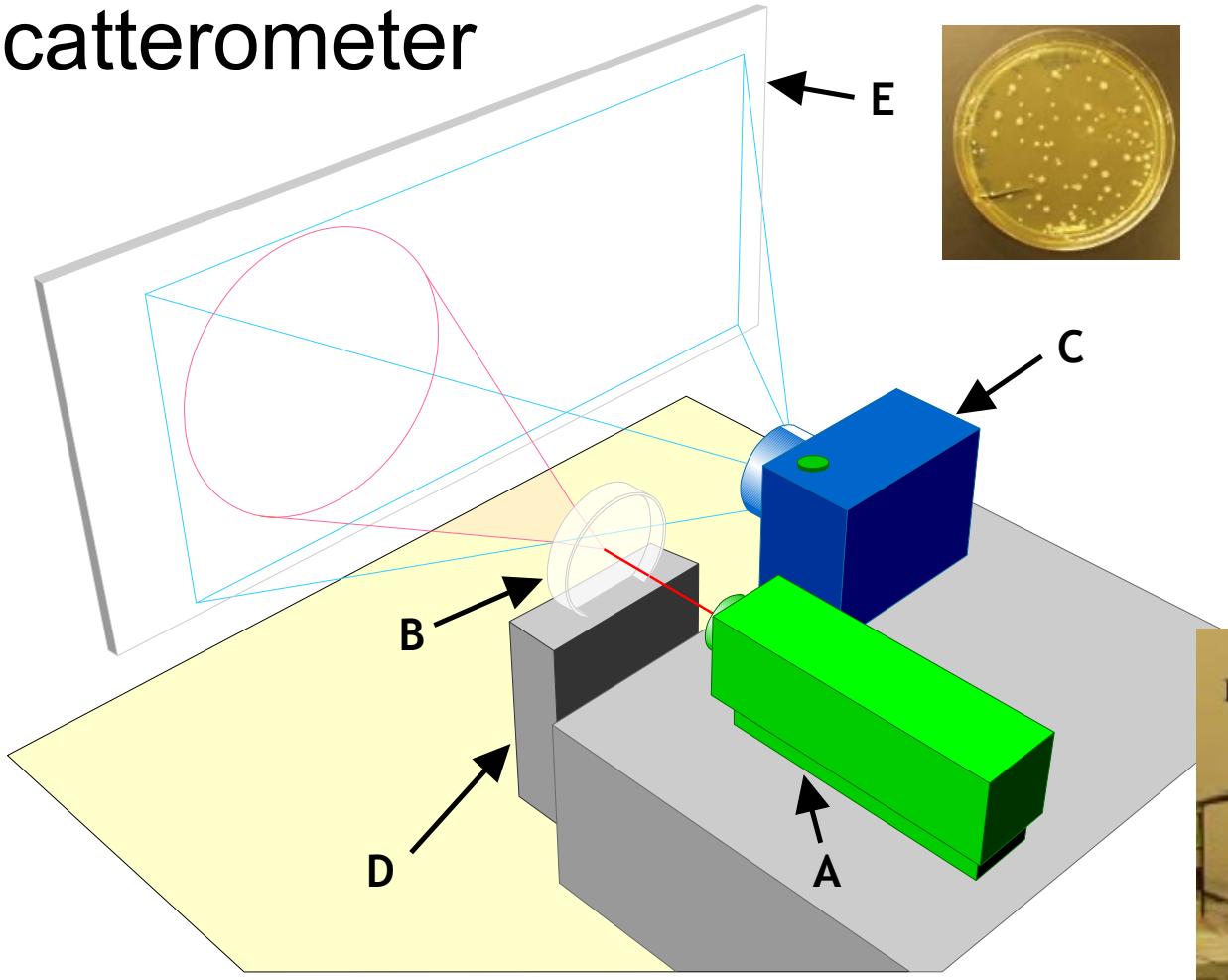


- Collect 32 channels of spectral data
- Collect 20-32 channels of calibrated scatter data
- Use scattering “fingerprint” analysis to extract information on size, shape, and “refractive index” distribution
- 1000-3000 particles/sec for 64 channel data

PUCL
cytometry laboratories

Bacterial classification by angular scattering fingerprints

Scatterometer



>1500 scatter patterns from cultures of 108 *Listeria* strains were measured and analyzed

69 – *L. monocytogenes*

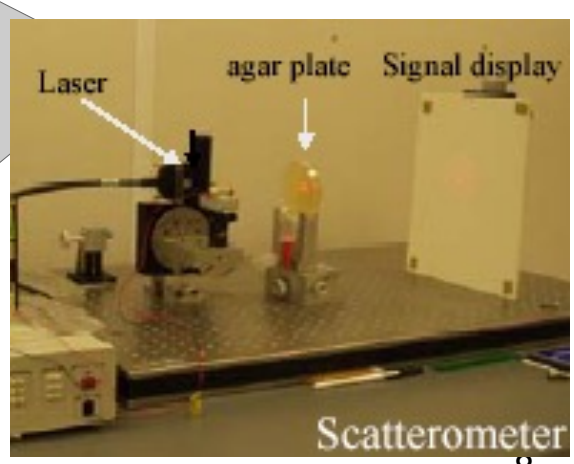
16 - *L. innocua*

12 - *L. ivanovii*

5 - *L. seeligeri*

3 - *L. welshimeri*

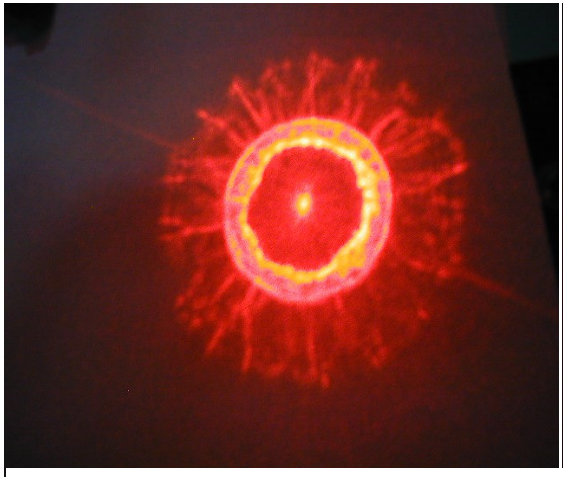
3 - *L. grayi*



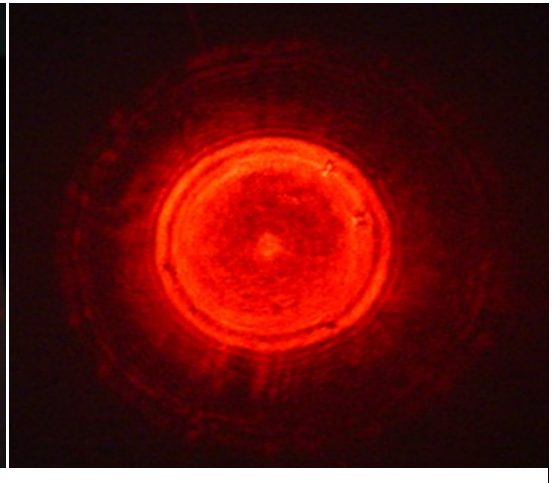
Robinson, Bhunia, Hirleman

PUCL cytometry laboratories

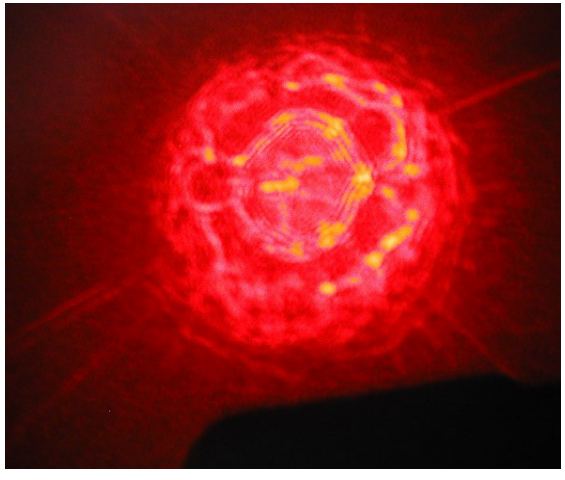
Every organism has a very specific scatter pattern



***L. monocytogenes* ATCC19113**



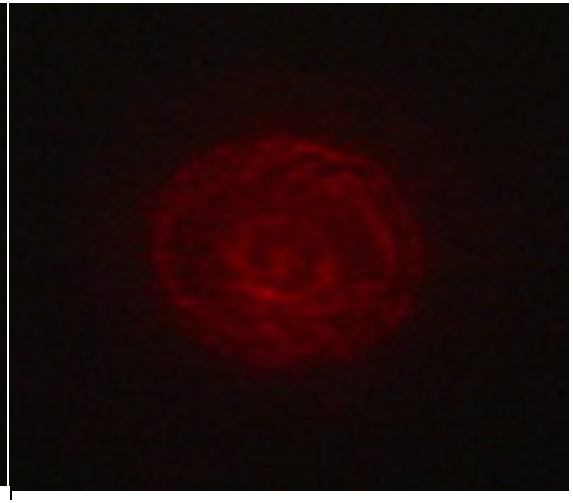
***L. innocua* F4248**



***L. ivanovii* ATCC19119**

***L. seeligeri* LA 15**

***L. welshimeri* ATCC35897**



***L. grayi* LM37**

PUCL
cytometry laboratories

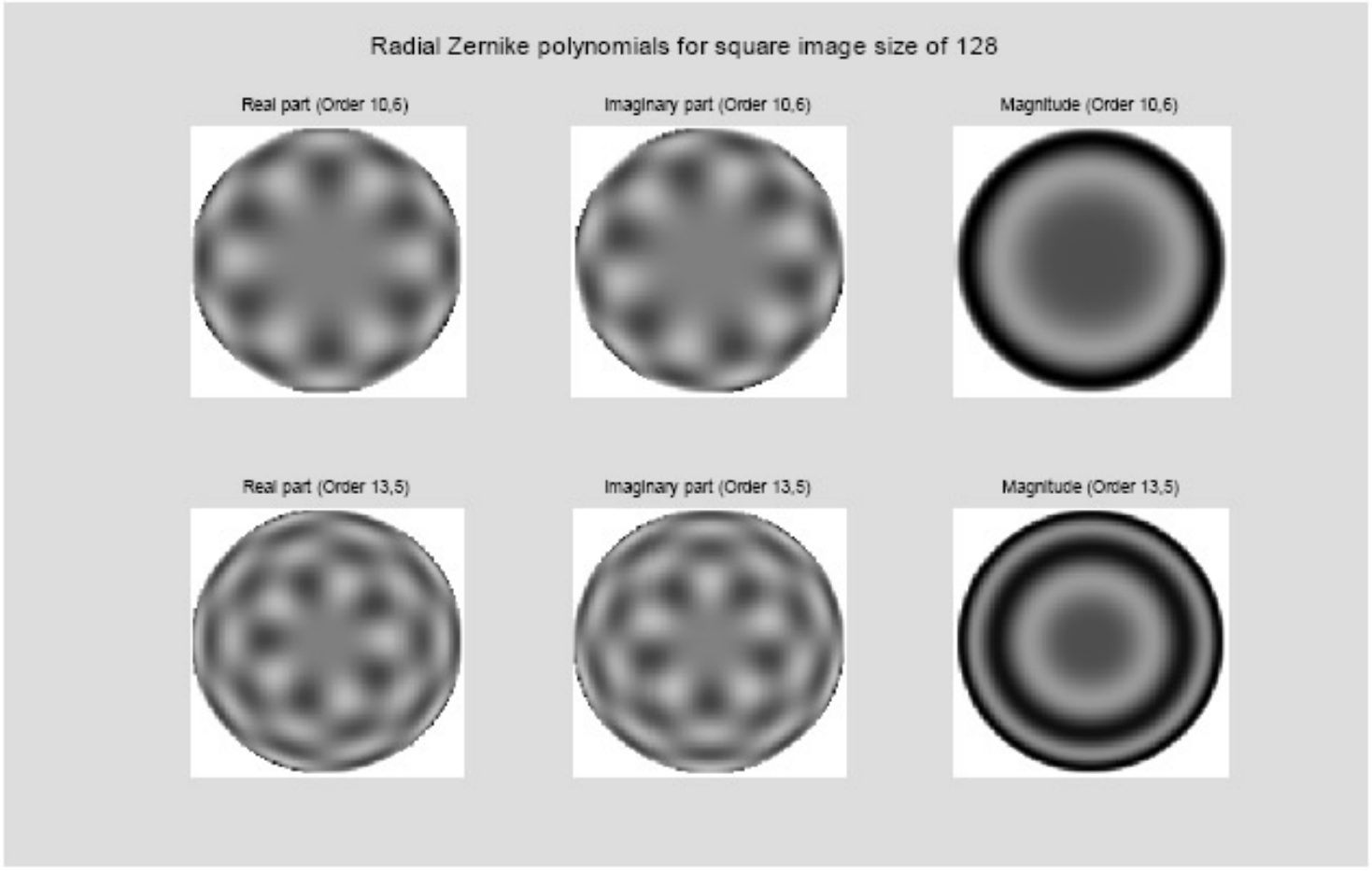
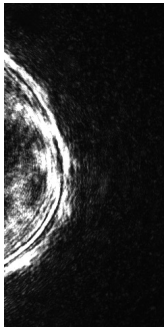
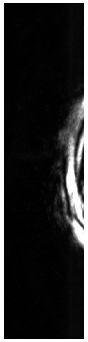


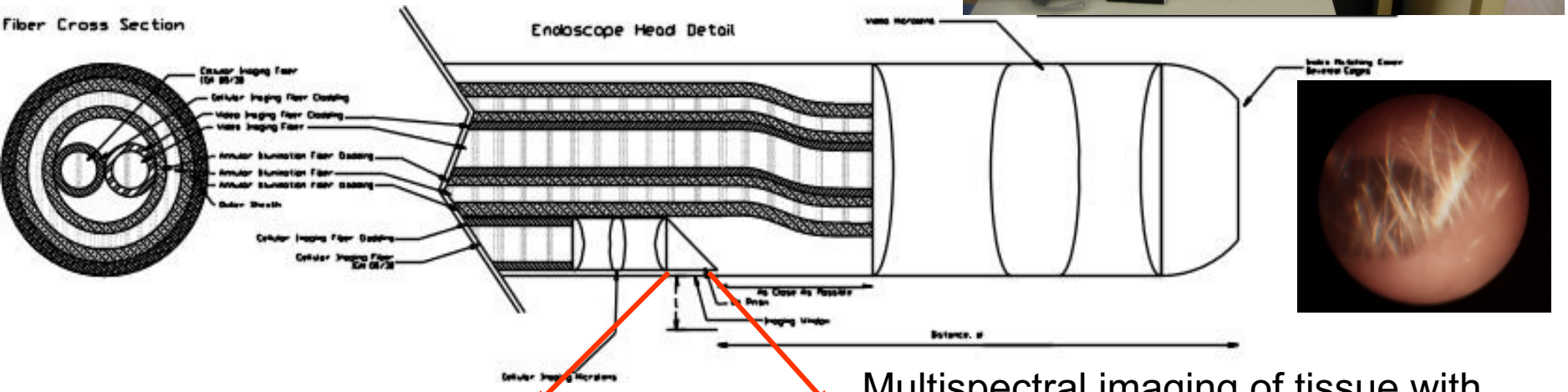
Fig. 1. Graphical representation of radial Zernike polynomials in 2D.

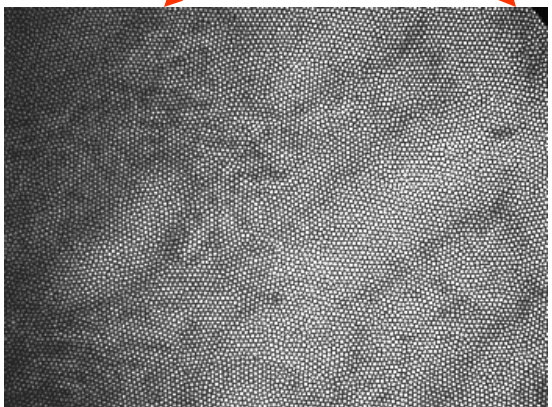


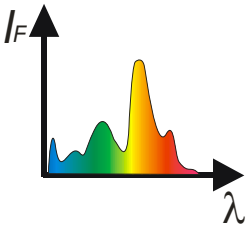


- Can we do the classification in real time

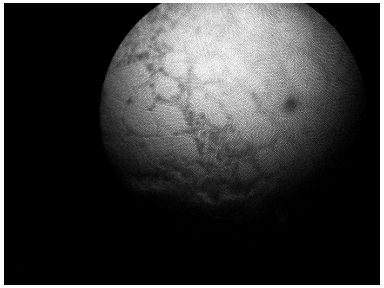
Multimode Endoscope







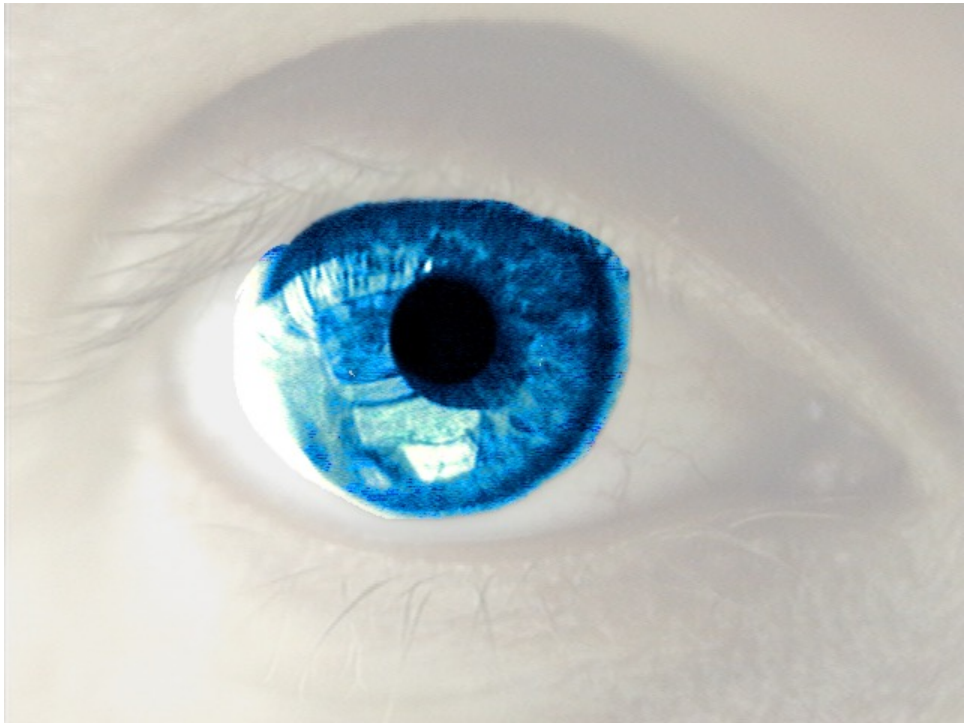
Multispectral imaging of tissue with simultaneous white light imaging



PUCL cytometry laboratories

Purdue University Patent Pending

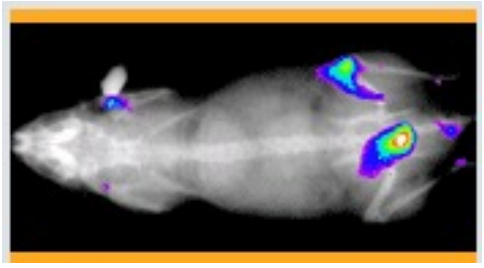
Calibrated Fluorescence Opthalmoscopy



PUCL
cytometry laboratories

Whole Animal Spectral Imaging

- Use AOTF based imaging system with a variety of cameras & light sources
- Multispectral imaging:
  - Use of multiple fluorescent probes.
  - Discrimination of autofluorescence.



PUCL
cytometry laboratories

Cell Analysis Tools

- Measurement of the optical properties of single cells
  - Example: blood cells – separation of white blood cell populations
  - Example: mixed bacterial suspensions –classification by species
  - Example: multiplexed beads – identification of analytes in fluids such as serum, CSF, cell culture medium, etc
  - Example: functional analysis of cell populations – oxidative metabolism, enzyme, cell cycle analysis

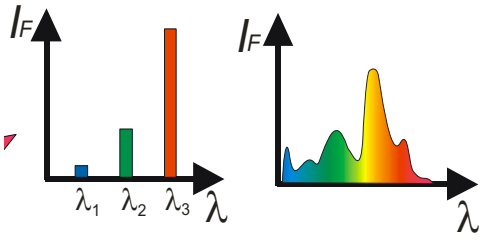
PUCL
cytometry laboratories

Integration of Technologies

- Redefine the fundamental basis for flow cytometry design by integration of principles of chemical analysis and image analysis
  - Use a spectral analysis technology as opposed to a fluorescence intensity profile analysis system
  - Implement capabilities for automated classification

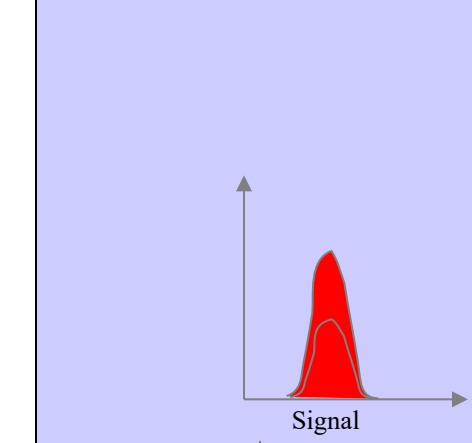
IMPACT:

- Next generation clinical diagnostics in real time
- Fast classification for any complex system

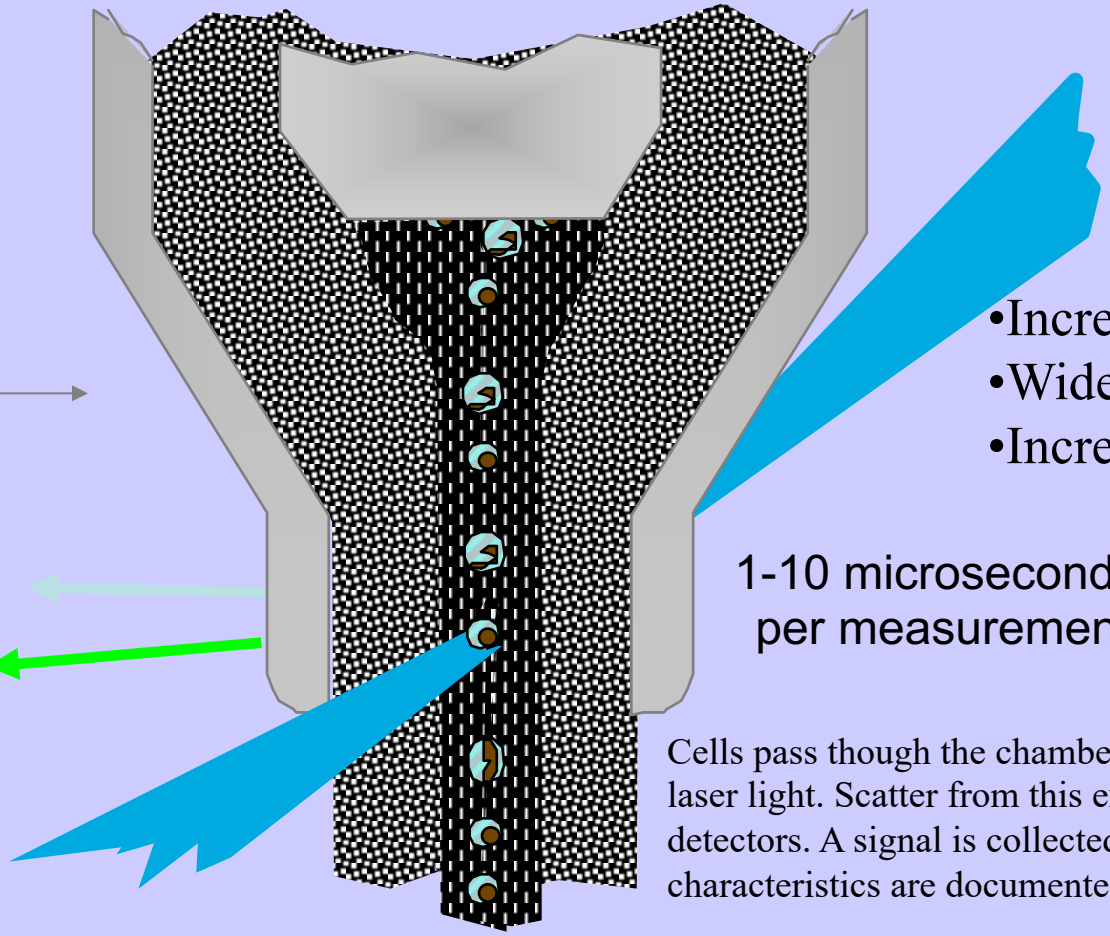


PUCL
cytometry laboratories

Hydrodynamically focused fluidics



Laser
Excitation
volume



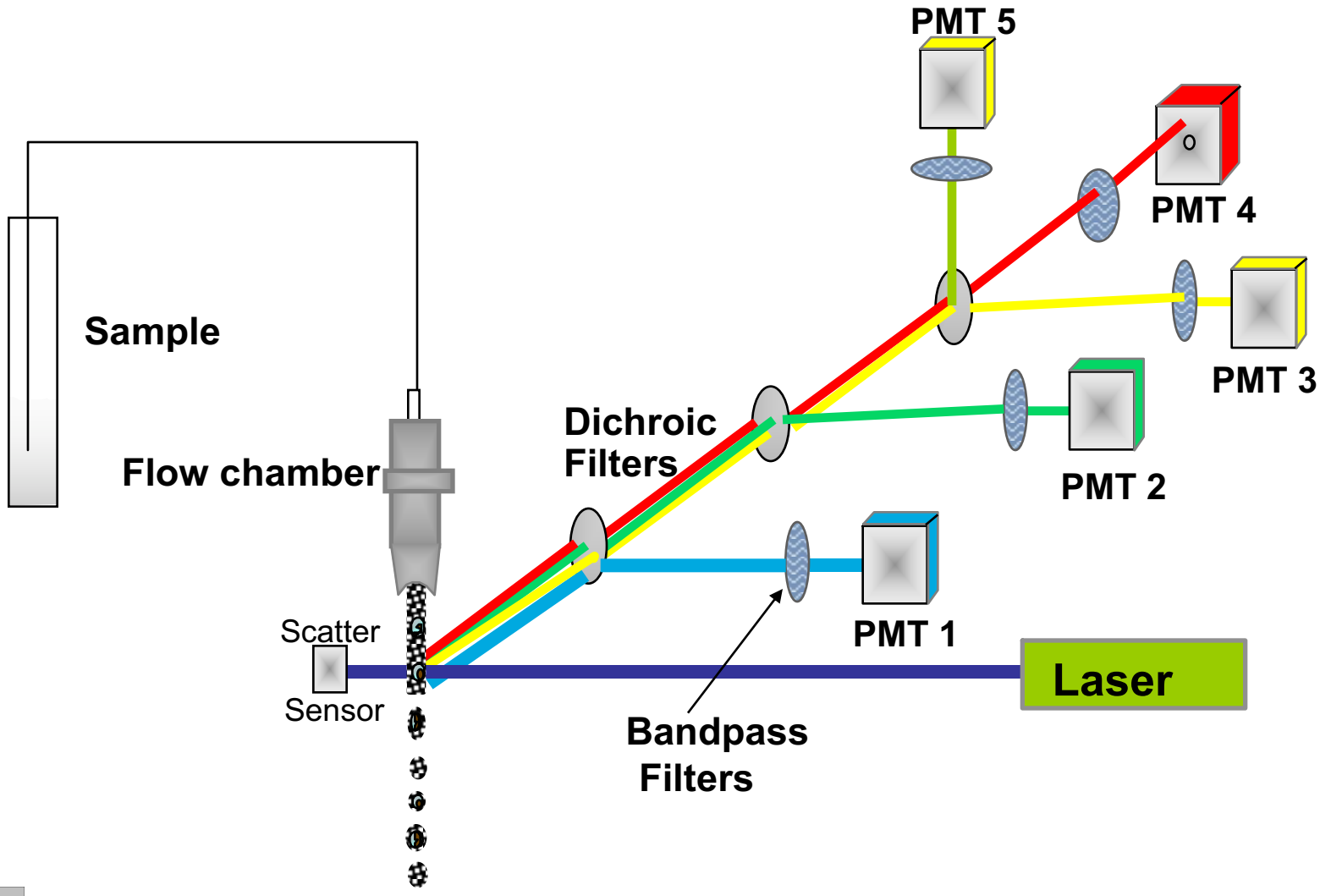
- Increase Pressure:
- Wider Core
- Increased Turbulence

1-10 microseconds
per measurement

Cells pass though the chamber and are excited by the laser light. Scatter from this excitation is directed to detectors. A signal is collected for each cell and its characteristics are documented as shown on left.

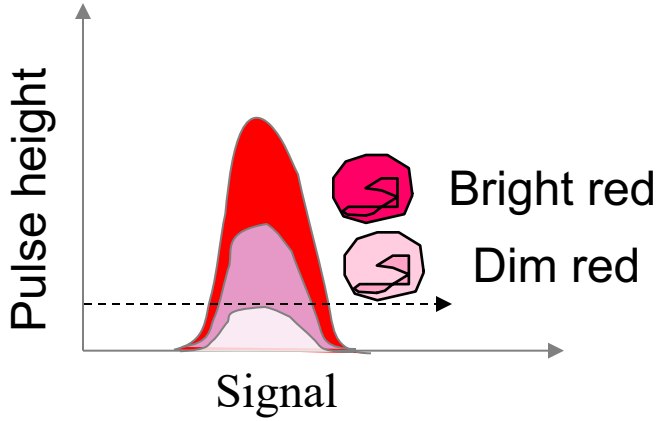
PUCL
cytometry laboratories

Optical Design of a basic flow cytometer

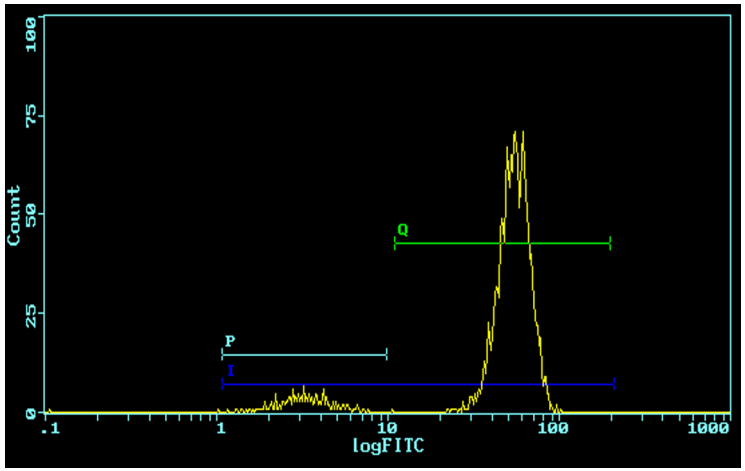


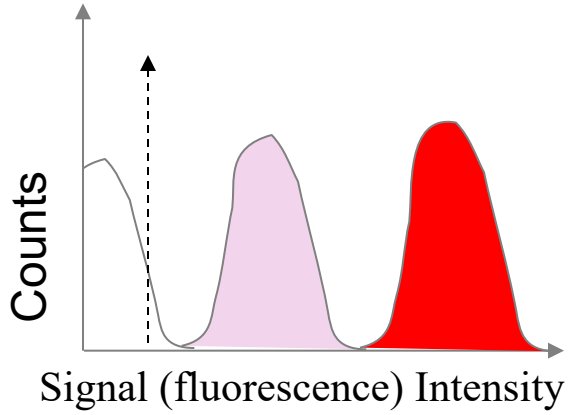
PUCL
cytometry laboratories

Review of the principles that govern current flow cytometry



Single band measurement

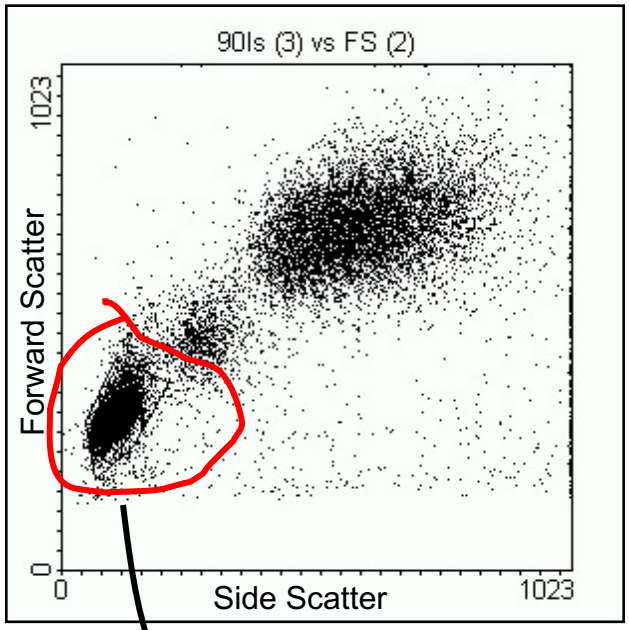




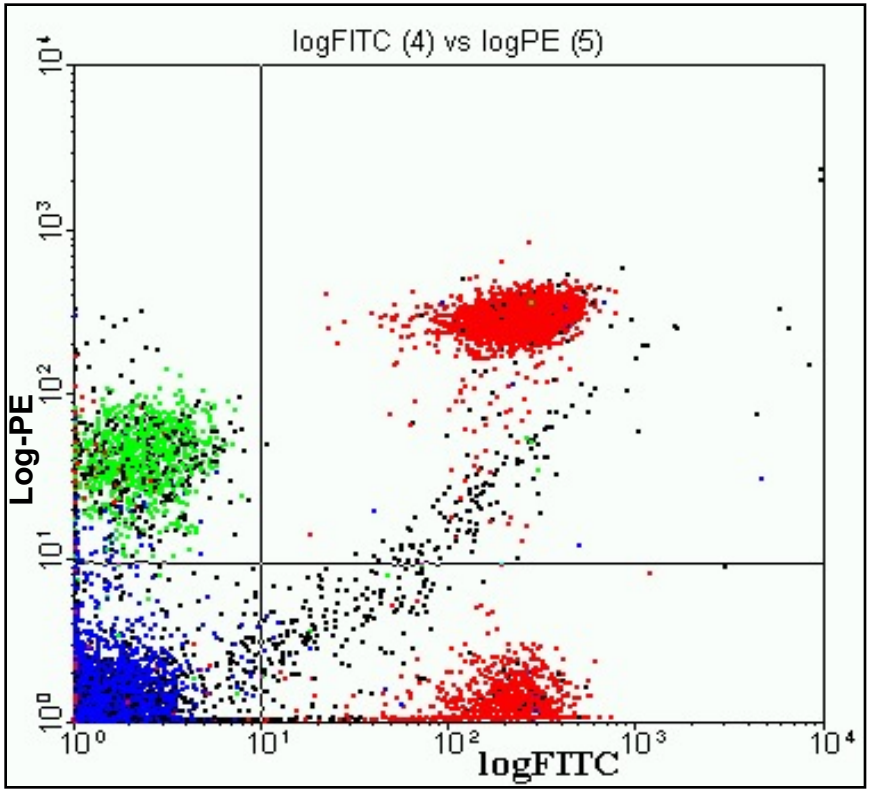
PUCL
cytometry laboratories

Basic gating and analysis
population identification

Variables 1 & 2

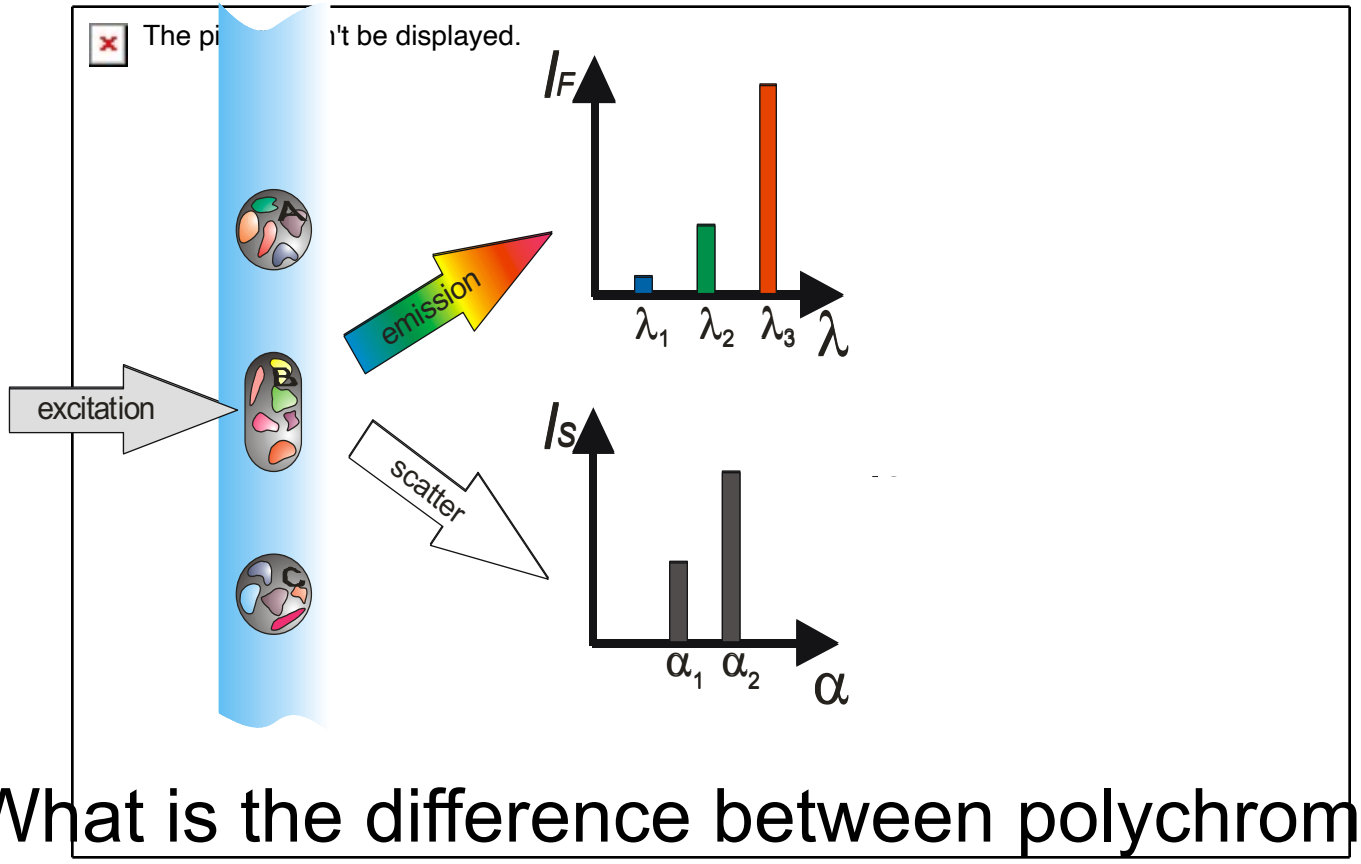


Variables 3 & 4



PUCL
cytometry laboratories

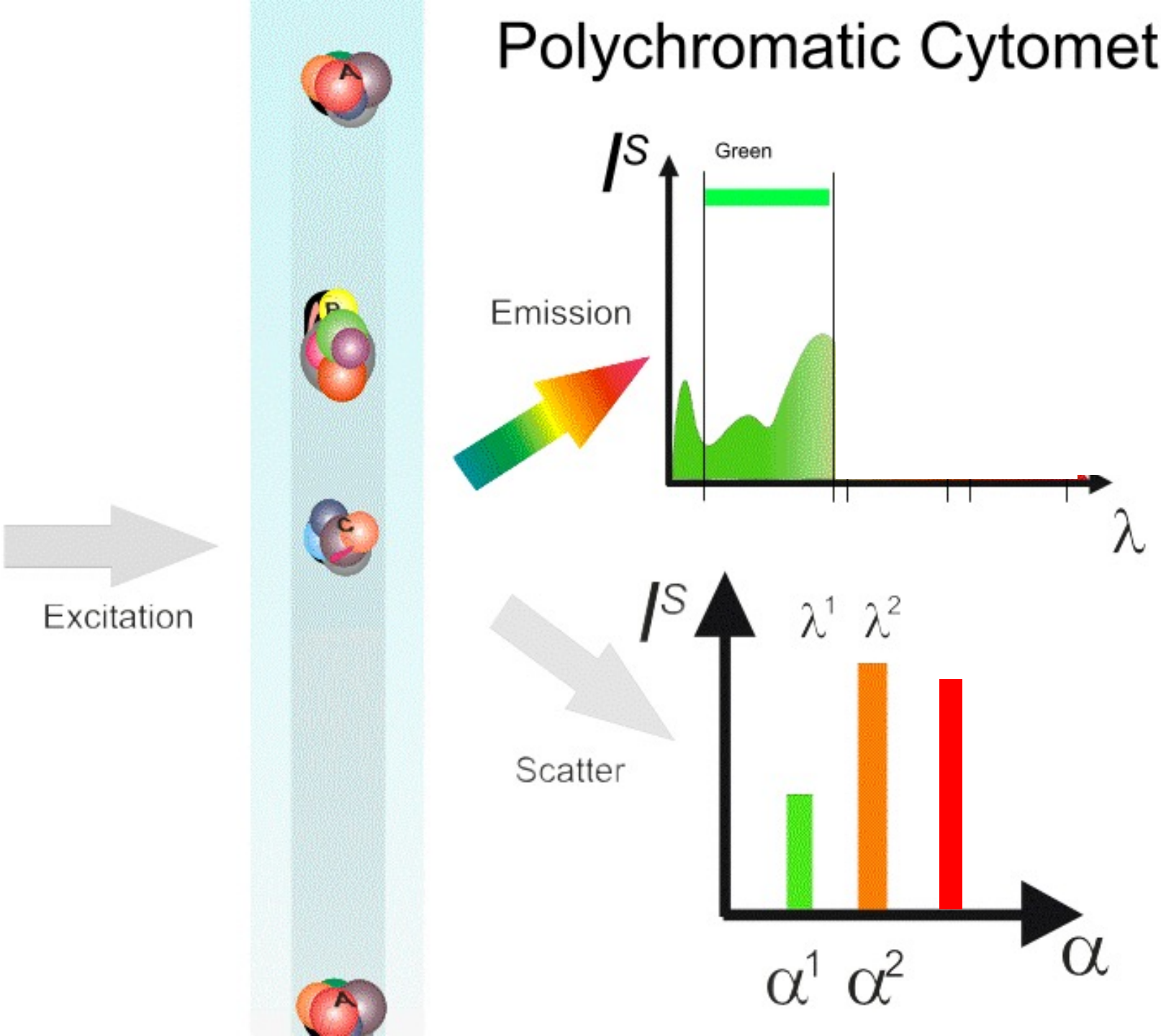
Multicolor (polychromatic) vs. multispectral cytometry (I)



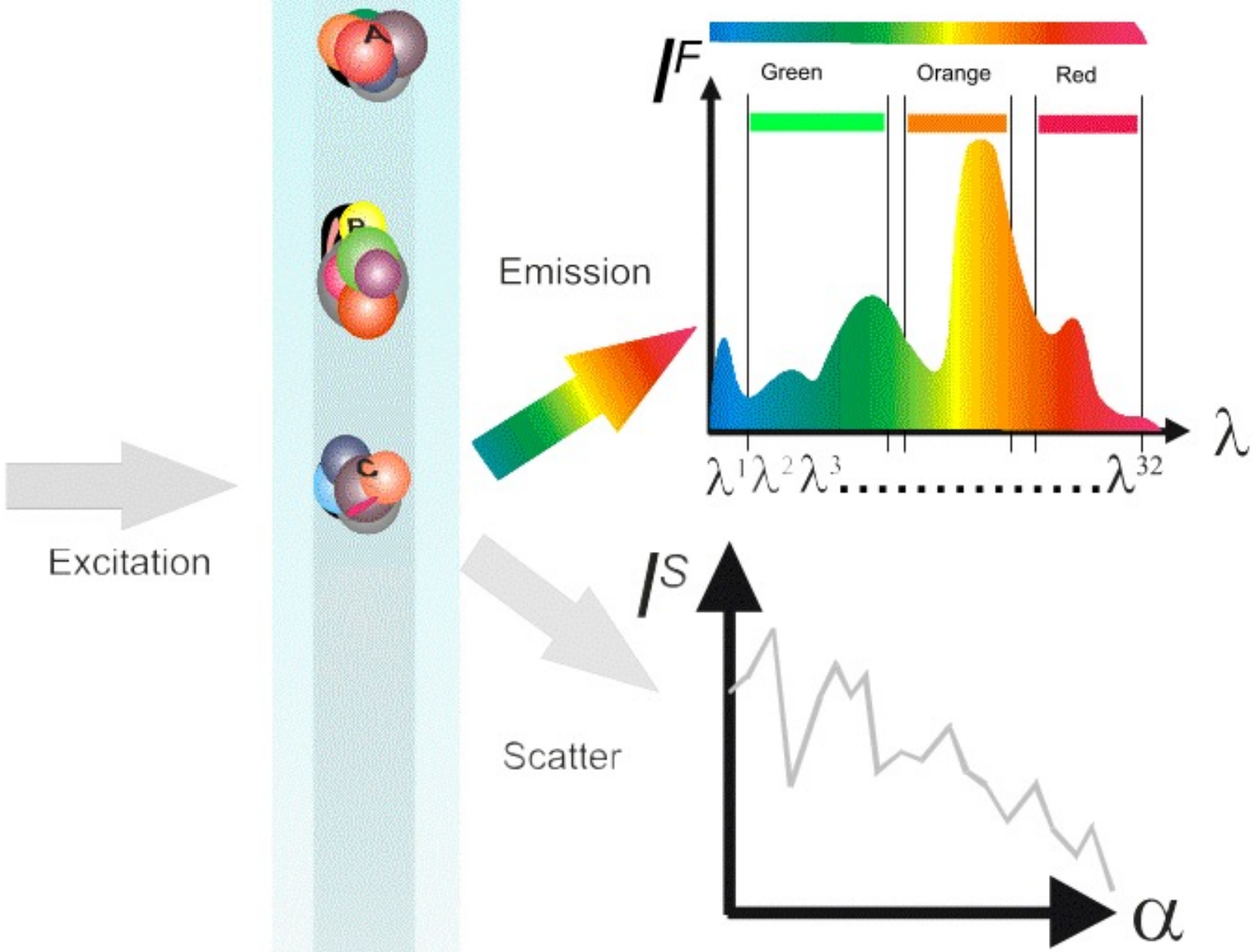
- What is the difference between polychromatic and multispectral cytometry.
- Is it the number of colors?

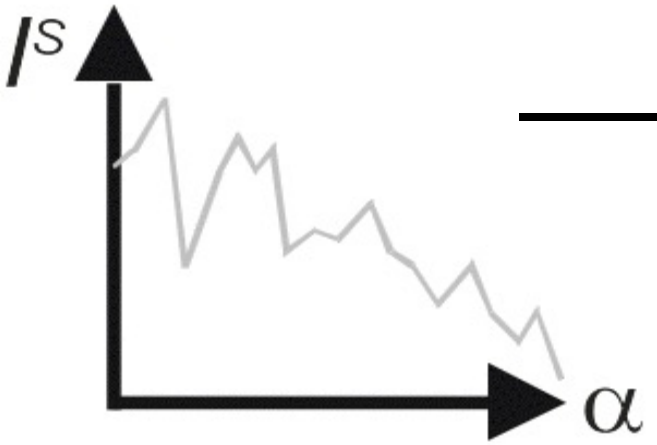
PUCL
cytometry laboratories

Polychromatic Cytometry

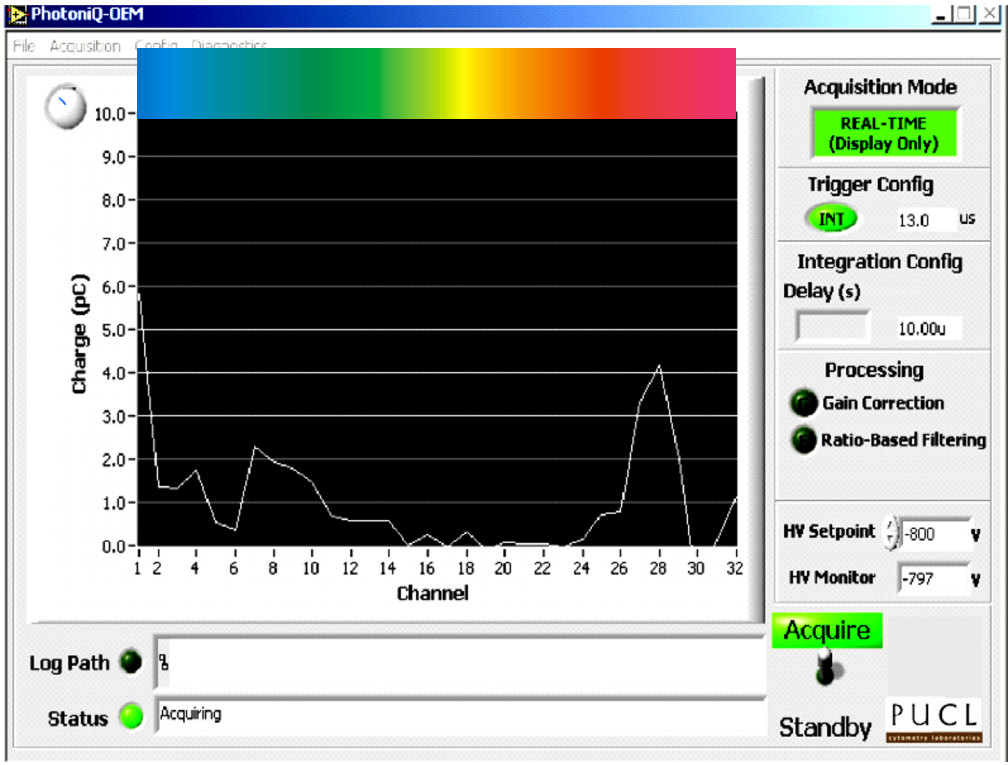


Multispectral Cytometry





One full spectra per particle



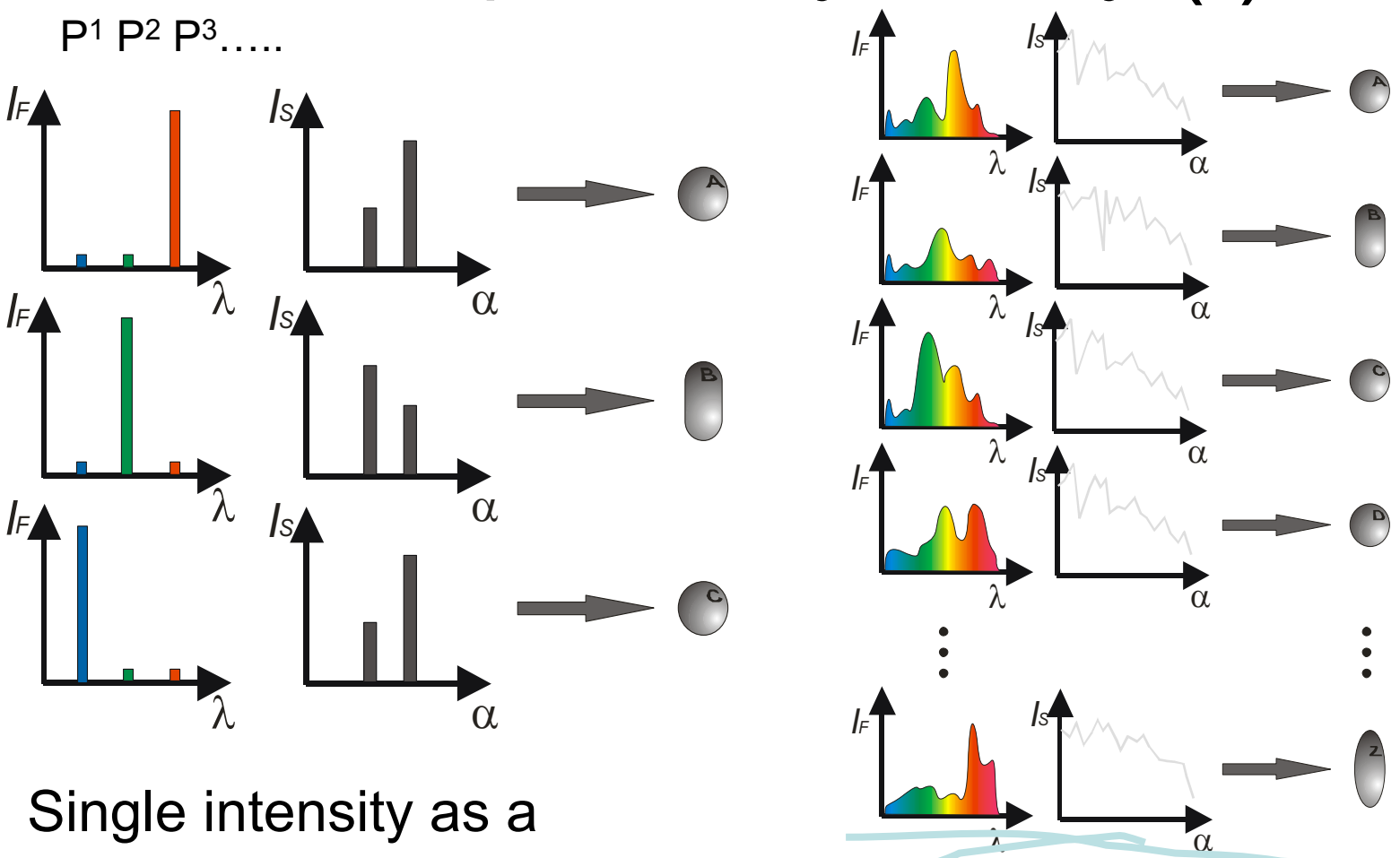
Spectral Signature

Match Signature

Resolution of Data Point

PUCL
cytometry laboratories

Multicolor (polychromatic) vs. multispectral cytometry (I)



Single intensity as a parameter (usually)

Spectrum as a parameter

PUCL
cytometry laboratories

History of Multispectral Imaging

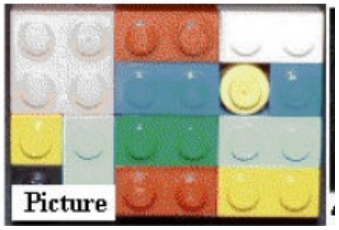
A Purdue solution – Prof David A. Landgrebe

The Laboratory for Applications of Remote Sensing (LARS)

- Launching of the first weather satellite, TIROS-1, on April 1, 1960
- In 1964, the U.S. Department of Agriculture's Economic Research Service agreed to fund a small grant at Purdue to have data from the optical portion of the electromagnetic spectrum collected over Purdue agricultural sites by the Univ. Michigan equipment and analyzed at Purdue.
- LARS was formed in 1966 to take an interdisciplinary team approach to the creation and use of space-based technology for observing and managing of agricultural resources world wide.
  - Goal to analyze land masses for agricultural uses from spacecraft altitudes
  - Major problem was spatial resolution too costly at the time
- It was thus decided to rely primarily upon the *spectral* response of the subject matter, thus creating a new type of spectroscopy.
- It became the basis for the LANDSAT series of Earth satellites and is the basis for a new generation of instruments to be carried on Space Station platforms in the 1990's.

Material Summarized from http://www.lars.purdue.edu/home/LARSHistory.html

Color composition is a mixture of spectral bands

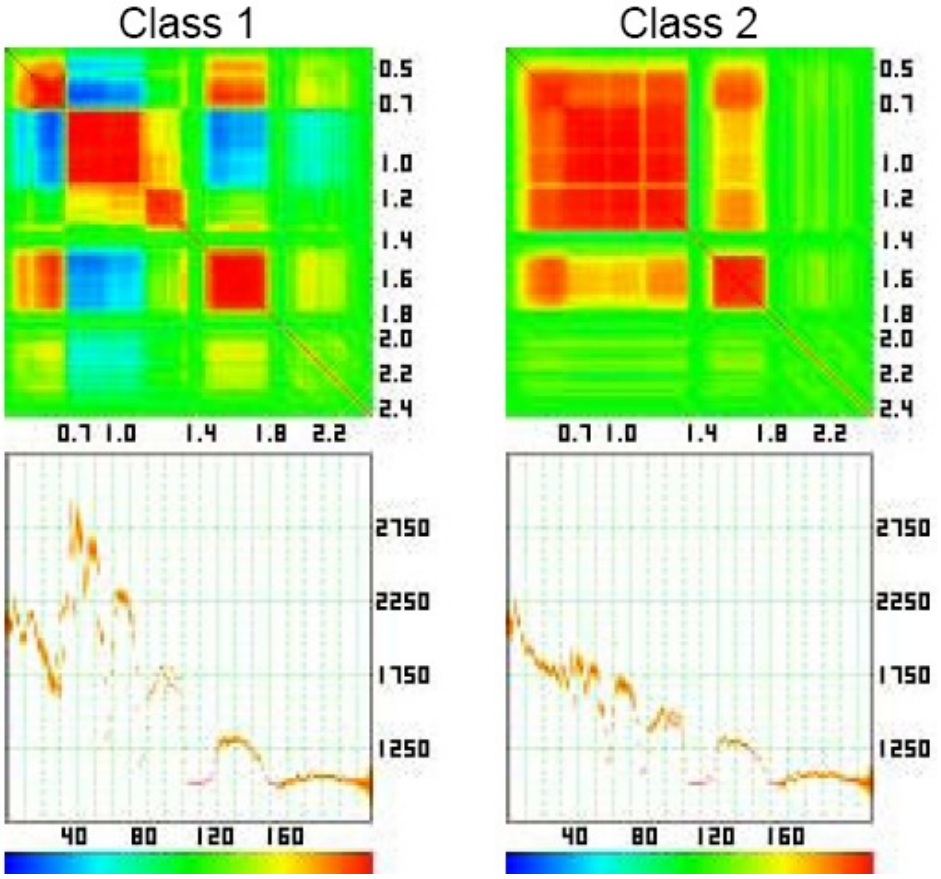


Proc. SPIE Vol. 4056, p. 50-64, Wavelet Applications VII, Harold H. Szu, Martin Vetterli; William J. Campbell, James R. Buss, Ed.

PUCL
cytometry laboratories

Images of soybean plants collected with Landsat system

Long Island, NY

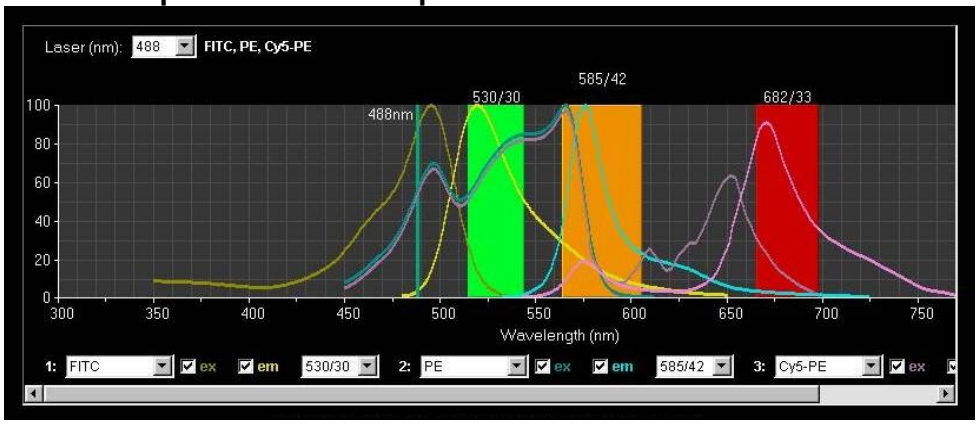


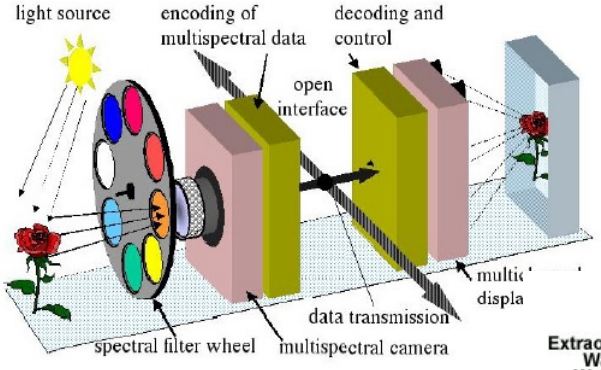
Images from LARS Laboratory at PURDUE

PUCL
cytometry laboratories

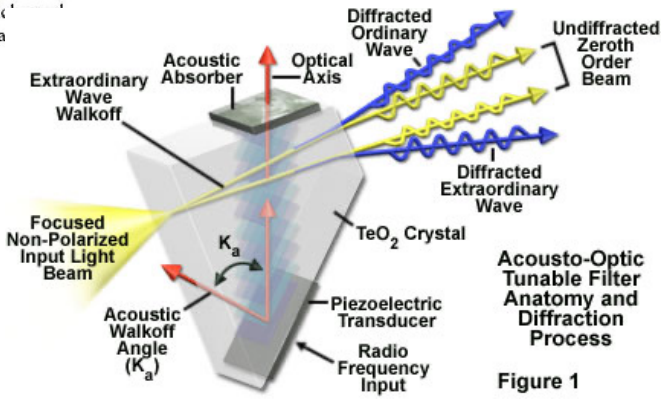
Multispectral detection tools

Multiple sets of optical filters

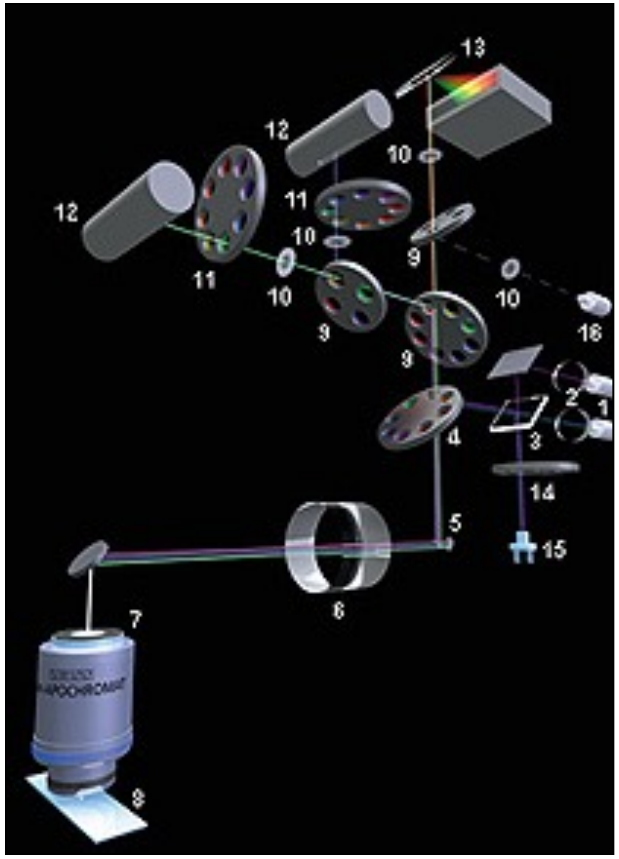




AOTF



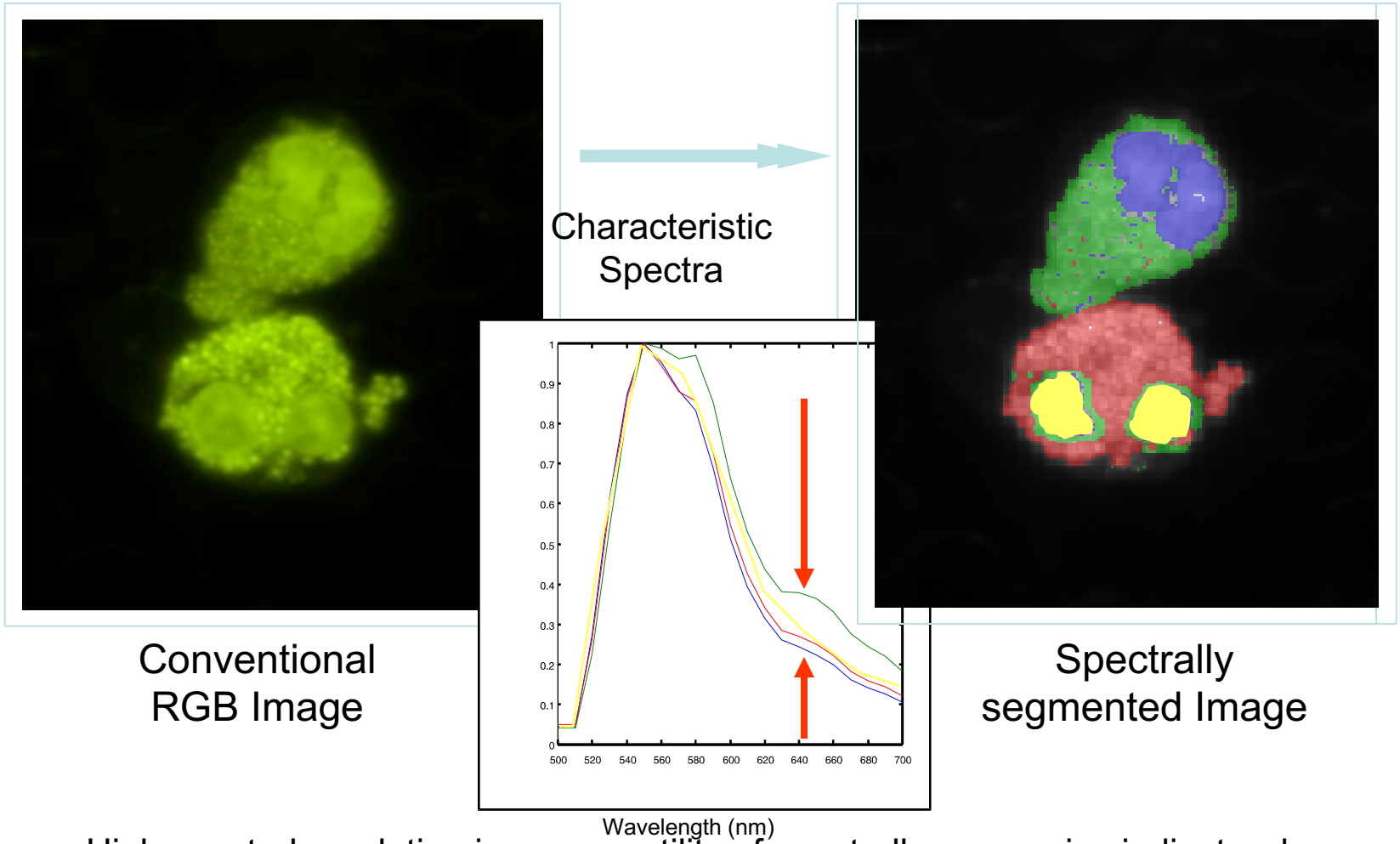
Gratings and multianode PMTs



Multianode PMT with variety of gratings

PUCL cytometry laboratories

High-resolution cytology segmentation



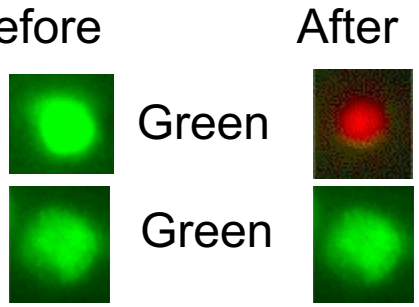
High spectral resolution increases utility of spectrally responsive indicator dyes

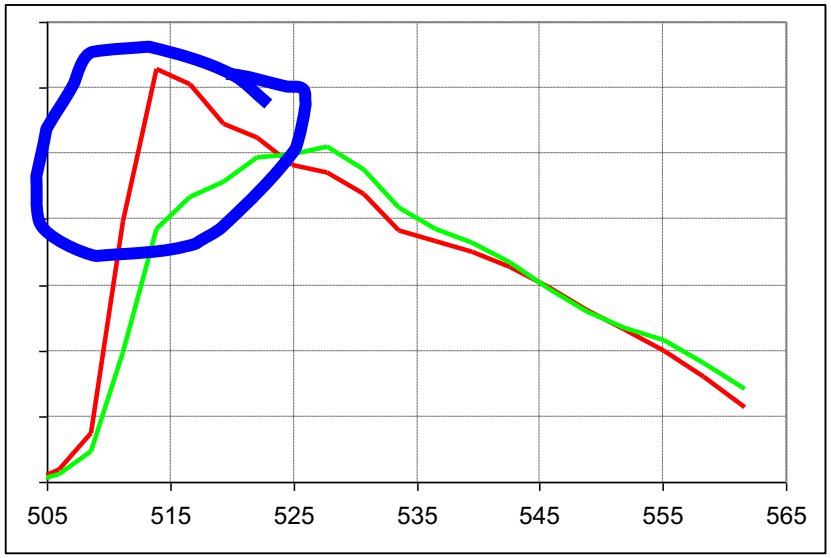
Slide from Dr. Richard Levenson, CRi, Inc.,35B Cabot Rd.,Woburn, MA 01801, www.cri-inc.com

PUCL cytometry laboratories

Spectral “unmixing”

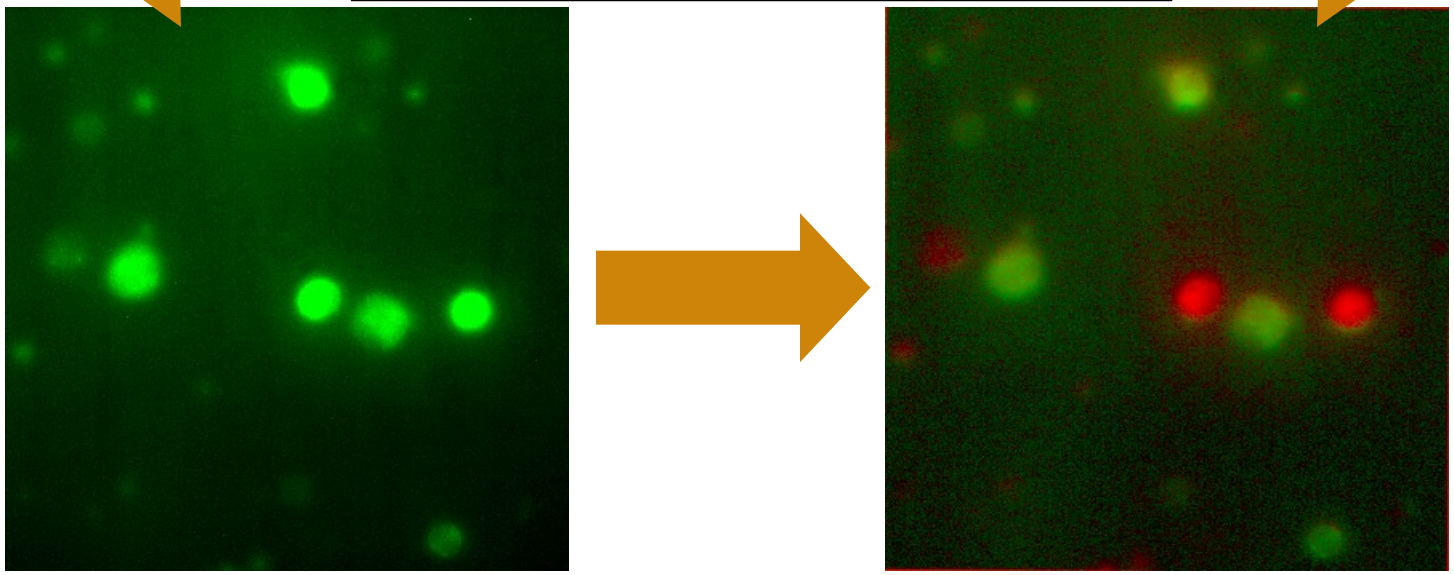
Dye 1: bis-(1,3-dibutylbarbituric acid)trimethine oxonol, $DiBAC_4(3)$
Dye 2: 3,3'-dihexyloxacarbocyanine iodide, $DiOC_6(3)$





FITC filter block

Unmixed
$DiOC_6(3)$
And bis-oxonol



PUCL
cytometry laboratories

Current cell analysis technologies

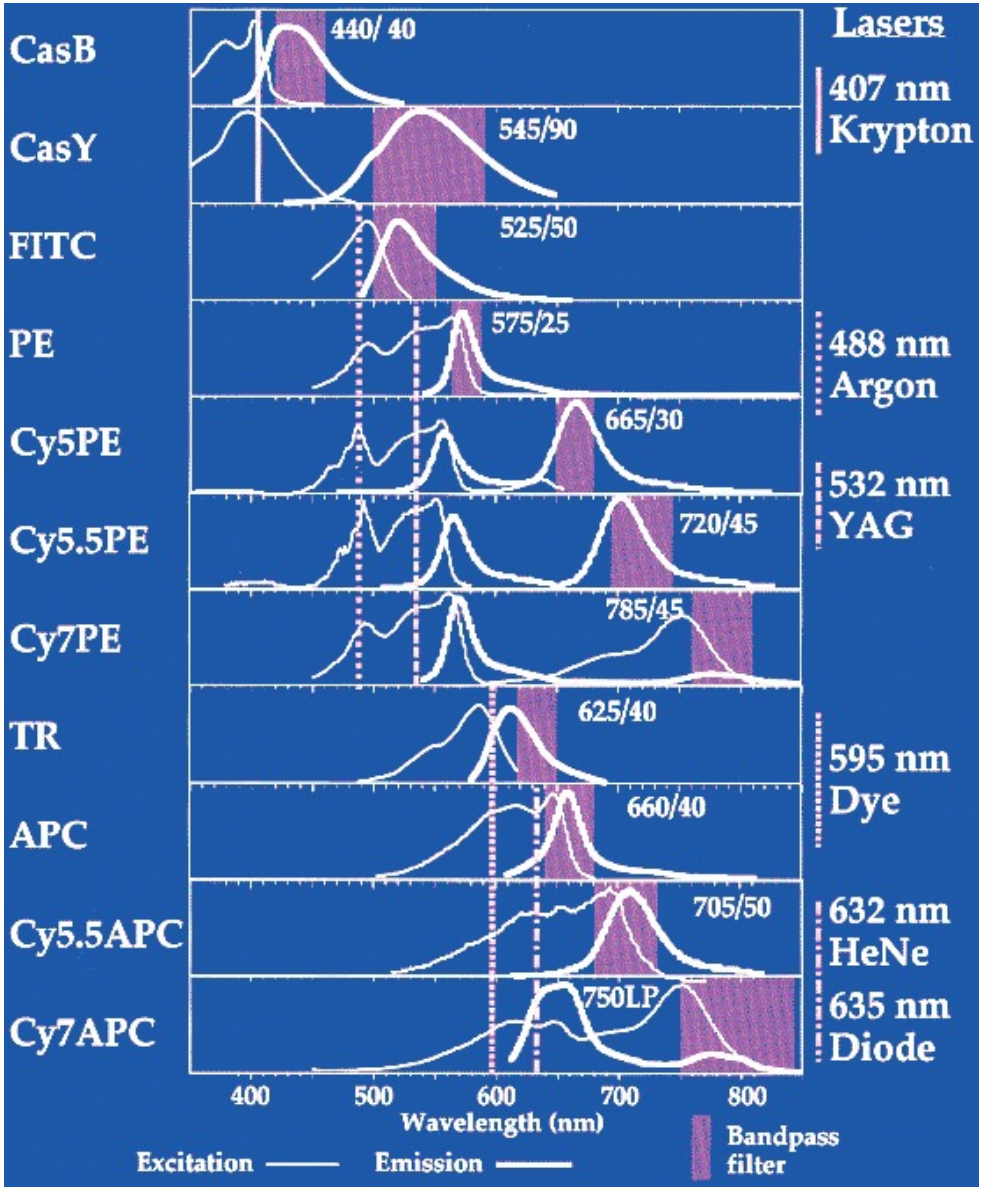
Traditional methodology of cytometry is univariate or bivariate - the intensity of fluorescence at one or two, three, etc., given excitation/emission wavelength pairs are used to identify a phenotype.

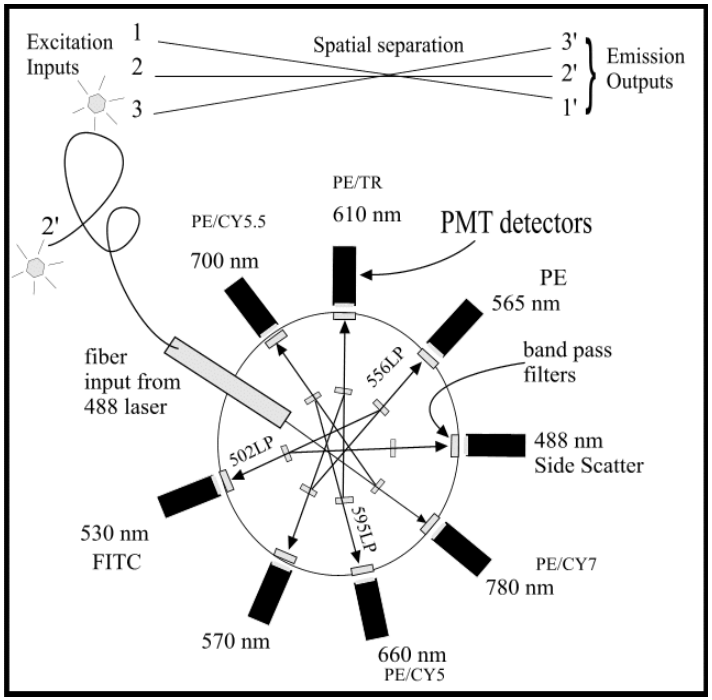
This type of analysis focuses on the variance of a single wavelength and does not take advantage of the information content of a complete spectrum.

Why multispectral cytometry?

PUCL
cytometry laboratories

Advanced polychromatic cytometry



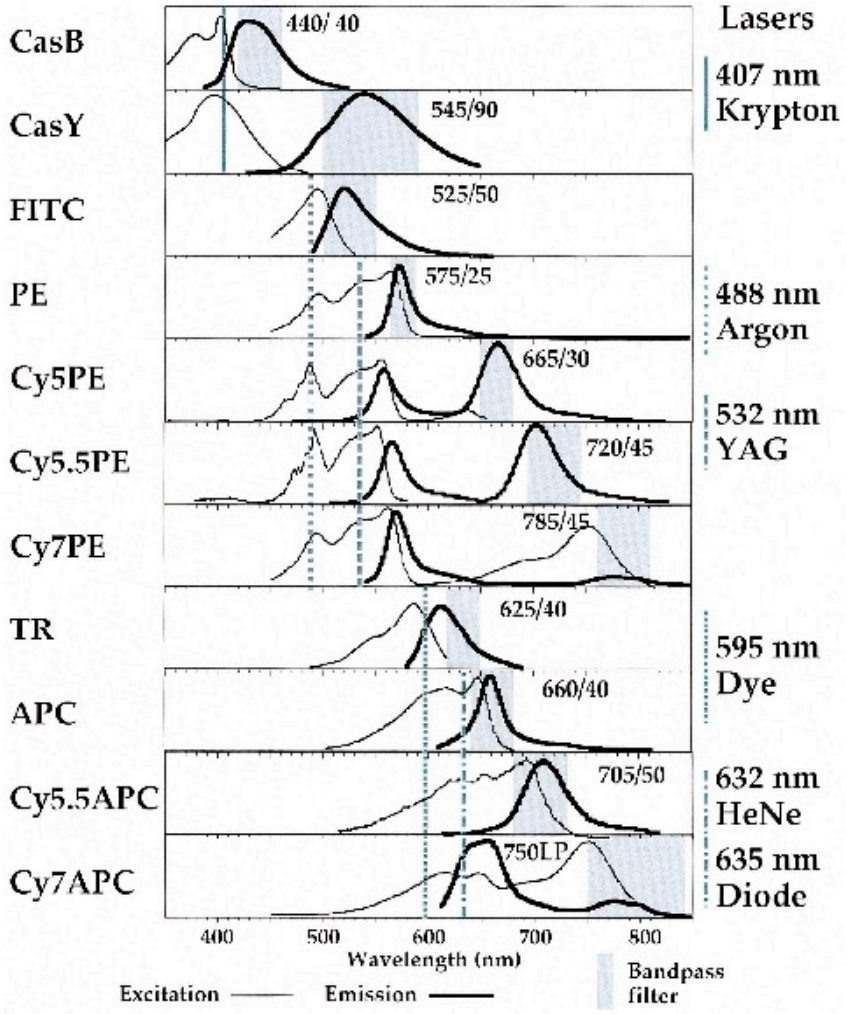


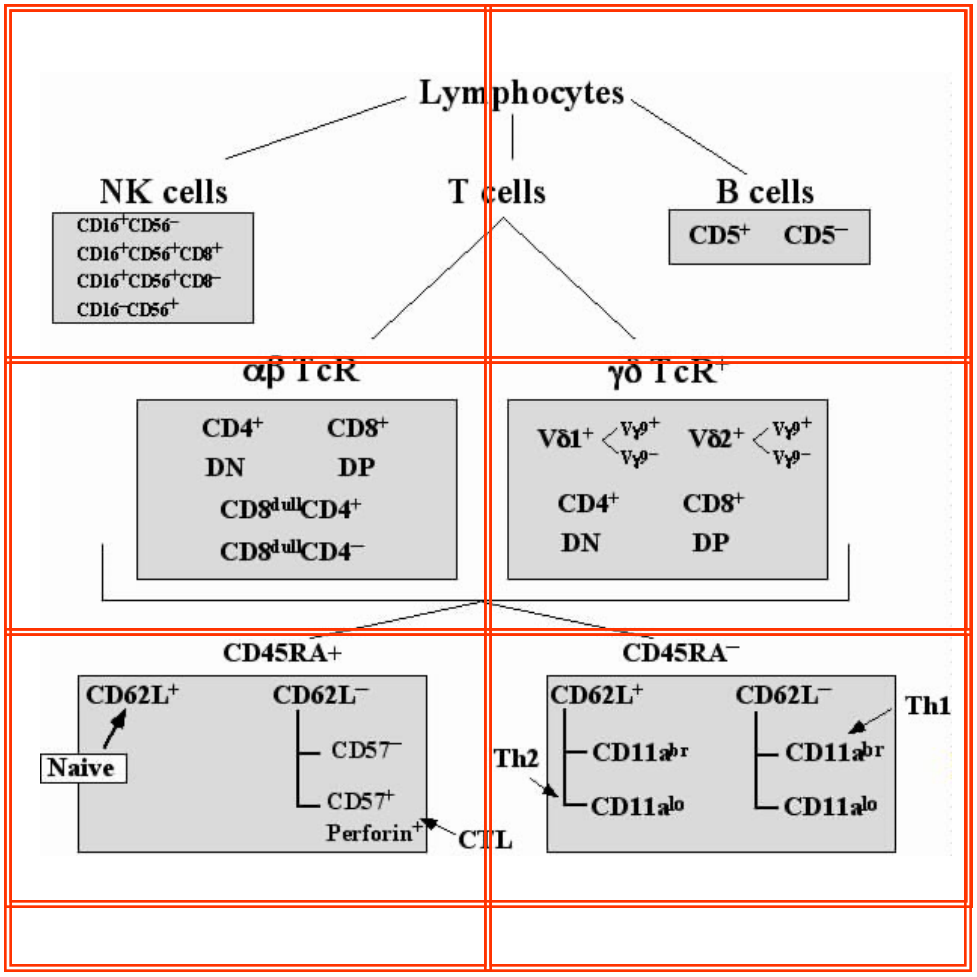
8 PMTs and 16 filters

Figure from Roederer et al

PUCL cytometry laboratories

Cell/system complexity can be reduced using tools such as flow cytometry





Figures from Roederer, et al

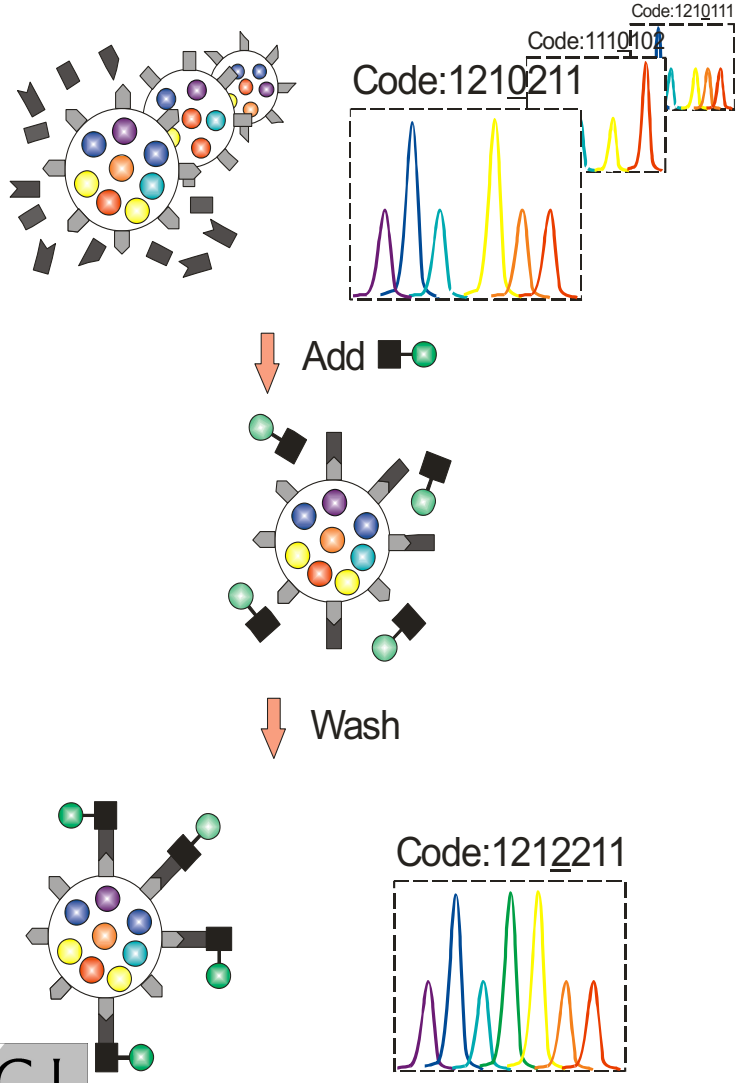
PUCL
cytometry laboratories

Multispectral Cytometry. Why?

- Identification of multiple spectrally overlapping stains (multiplexing)
- Spectral barcoding
- Potential capability of spectral un-mixing (multiple stains in a single particle)
- Identification of intrinsic fluorescence (autofluorescence classification)
- Opportunity for intelligent systems approach to classification

PUCL
cytometry laboratories

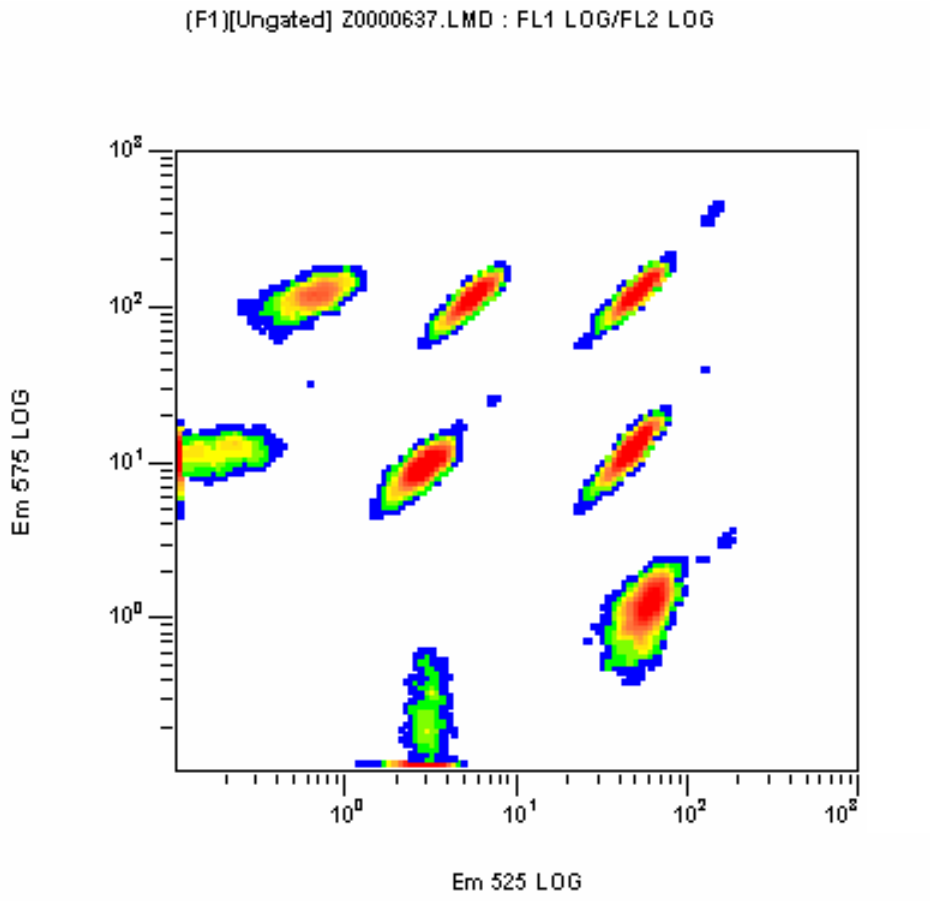
Nanocrystals/Micro-Dots multiplexed systems

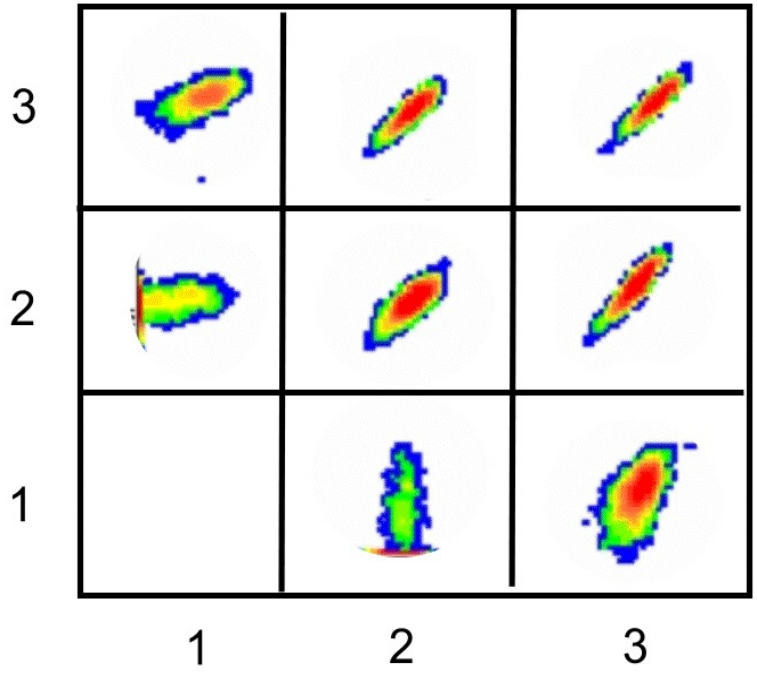


- New probes
- Potentially 1000's of combinations
- Sensitive, long lived, less bleaching
- Difficult to make
- Will require some advanced classification

PUCL
cytometry laboratories

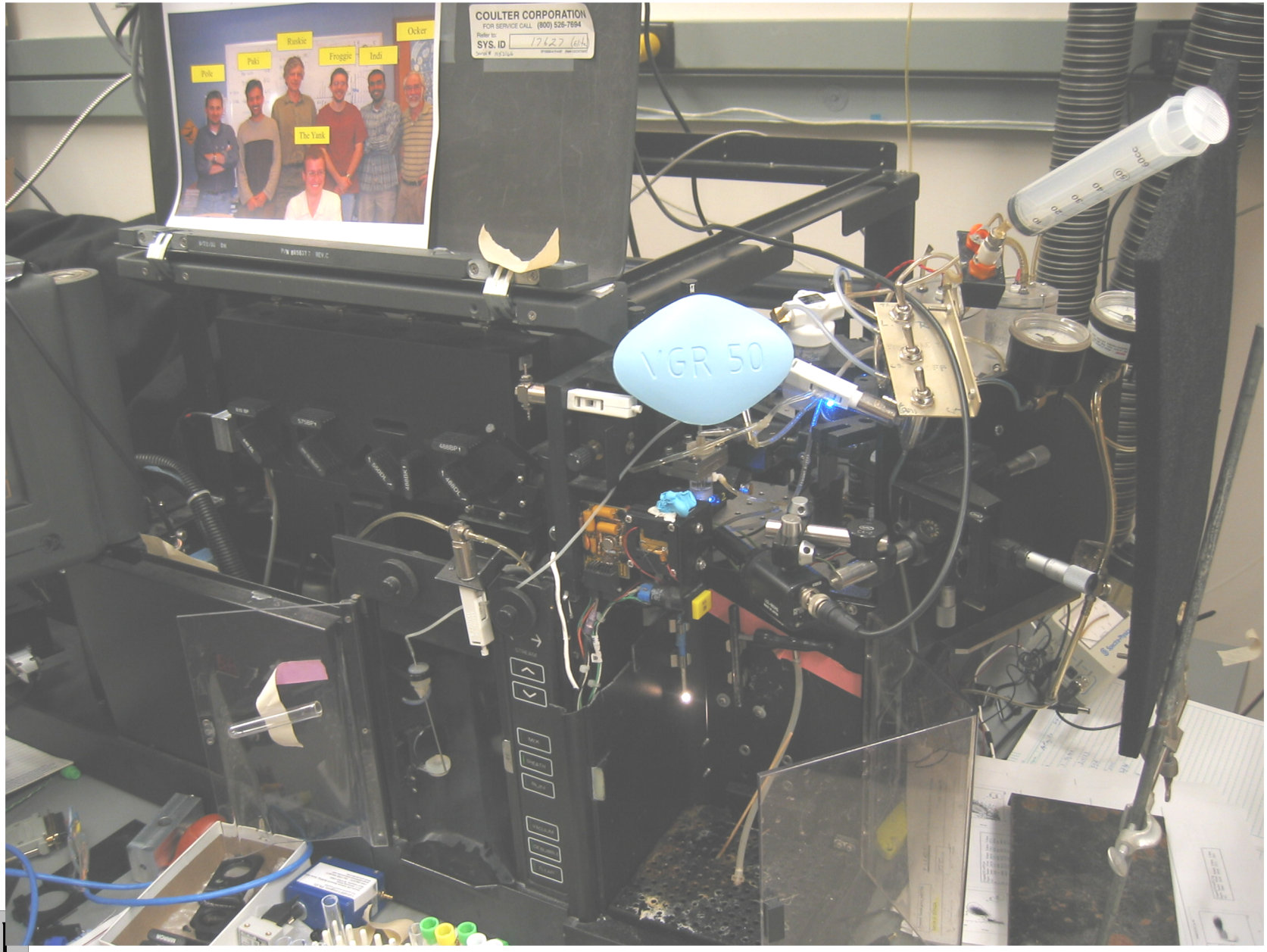
Nano-crystals – multicolor sets





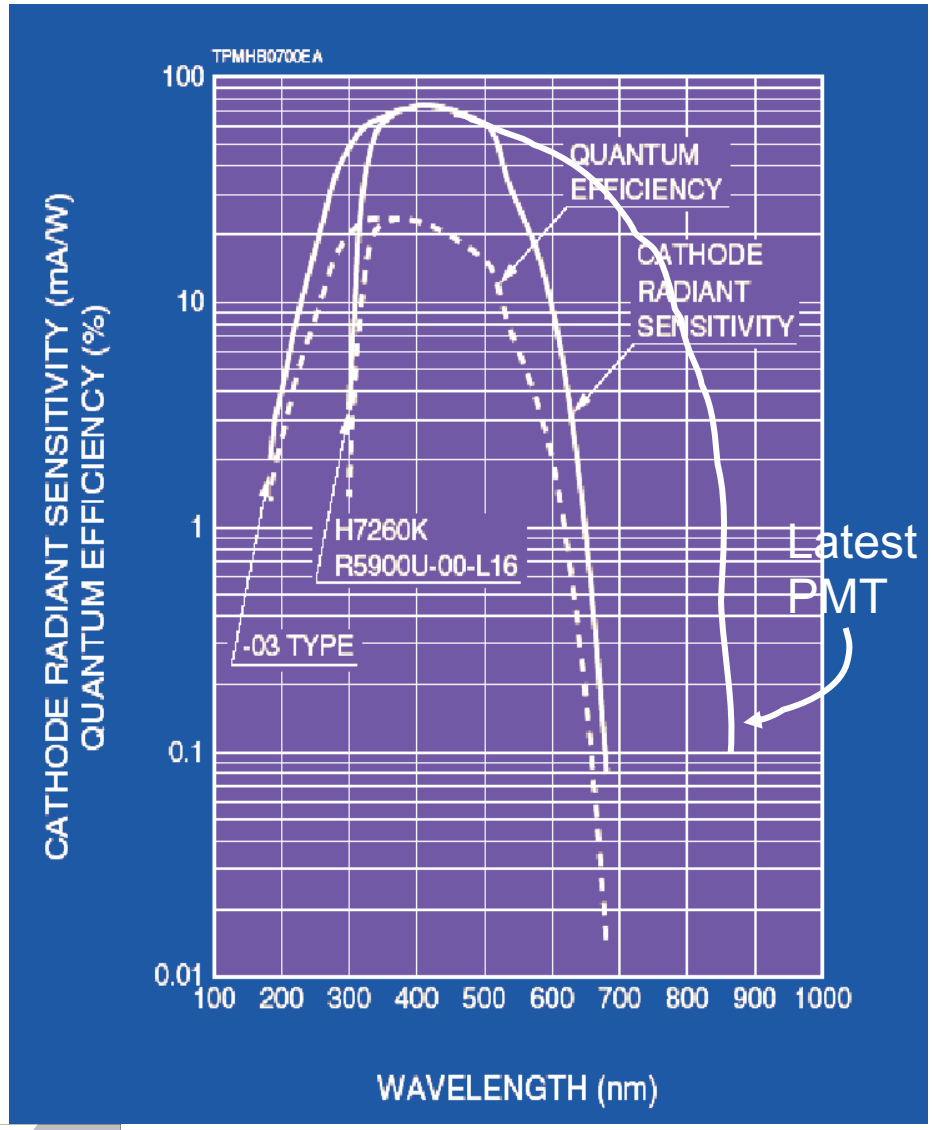
Nano-crystals were generously supplied by Crystalplex

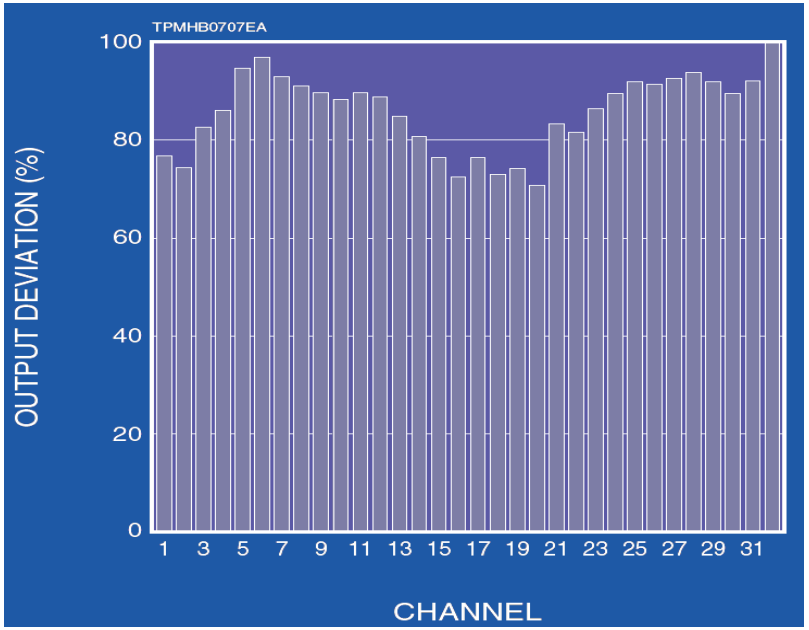
PUCL
cytometry laboratories



PUCL
cytometry laboratories

Multianode PMT – sensitivity and uniformity

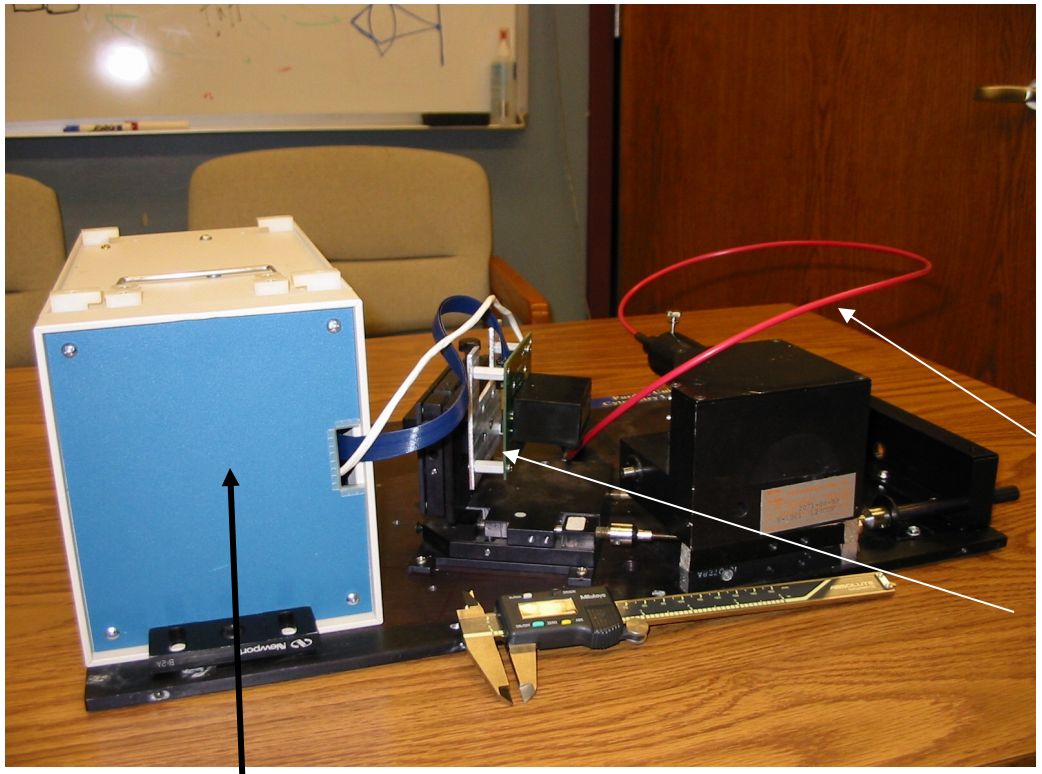




Hamamatsu 32 Ch PMT

PUCL
cytometry laboratories

Particle Spectrometer



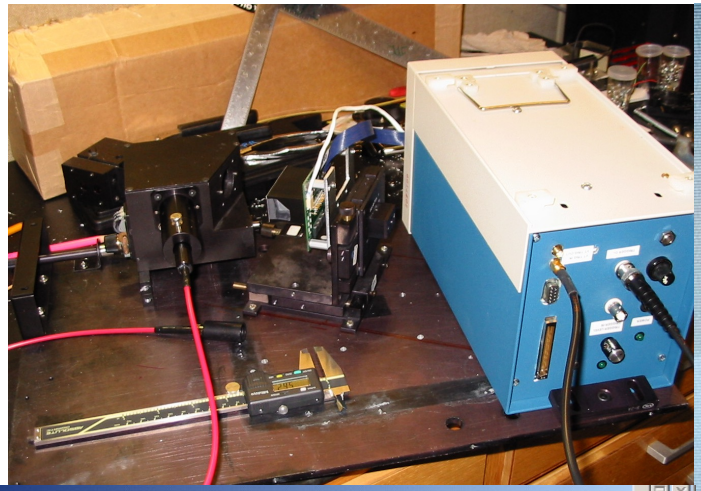
Fiber to photon source

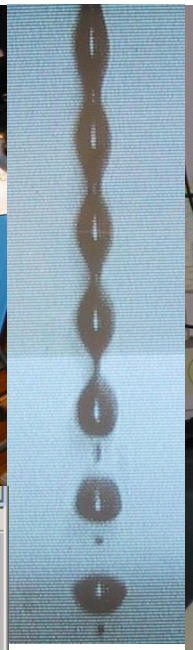
Grating

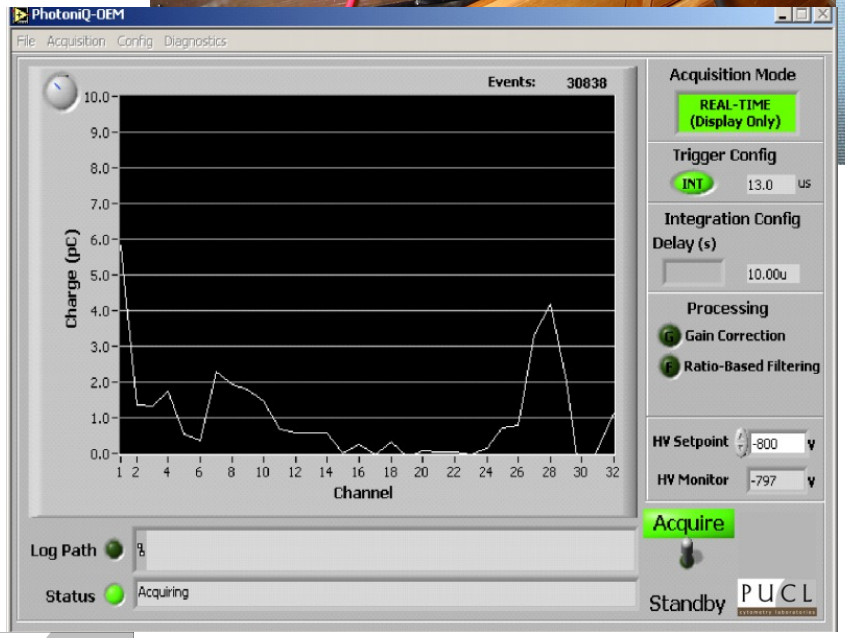
Power supply and high-speed electronics for 32 channel collection (64 channel in new instrument)

PUCL cytometry laboratories

A 32-channel multispectral flow cytometer



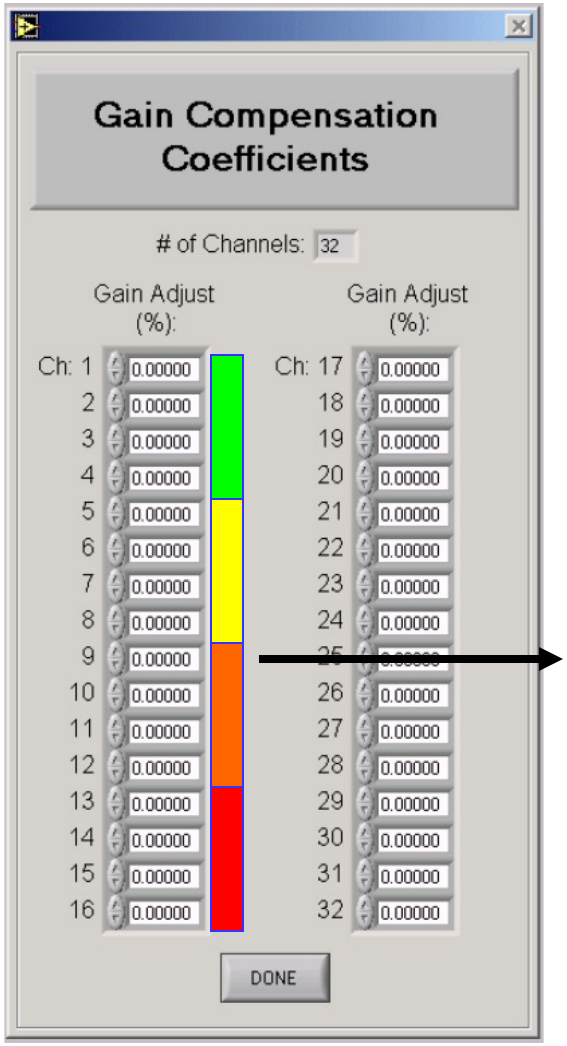




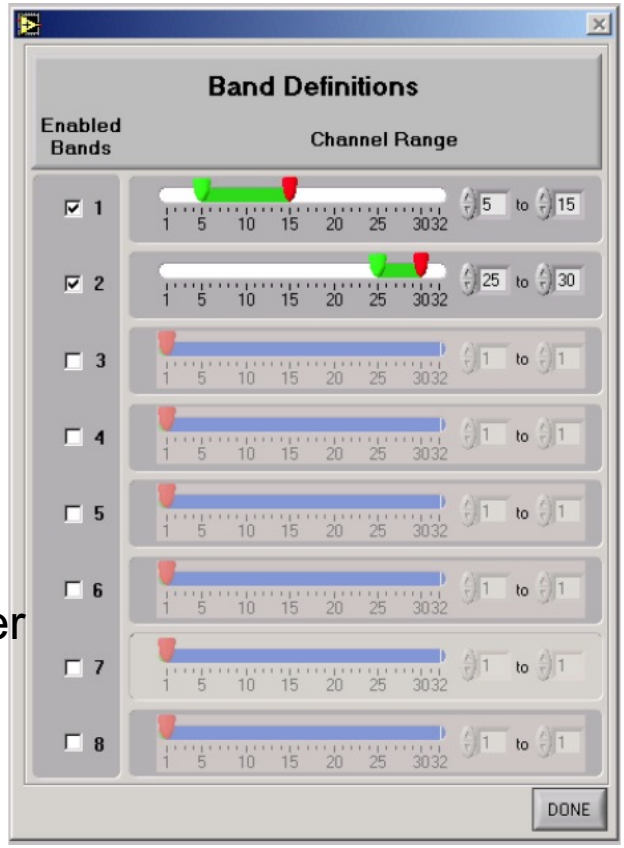
PUCL cytometry laboratories

Initial Spectral data

Multianode PMT – gain and spectral filtering

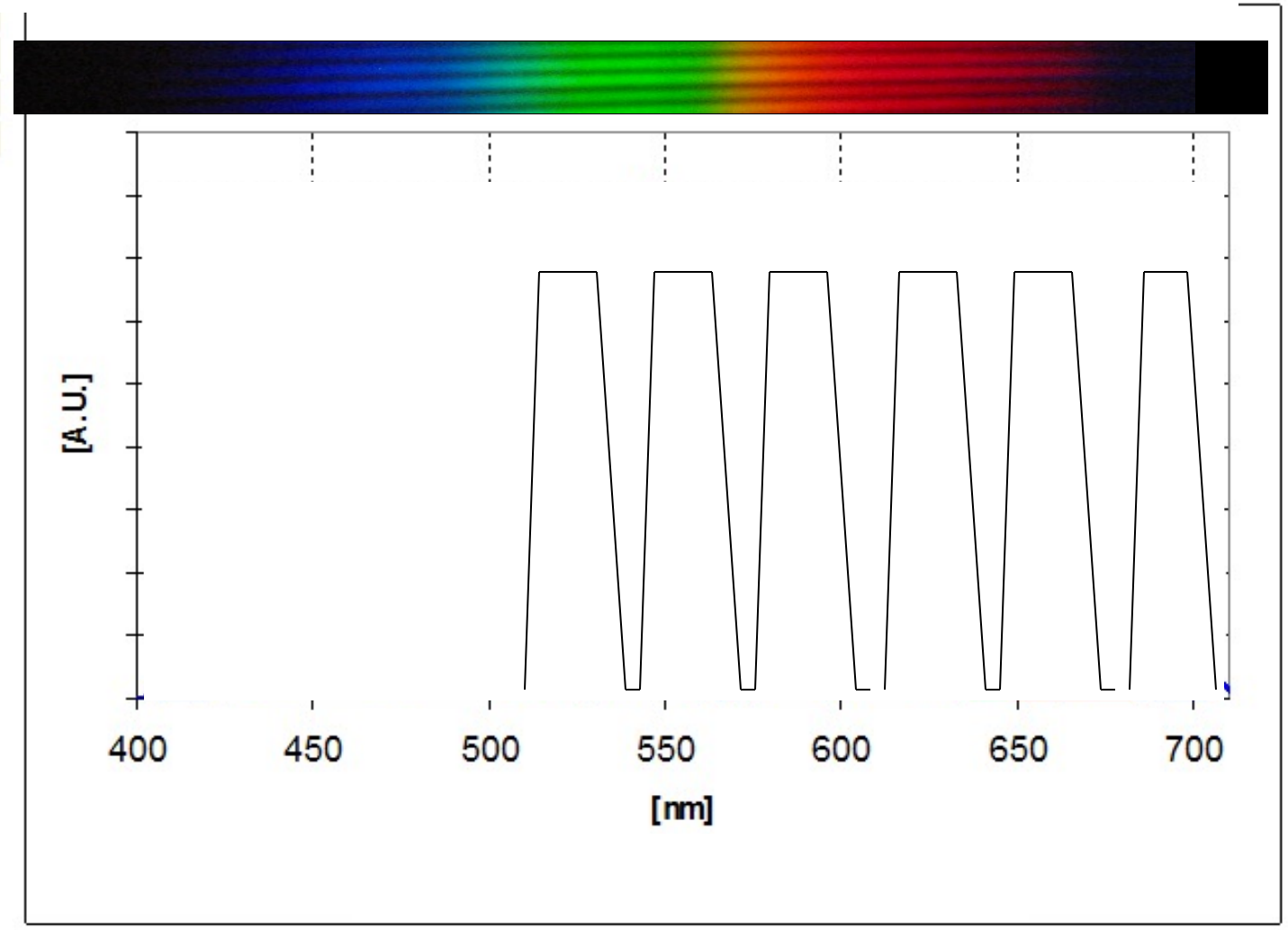


Now a simple 4 color cytometer

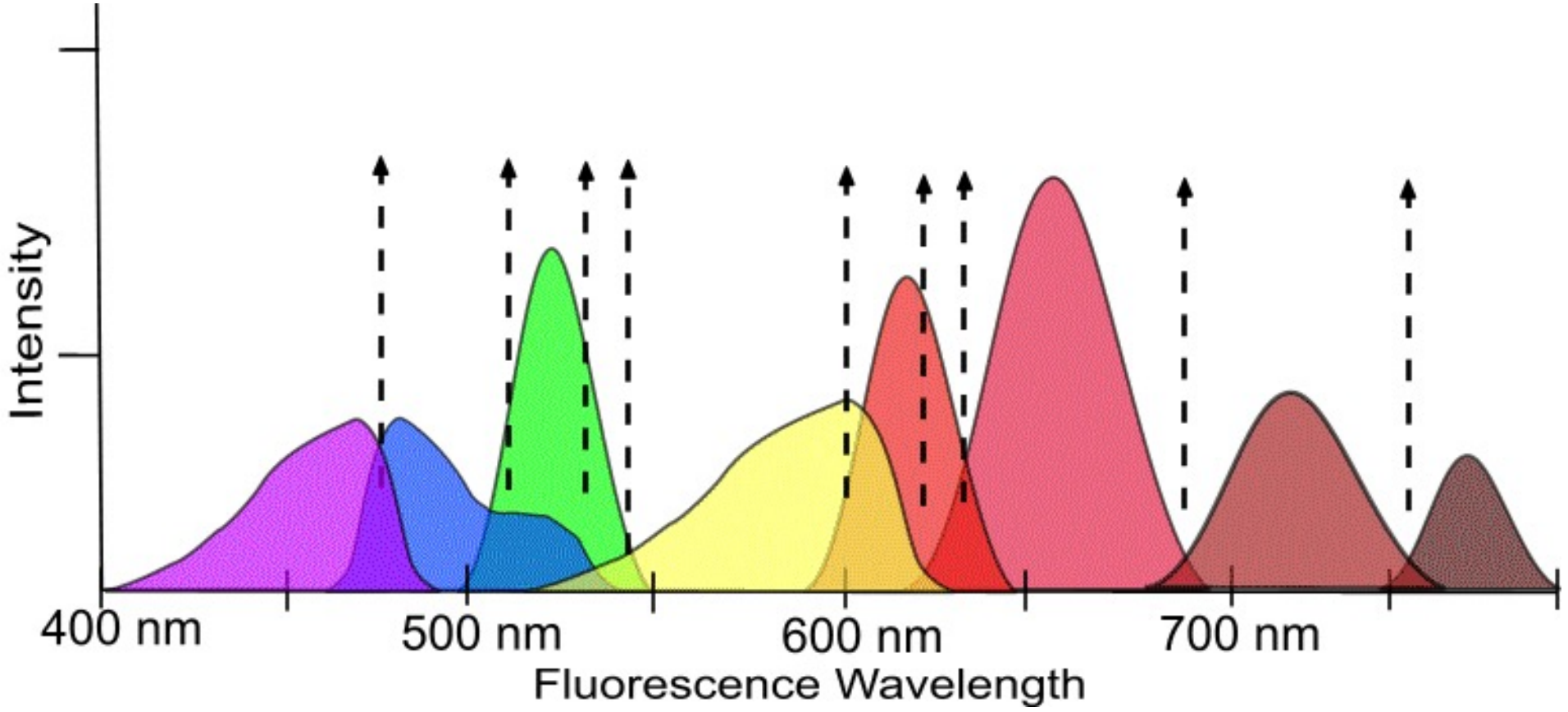


PUCL cytometry laboratories

Basic flow Cytometry Systems

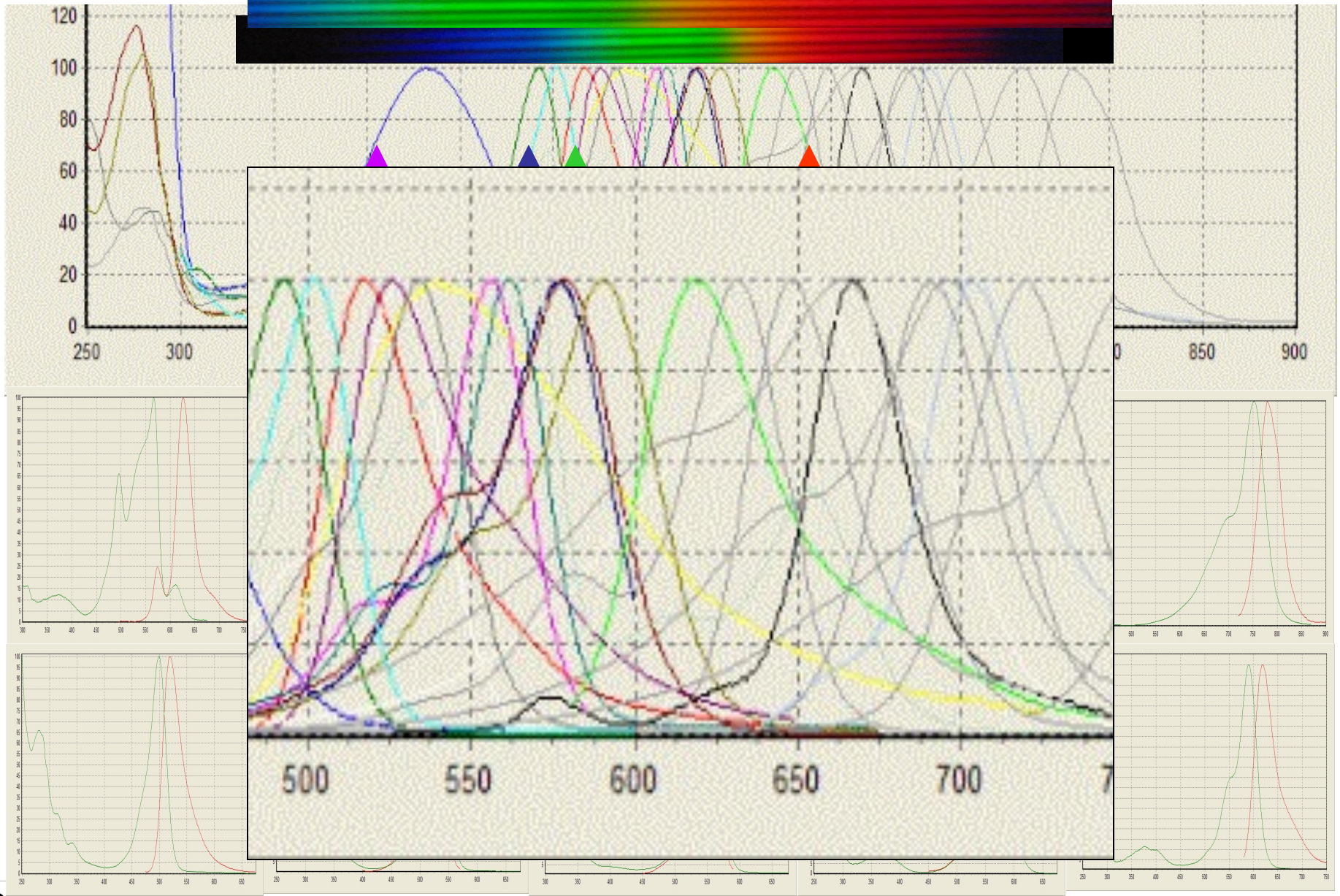


Spectral Overlap makes for very complex analysis



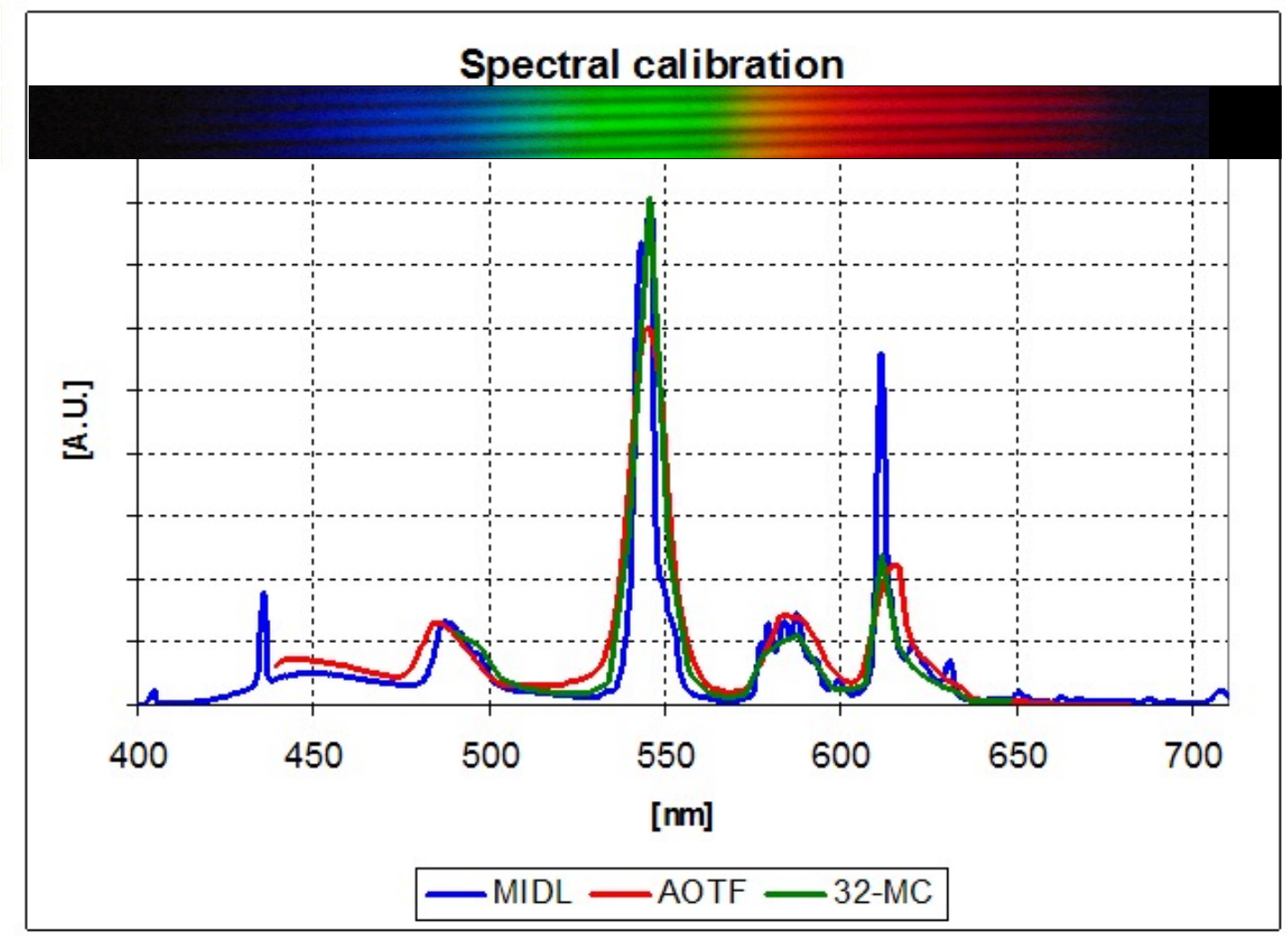
PUCL
cytometry laboratories

Overlapping Spectra



PUCL
cytometry laboratories

Calibration: AOTF vs 32-MC

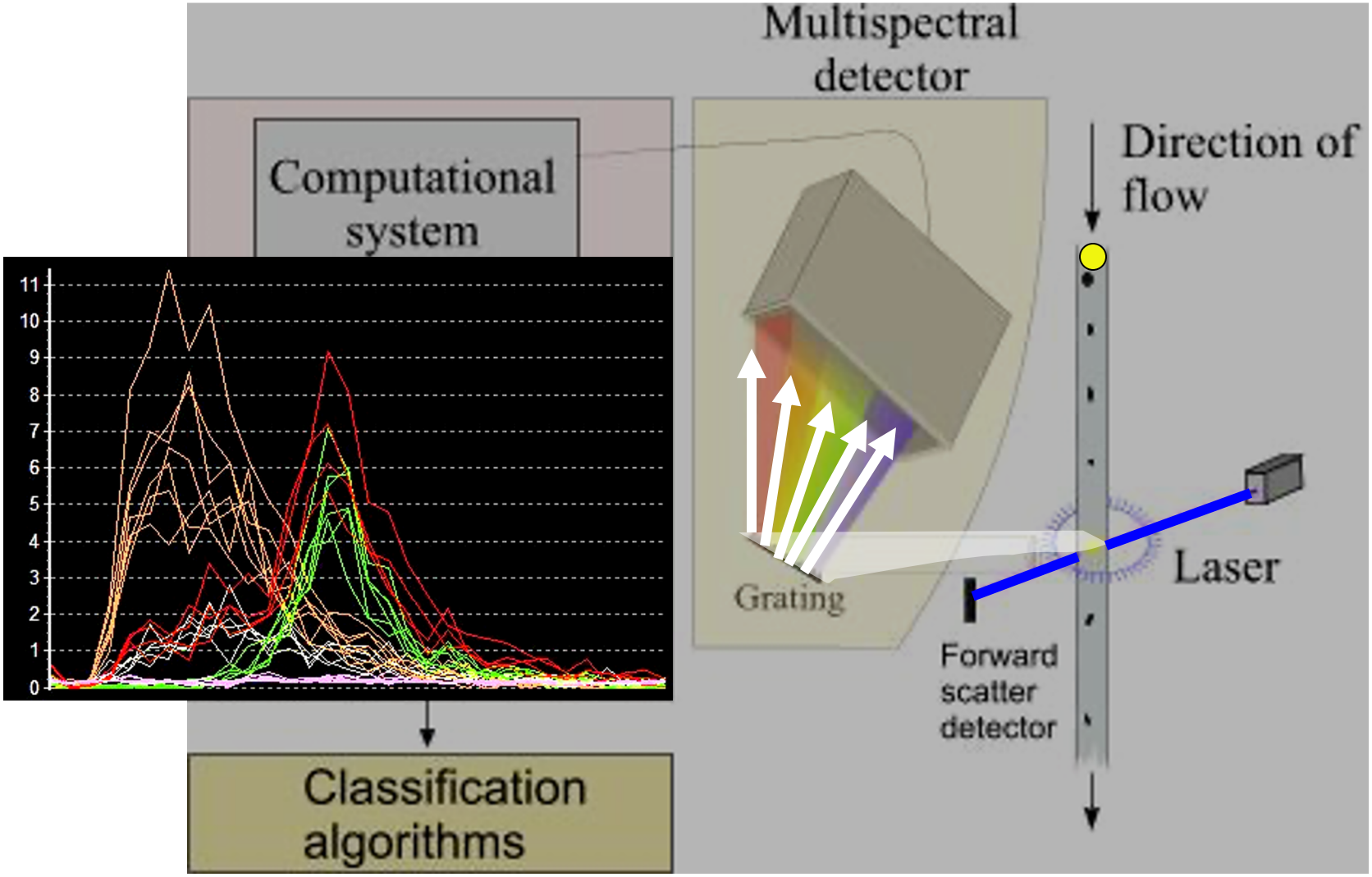


Calibration is accurate and against an easily obtainable calibration lamp

($300 lamp is from Lightform, Inc www.lightform.com)

PUCL
cytometry laboratories

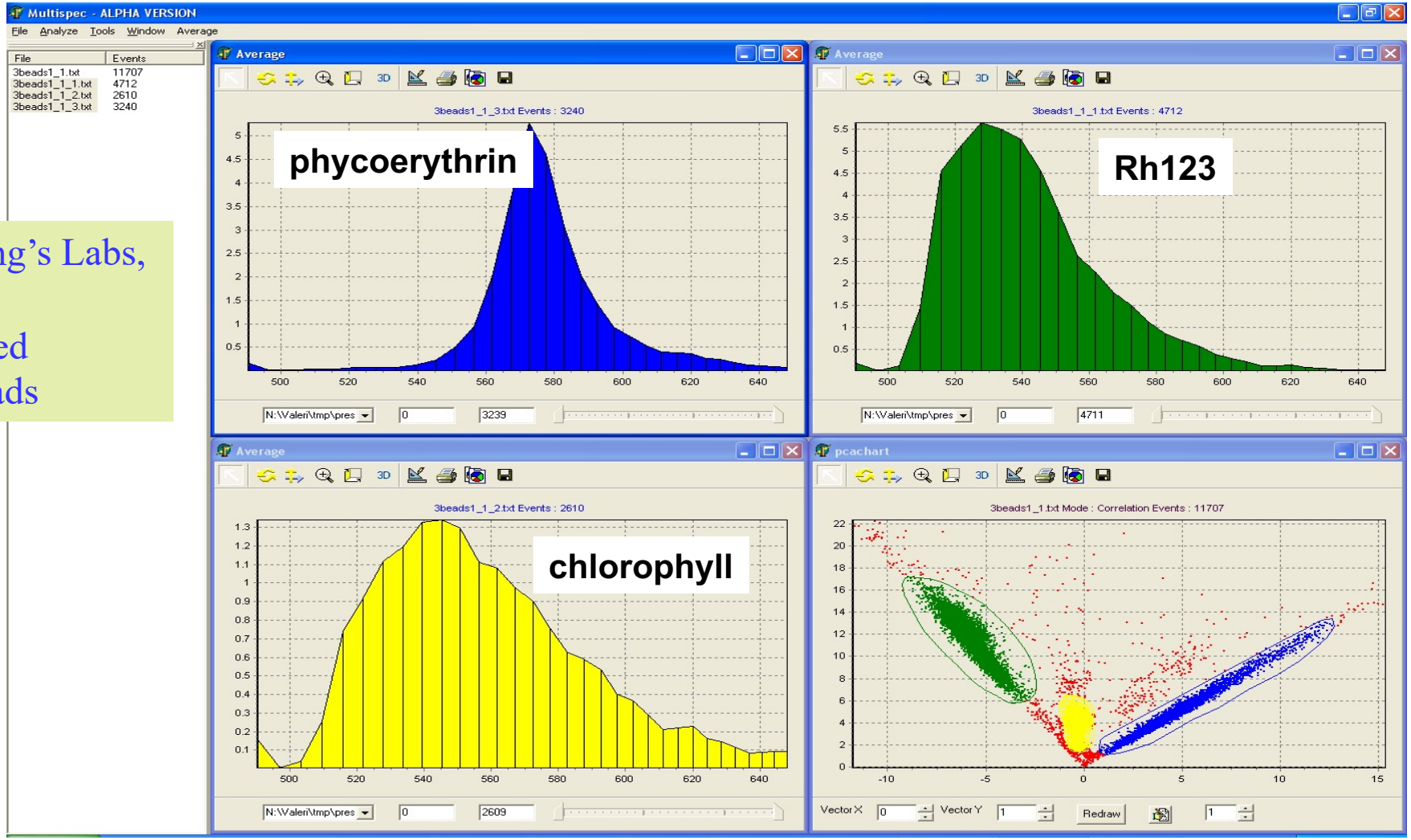
Principle of Operation



PUCL cytometry laboratories

US & foreign patents pending

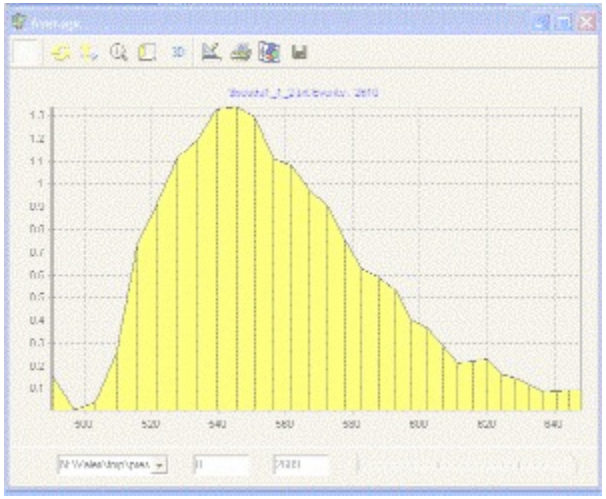
Spectra of gated zones

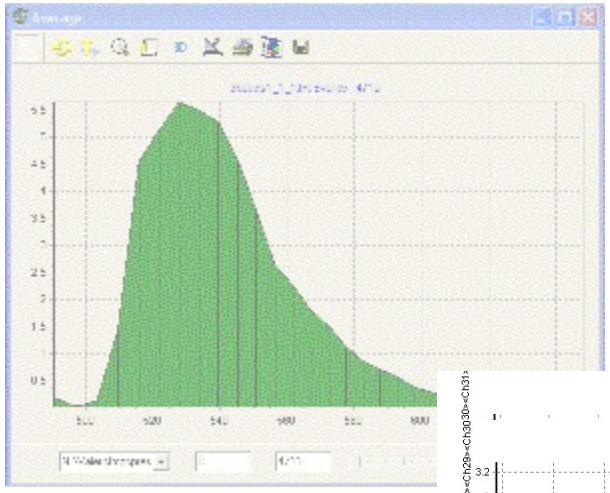


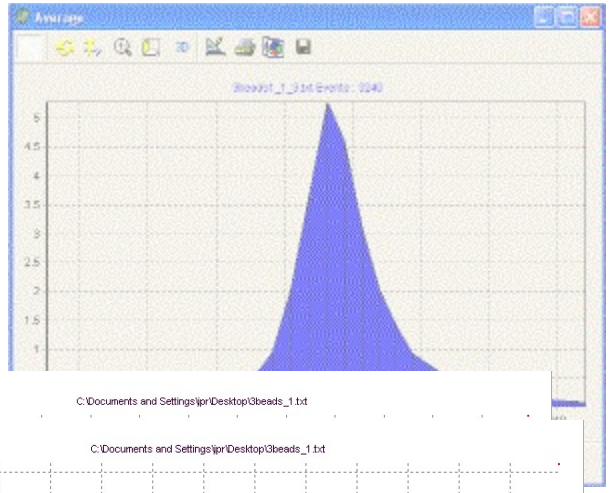
Bang's Labs, Inc
Dyed Beads

PUCL
cytometry laboratories

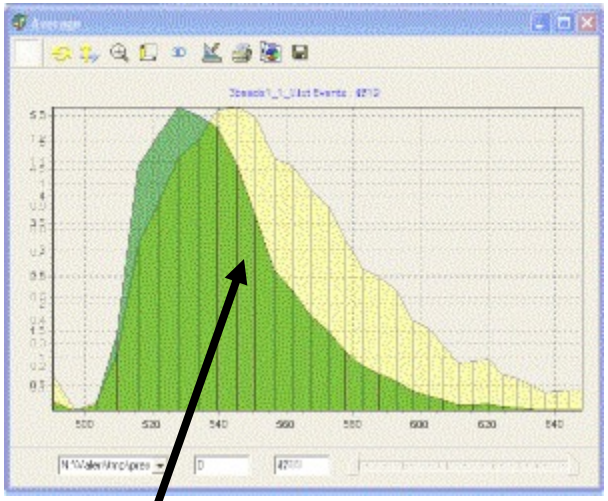
Chlorophyll Rh-123 PE

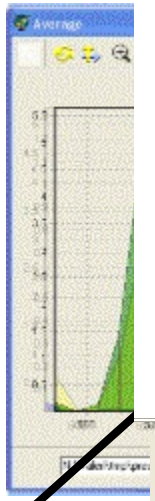


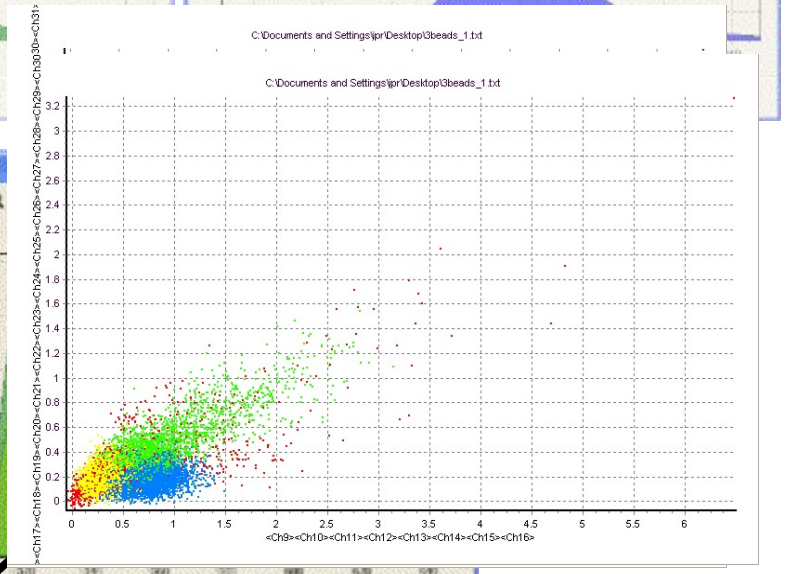


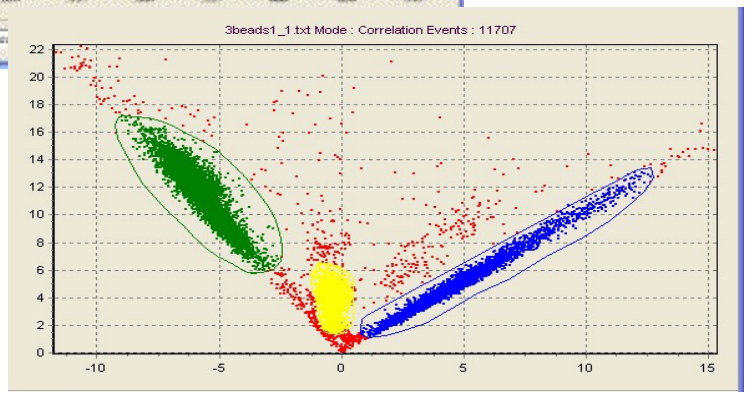


Bang's Labs Dyed Beads*







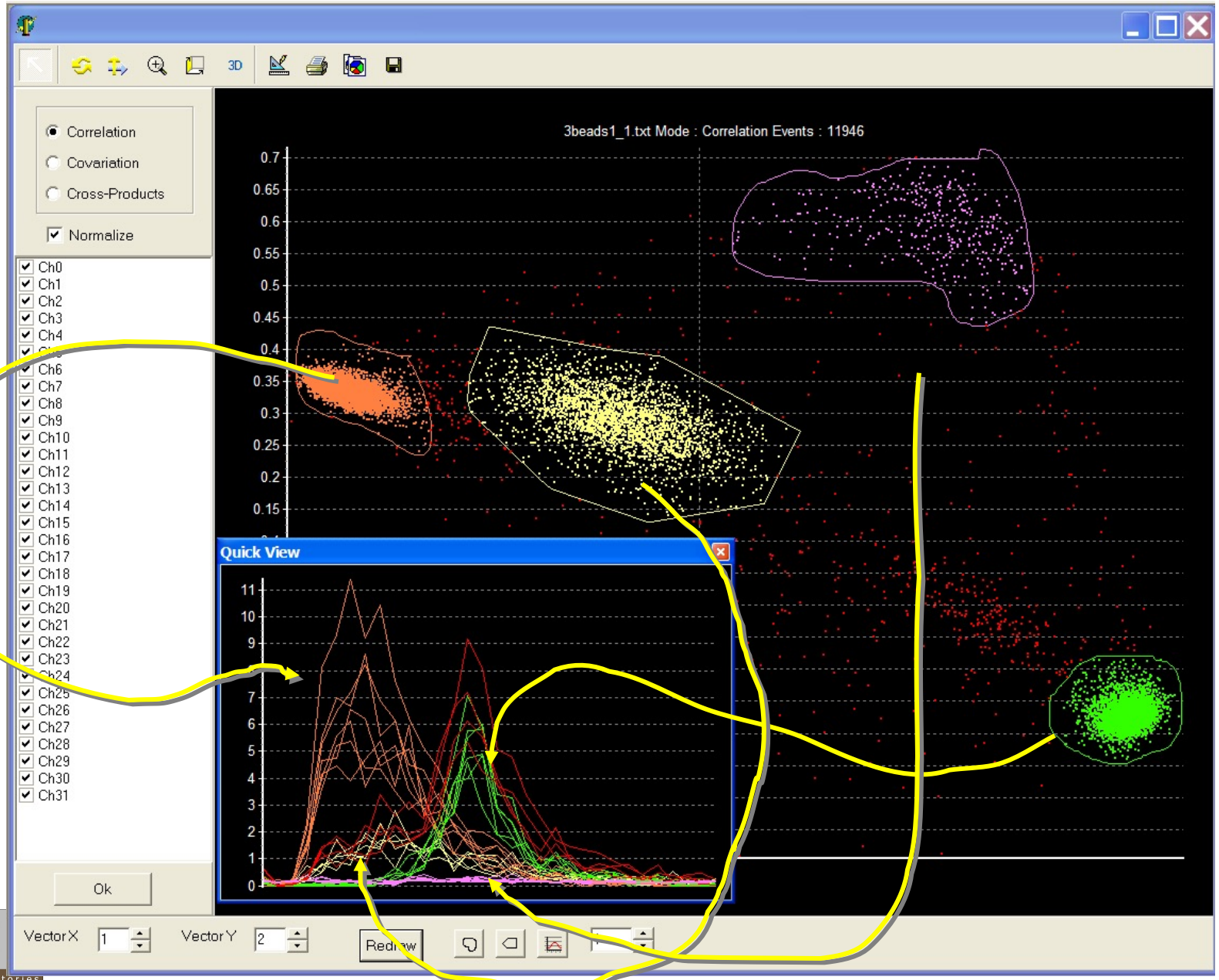


These CANNOT be resolved

Except using advanced Classification tools like PCA

PUCL cytometry laboratories

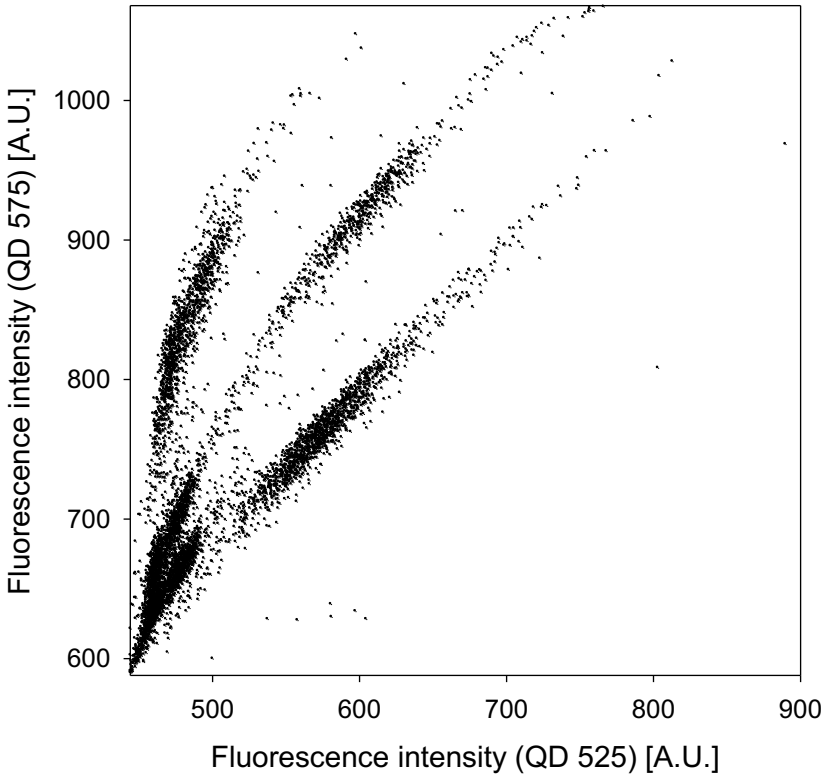
Spectral analysis allows classification



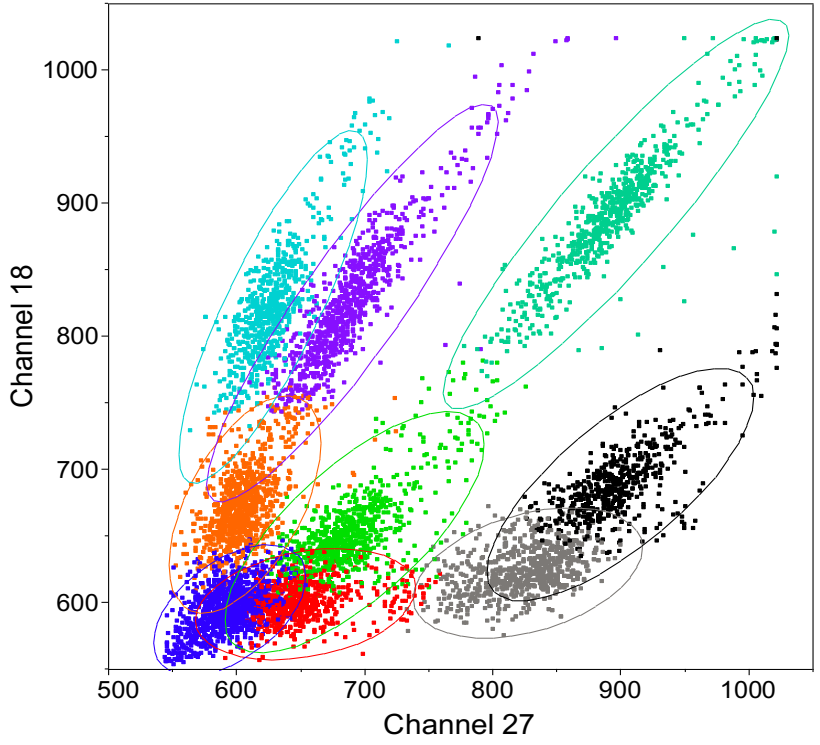
PUC cytometry laboratories

Develop signal processing tools suitable as a foundation for clinical instruments

Analysis of complex samples (mixed nanocrystals)



2 color system



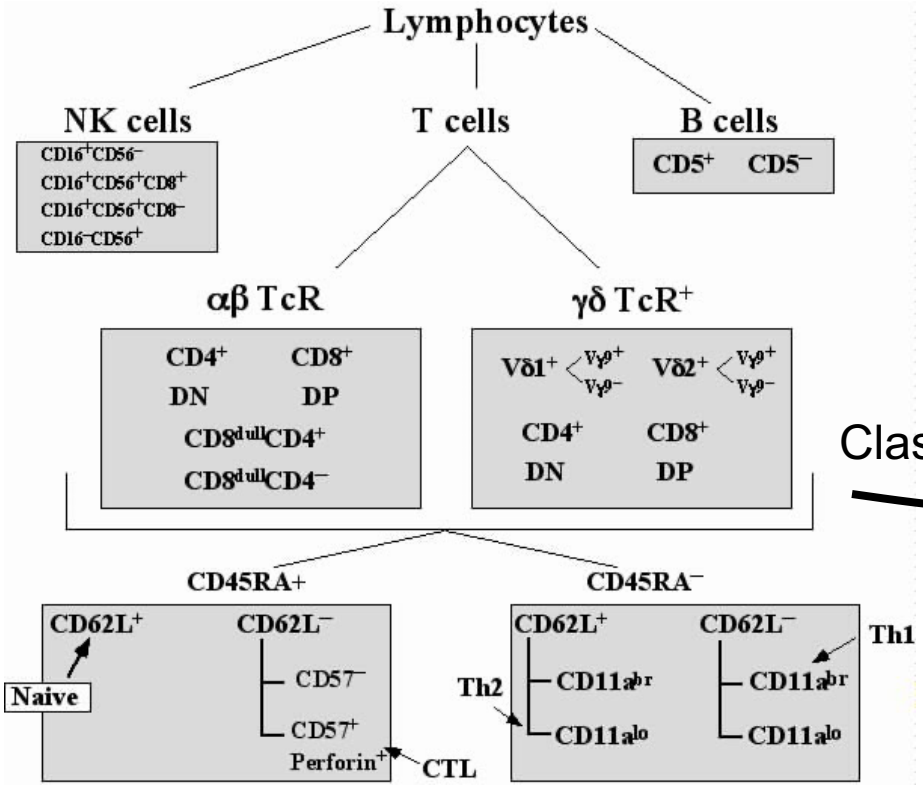
32 channel system

PUCL
cytometry laboratories

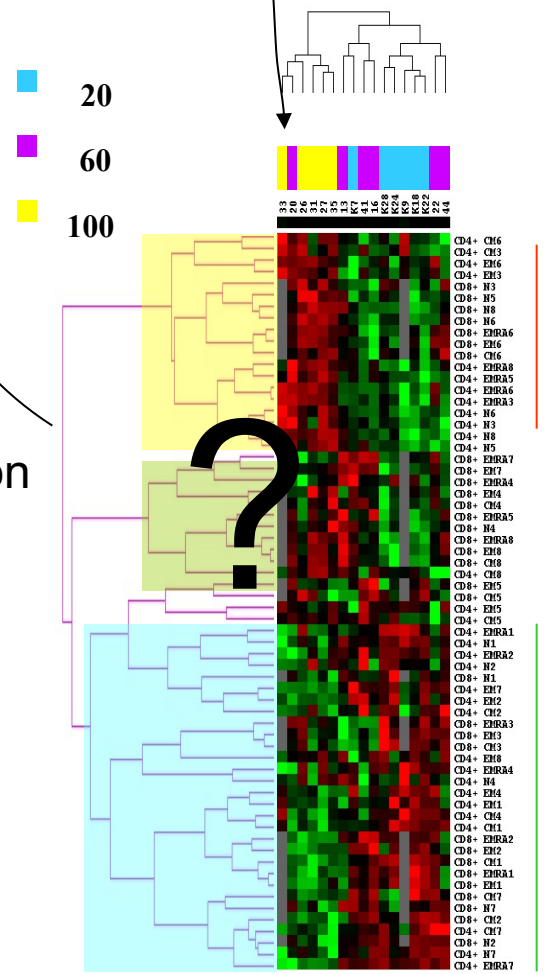
Problems?

- Problems with dispersion elements
- Low sensitivity - narrow bandwidth = high spectral resolution AND low numbers of photons
- Data collection – relatively expensive electronics is required at present
- Data analysis – traditional methods fail.
- Quantitation is no doubt more complex
- Standards development will be required

How do we bring clinical problems to the table?



Classification



Enrico Lugli et al, *Università di Modena e Reggio Emilia*

Oral Presentation Immunology
(Group of Andrea Cossarizza, University of Modena)

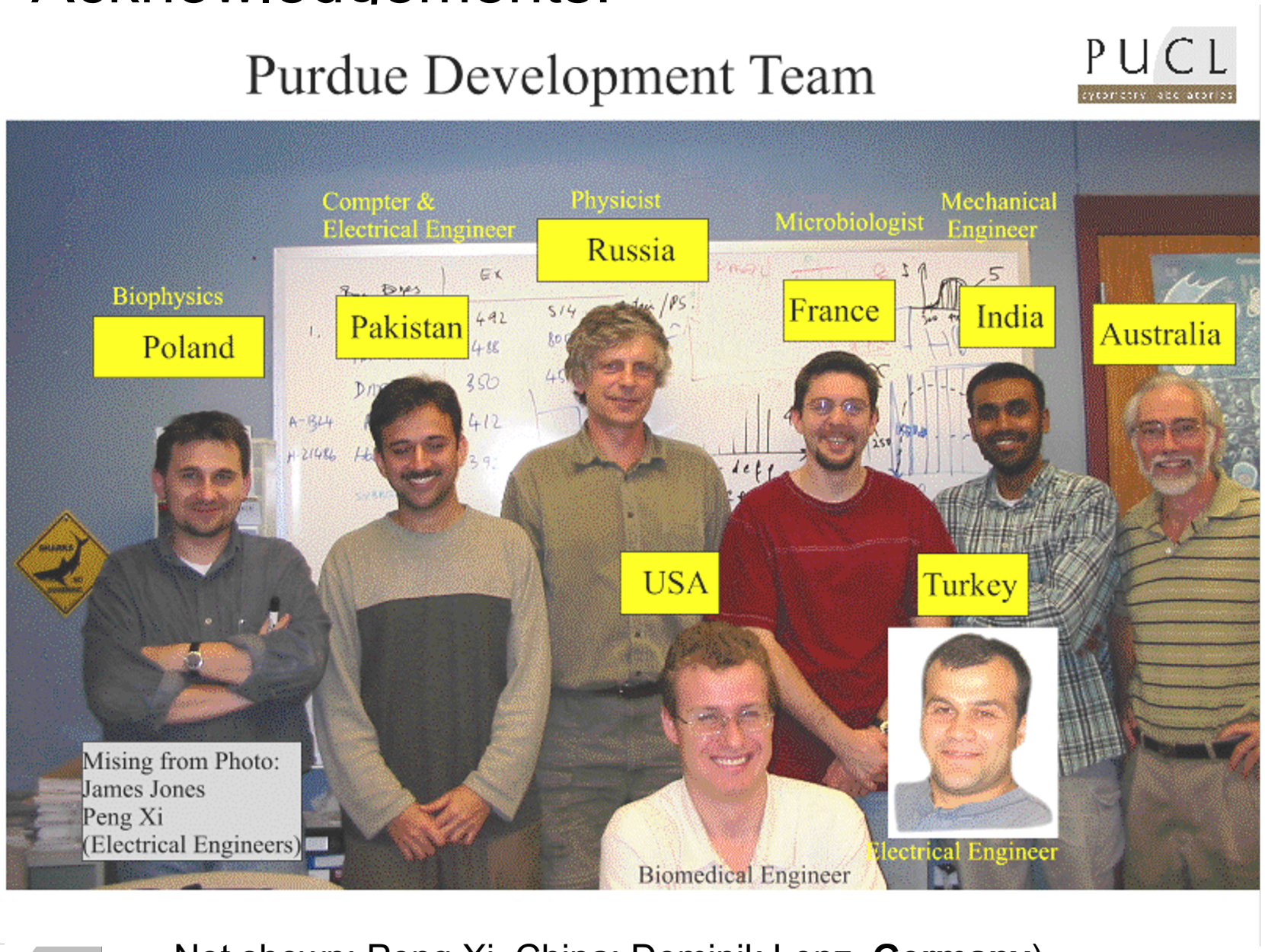
PUCL cytometry laboratories

Conclusions

- Spectral cytometry may fundamentally change the current concepts of cytometry
- Spectral cytometry may be a far better alternative for **some specific applications requiring classification**
- Spectral cytometry can separate probes of “apparently” the same emission (eg FITC, DIOC5, DIOC6, Oxonol, etc)
- There is less dependence on differences in intensity as there are in the spectral changes
- Traditional spectral compensation (hard to do) is not used
- There are complex problems to solve mathematically
- Much larger number of fluorochromes can be separated using the spectral cytometer (???)
- May be the choice for clinical diagnostic instrumentation

PUCL
cytometry laboratories

Acknowledgements:



Not shown: Peng Xi, China; Dominik Lenz, **Germany**)
Tytus Bernas, Poland; Sang Youp Lee, **S. Korea**)

Acknowledgements

Contributors – Collaborators

- Dan Hirleman (ME)
- Yinlong Sun (CS)
- Kinam Park (IP)
- Eli Asem (BMS)
- Sophie LeLevrie (BMS)
- Arun Bhunia (FS)
- Gerald J. Gregori (CNRS)

Senior Scientist

Bartek Rajwa (Biophysics)

Postdocs

- Valery Patsekin (Photonics)
- Tytus Bernas (Proteomics)
- Sang Youp Lee (Fluidics)
- Dominik Lenz (Cytometry)
- Krishna Mishra (Molecular Biology)

Graduate Students

- Wamiq Ahmed (Computational)
- Murugesan Venkatapathi (M.Eng)
- Bulent Bayraktar (Computational)
- Silas Leavesley (BioEng)
- Jia Liu (Cell Biology)
- Connie Paul (Pharmaceuticals)

Staff

- Jennie Sturgis (Imaging)
- Kathy Ragheb (Flow)
- Cheryl Holdman (Flow)
- Gretchen Lawler (CPC)
- Steve Kelley (Network)

Departments & Centers

Purdue University Cytometry Labs
Basic Medical Science
Biomedical Engineering
Electrical & Computing Engineering
Mechanical Engineering
Bindley Bioscience Center
Discovery Park
Purdue Cancer Center

Funding:
NIH, NSF, USDA, Purdue University
Corporate: Beckman-Coulter, Becton Dickinson, Point-Source, Parker-Hannifin, Bio-Rad, Polysciences, Bangs labs, MediaCybernetics,