Historical Perspective of Cytometry: Past successes and Future Opportunities

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“3rd Turkish-US Cytometry Workshop”
Akdeniz University, Antalya, Turkey

www.cyto.purdue.edu (science link)
www.cyto.purdue.edu/trackpaul (fun link)
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Goals of this lecture

1. Give a historical background to cytometry
2. Show 3 developing technologies that will have a major impact on the field – both clinical and research
Changes in Technology

- Early History
  - Visual observation - Color and color correction (apochromatic) - dyes for staining
    - 2600 BC Earliest written record of the use of dyestuffs in China
    - 715 BC Wool dyeing established as craft in Rome
    - 1327-1377 Edward III, "Royal Wool Merchant" offered protection to all foreigners living in England and to all who wanted to come to help improve the textile industry
    - 1646 Athanasius Kircher, a German Jesuit priest, recorded an interesting observation of the wood extract of *Lignum nephriticum*. An aqueous infusion of this wood exhibited blue color by reflected light and yellow color by transmitted light
    - 1856 William Perkin, an English chemist, synthesized a coal-tar dye, aniline purple
    - 1858-59 Magenta (fuchsia) discovered by Verguin the 2nd basic dye
    - 1861 Methyl violet, basic dye, by Lauth
    - 1862 Hofmann's Violet, Hofmann was one of the great dye chemists of all time
    - 1862 Bismarck Brown developed by Martius and Lightfoot, first soluble azo dye
    - 1863 Aniline Black, developed by Lightfoot, a black produced by oxidation of aniline on the cotton fiber.
    - 1866 Methyl Violet, basic dye
    - 1871 Adolph Von Baeyer, a German chemist, synthesized a fluorescent dye, fluoresceine
    - 1872 Methyl Green by Lauth and Baubigny,
    - 1876 Caro a chemist, discovered Methyl Blue
    - 1877 Malachite Green, basic dye by Dobner and Fisher
    - 1882 Paul Erlich, a German bacteriologist, employed the fluorescent dye uranin (sodium salt fluorescein) to track the pathway of secretion of aqueous humor in the eye. *This is the first case of the use of in vivo fluorochrome in animal physiology.*
    - 1884 The Gram stain, gentian violet an essential component was developed by Hans Gram, a Danish physician.
    - 1887 Rhodamine B (brilliant red-violet) basic dye

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Changes in Technology

Detection systems

- 1875, American, **G.R Carey** invented the phototube
- 1878, Englishman **Sir William Crookes** invented the 'Crookes tube', an early prototype of cathode-ray tube
- 1895, German, **Wilhelm Roentgen** invented an early prototype X-ray tube
- 1897, German, **Karl Ferdinand Braun** invents the cathode ray tube oscilloscope
- 1904, **John Ambrose Fleming** invented the first practical electron tube called the 'Fleming Valve'. Leming invents the vacuum tube diode
- 1922, **Philo T. Farnsworth** develops the first tube scanning system for television.
- 1923, **Vladimir K Zworykin** invented the iconoscope or the cathode-ray tube and the kinescope.
- 1926, **Hull and Williams** co-invented the tetrode electronic vacuum tube.
- 1938, Americans **Russell** and **Sigurd Varien** co-invented the klystron tube
- 1938 **Frits Zernike** built a microscope based on the principle in 1938 and received the Nobel Prize in 1953
- 1947 - The transistor was invented at Bell Telephone Laboratories by a team led by physicists **John Bardeen, Walter Brattain, and William Shockley**
- **1947 Gabor** invented the basic wave-front reconstruction technique of holography
- 1953 -Marvin Minsky invented (patented) the confocal microscope
Cytometry has a history of over 100 years

- **Ehrlich** 1880s - used acidic and basic dyes to identify acidophilic, eosinophilic, basophilic and neutrophilic leukocytes 1880’s to study the dynamics of ocular fluids - used fluorescein for first time
- **Robert Feulgen** (1925) - demonstrated that DNA was present in both animal and plant cell nuclei - developed a stoichiometric procedure for staining DNA involving a derivatizing dye, (fuchsin) to a Schiff base
- **Torbjorn Caspersson** - (1938-1998) - 1941 - “demonstrated that nucleic acids, far from being waste products, were necessary prerequesites for the protein synthesis in the cell (published in Naturwissenschaften in January 1941) and that they actively participated in those processes.” [“History of the Development of Cytophotometry from 1935 to the present” in Analytical and Quantitative Cytology and Histology, pp2-6, 1986]
- Early interests in cancer diagnosis from the times of **Papanicolaou & Traut** (1941)
- **Wallace Coulter** (1953-56) – Coulter principle
- Tremendous technology developments in the 1960s – **Fulwyler** – Cell Sorter, **Sweet**, Inkjet printer
- Automation in the 1990s led to vast numbers of cytometry instruments
- 2000’s – next generation technologies with advanced bioinformatics
Cell analysis technology *state-of-the-art*...?

- 1930-40s • Cell cytochemistry & staining
- 1950s • Cell counting
- 1960s • Cell sorting
- 1970s • Cell detection
- 1980s • Cell separation/classification (MABs)
- 1990s • Polychromatic (multicolor) cytometry
- 2000s • Automated imaging, cytomics, metabolomics
- 2010s • Technology Integration
  • Mass Cytometry

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Pathways for Discovery

1. Biological problem
2. Hypothesis
3. Experimental approach
4. Use available tools
   - Solution
   - Failure
5. Create new tools
6. Multidisciplinary
   - Biology
   - Engineering
7. New technology
   - Maybe mass-cytometry fits here

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“Provided there is no turbulence, the wide column of particles will then be accelerated to form a narrow column surrounded by fluid of the same refractive index which in turn is enclosed in a tube which will not interfere with observation of its axial content.”

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Wallace Coulter

Wallace Coulter - Coulter orifice - patent 1953
Commercialized in 1956 -

Measured changes in electrical conductance as cells suspended in saline passed through a small orifice

The first commercial version of the Coulter Counter was sold in 1956

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1st Example of New Technology

1. **Invention of cell sorting - flow cytometry**

   • **Problem:**
     - pathologist uses new technology (Coulter counter late 1950s)
     - Tries to use technology to interpret biology but does not understand it and makes fundamental error

   • **Solution:**
     - New technology had to be developed to test hypothesis
     - Pathologist proved wrong
     - Others identify new uses for invented technology
The beginning of the “cell sorter”

“After giving your ingenious cell-sorting scheme additional thought, my only conclusion is that it will surely work.”
From Character recognition to automated cell recognition

LA Kamentsky & CN Liu, Computer-automated design of multfont print recognition logic, IBM J. Research & Development 7, 1963

Slide kindly provided by Compucyte

High Content Screening

J. Paul Robinson, Purdue University
August 14, 1964

Mr. Mack J. Fulwyler
Los Alamos Scientific Laboratory
P.O. Box 1663
Los Alamos, New Mexico 87544

Dear Mr. Fulwyler,

I want to thank you for a most interesting and stimulating discussion during your visit yesterday. After giving your ingenious cell-sorting scheme additional thought, my only conclusion is that it will surely work. I certainly hope that you are successful in developing this and am looking forward to hearing more about it.

The amplifier and transducer driving schematics are enclosed. If there is any other information that I have that would be of use to you, please let me know.

Best Regards,

Richard Sweet
Research Associate
Systems Techniques Lab
January 24, 1966

Mr. R. A. Sweet  
Stanford Electronics Laboratories  
Stanford University  
Stanford, California

Dear Mr. Sweet:

I am enclosing a reprint of the separator article which appeared in the November 12, 1965, issue of Science. If you are interested, I will be glad to give you more information on the device.

Dr. Leonard Herzenberg of the Genetics Department at the Stanford Medical School is considering building a separator for research involving the biology of cells.

Did you receive your ink drop gun in good condition? What about the droplet pictures?

Sincerely yours,

Mack J. Fulwyler

February 25, 1966

Mr. Richard Sweet  
Stanford Electronics Laboratories  
Stanford University  
Stanford, California

Dear Mr. Sweet:

Dr. Leonard Herzenberg in the Department of Genetics of Stanford Medical School has a set of drawings of the droplet generator system. I understand that he is beginning to construct a separator.

I do not attempt to avoid formation of satellite droplets; as long as they coalesce quickly, they do not affect the separation efficiency. Stabilizing the length of the fluid jet is of concern, but after a few minutes of operation this length is fairly stable.

As you will see from the blueprints, a good deal of effort has gone into features secondary to droplet formation such as the flushing system, removable apertures, etc. The latest design will, hopefully, withstand autoclave sterilization.

We are pressing biological applications of the device as quickly as time and personnel permit. We are also investigating optical particle sensors with the hope of measuring optical characteristics of cells and separating on this basis.

Unfortunately, I am unable to give you the drop-to-drop spacing, etc., which you requested. I fear I have lost the record of operating conditions at which these photographs were taken.

Perhaps I will be able to see you next time I am in Palo Alto. If you are ever in this area, please plan to visit us; I am sure you would find our work interesting.

Is your ink writing oscillograph patented in such a way that this must be considered by a commercial company manufacturing the cell separator?

If, after talking to Dr. Herzenberg, you have questions, please feel free to write.

Sincerely yours,

Mack J. Fulwyler

Enc. sketch
Fulwyler’s original cell sorter – a 1967 model
Example of New Technology

2. Technology Integration Example

1. Pap smear: Situation:
   – Pathologist can read and interpret slide

   Problem:
   – Pathologist wants to automate technology

   Solution:
   – Engineer brings new imaging/cytometry technology to pathology problem
Two analytic instruments were built and one was delivered to LA Herzenberg at Stanford University in 1967.
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Integration of Technologies

• Fundamental redesign of flow cytometry technology by integration of principles of chemical analysis, image analysis & informatics
  – Implement capabilities of instruments so that we get quantitative results
  – Integrating technologies to create new capabilities
  – Ability to separate populations and create multiparameter analysis
  – New computers and software tools

**IMPACT:**

Highly organized systems – particularly in the clinical domain
  – Potential for creating automated systems
  – Almost real time analytical tools now available
  – High numbers of variables (parameters) in nicely designed instruments
Let's move to the future – what are some of the next-generation tools

1. Hyperspectral flow cytometry
2. High throughput Cytometry
3. Mass Spectroscopy – CyTOF – Very High Content
   20 to 100 parameters!!!
• Used by NASA in the LandSAT program.
• Many applications in biology and medicine.
• Started at Purdue University in the 1960’s by Professor David Landgrebe
Basic imaging...

Greyscale image → Color image → Multispectral image
Color composition is a mixture of spectral bands
Absorption Example

- Dutch Boy paint cards
- Colors difficult to distinguish by visual inspection
Optical Design of a basic flow cytometer

Sample

Flow chamber

PMT 1

PMT 2

PMT 3

PMT 4

PMT 5

Laser

Dichroic Filters

Bandpass Filters

Scatter Sensor

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Spectral Overlap makes for very complex analysis
Spectral Overlap makes for very complex analysis
Polychromatic Cytometry

Excitation

Emission

Scatter

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Advanced polychromatic cytometry

14 PMTs
41 filters
Multispectral Cytometry

32 Channel Pulse
Describes the
Entire spectrum

Spectrum becomes a parameter

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Asahi Spectra USA inc.
6-channel detection unit

- 620/30 BP (PE-Texas Red)
- 525/30 BP (FITC)
- 767/30 BP (PE-Cy7)
- 675/30 BP (PE-Cy5)
- 575/30 BP (PE)

32 channel-PMT

- Focusing lens
- Grating
- Side scatter PMT

Mirror

50/50 beam splitter

10/90 beam splitter for alignment or 488 LP filter for analysis

Laser beam

Epics Elite Flow cell

jet-in-air

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A 32 Ch PMT detector
What!! No need for gates???

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White Blood Cells

FITC

PE

ECD

CY5

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White Blood Cells
A blood sample incubated with:
- CD45 FITC
- CD4 PE
- CD8 ECD
- CD3 PC5

is analyzed both with the 32PMT and the 6PMTs. A PCA is run on both data. Lymphocytes are gated out from SSC vs FS.

Comparisons of PCA results:

**32 channels PMT**

PCA (Correlation) on all the parameters: 32 channels + SSC+ FS(sum)+ RingB+ RingC+ RingD+ RingE,

PCA (Correlation, Hyperspherical) on all the parameters: 32 channels + SSC+ FS(sum)+ RingB+ RingC+ RingD+ RingE,

**6 PMTs device**

PCA (Correlation) on 4 channels + SSC+ FS(sum) + RingB+ RingC+ RingD+ RingE,

PCA (Correlation, Hyperspherical) on 4 channels + SSC+ FS(sum) + RingB+ RingC+ RingD+ RingE,

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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

Add

Wash

Code: 121 211 2

Code: 111 102 0

Code: 121 211 0

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2. Automation & Automated Analysis

- Will improve accuracy because of standardization
- Will decrease time to analysis
- Will identify subsets otherwise missed
- Current software cannot analyze huge data sets
- Concept lends itself to more automation
- Only way to make flow cytometry a systems biology tool

So here is the problem....
A complicated mess...
And perhaps hundreds of files
So several questions arise...
So several questions arise…

• What kinds of analytical tools do you need and how do you efficiently achieve an analytical solution?
• How do you handle huge data sets?
• What is the place of automation?
• Is it better to collect more variables/parameters on fewer cells..
• Or less variables/parameters and lots of cells…. 

and most people answer …
and most people answer …

• We want a lot of parameters and a lot of cells…..fast…..and easy…..

So 2 examples of very large data sets…. 
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Primary T-Lymphocyte Data

Conditions (96 well format)

- Perturbation a
- Perturbation b
- Perturbation n

11 Color Flow Cytometry

- 9 phosphoproteins, 2 phospholipids
- 600 cells per condition
  - 5400 data-points
- Primary human T-Cells
- 9 conditions
  - (6 Specific interventions)

Sachs et al, Science, 2005

Slides kindly supplied by Gary Nolan
HyperCyt system added to flow cytometer

HyperCyte diagram kindly supplied by Larry Sklar

384 wells/10 min
1 µl/sample  5000 cells/µl

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Too much information to comprehend as small pieces....
Systems biology is looking at the big picture...

Automation is not the process of having everyone in the lab work really fast!

Automation that is slow and painful has little to no real value!
Automated Cytomat Incubator
Automated Biomek Preparative system
Automated HyperCyte Sample Delivery
Cyan flow cytometer

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Multiparametric cytometry and multifactorial HTS

Number of cells is measured, Fluorescence intensity is a feature

\[ c_1 = [m_1, m_2, m_3, \ldots, m_n] \]
\[ c_2 = [m_1, m_2, m_3, \ldots, m_n] \]
\[ c_3 = [m_1, m_2, m_3, \ldots, m_n] \]
\[ \vdots \]
\[ c_n = [m_1, m_2, m_3, \ldots, m_n] \]

Define multiple populations in the feature space

Find the features describing the populations \( f_1, \ldots, f_n \)

Flow cytometry

Perform \( m \times n \) single measurements

HTS

Find the features (such as IC\(_{50}\))
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**Multiparametric and multifactorial HT cytometry**

Define multiple populations in the feature space

\[ c_1 = [m_1, m_2, m_3, \ldots, m_n] \]
\[ c_2 = [m_1, m_2, m_3, \ldots, m_n] \]
\[ c_3 = [m_1, m_2, m_3, \ldots, m_n] \]
\[ \vdots \]
\[ c_n = [m_1, m_2, m_3, \ldots, m_n] \]

Find the features describing the populations \( f_1, \ldots, f_n \)

\[ g(c) \]

Create \( n \times m \) array of features, where every \( m \) could be a vector \([f_1, \ldots, f_n]\)

Find the features (such as IC\(_{50}\))

HT flow cytometry

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Problem 1: eliminate operator's input in analysis

Measured sample

Calculate fraction of "positive" and "negative" cells

Well... plenty actually
• **Method I**
  – Sort the apples (“gate” the green and red populations, or red and non-red)
  – Count red and green (non-red)
  – Compare the counts

• **Method II**
  – Find a bucket full of red apples only
  – Find the distribution
  – Take a bucket with red and green apples and find the distribution
  – Compare the distribution

- **Heuristic methods**
  – Minkowski-form
  – Weighted-Mean-Variance (WMV)

- **Distance functions used in nonparametric tests**
  – $\chi^2$ (Chi Square)
  – Kolmogorov-Smirnov (KS)
  – Cramer/von Mises (CvM)

- **Information-theory divergences**
  – Kullback-Liebler (KL)
  – Jeffrey divergence (JD)

- **Spectral measures**
  – Spectral angle mapper
  – Bhattacharyya distance

- **Ground distance measures**
  – Histogram intersection (Overton)
  – Quadratic form distance (QF)
  – Wasserstein-Rubinstein-Mallows distance (Earth Movers Distance)
Graphical data processing environments

IBM Data Explorer

Khoros

Orange

AVS

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Graphical cytometry analysis pipeline builder

We call this a Logic Map

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Membrane Potential, Glutathione, Viability, Superoxide

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JC-1
mbbr
Calcein
Mitosox [1]
384 Well Plate Format For drug screen - Redox

- Redox 3 color assay
- Negative control wells
- Ethanol positive controls
- 3-5,000 cells per well (1 to 2 x 10^6 cells/plate)

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The reason to do all this is to get graphs like this!!

3-5,000 cells per well (1 to 2 x 10⁶ cells/plate)

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Logic Process

1. Biomek automated plate preparation
2. Biomek to Hypercyte/Cyan
3. 1 Plate is run
4. Technician runs Plate Analyzer in semi-automatic mode to check assay integrity
5. Data OK?
   - Yes: Next Plate is run
   - No: Repeat

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Plate Analyzer – all the parts

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Instantly see any well or combination of wells with any parameter or combination of parameters Bindley Bioscience Center.
Highlight the 3D Display Format.
Direct reporting of IC50 Results

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Logic Map for 3 color Redox assay in 384 well plate – 96 dose response curves in 1 click
3 functional assays, 32 drugs, 96 curves, 10 points/drug

One 384 well plate, 8 minutes collection time
Each Drug is a different color

- mBBr
- Calcein
- Mitosox

Gray represents a dose response of mBBr with each color representing a different drug.

mbbr IC50 curves (32 drugs, 10-point curves)

Gray represents a dose response of Calcein with each color representing a different drug.

Calcein IC50 curves (32 drugs, 10-point curves)

Gray represents a dose response of Mitosox with each color representing a different drug.

Mitosox IC50 curves (32 drugs, 10-point curves)
‘Things don’t change. You change your way of looking, that’s all.’

Carlos Castaneda

Time

Circa 1990

An Innovation in Flow Cytometry Data Collection and Analysis Producing a Correlated Multiple Sample Analysis in a Single File

J. Paul Robinson, Gary Durack, and Steve Kelley
Purdue University Cytometry Laboratory and Department of Veterinary Physiology and Pharmacology, Purdue University, West Lafayette, Indiana 47907
Received for publication April 17, 1990; accepted August 26, 1990

Time Interval Gating for Analysis of Cell Function Using Flow Cytometry

Gary Durack, Gretchen Lawler, Steve Kelley, Kathy Ragheb, R.A. Roth, P. Ganey, and J. Paul Robinson
Purdue University Cytometry Laboratory and Department of Veterinary Physiology and Pharmacology (G.D., G.L., S.K., R.R., J.P.R.), Purdue University, West Lafayette, IN 47907 and Department of Pharmacology and Toxicology (R.A.R., P.G.), Michigan State University, E. Lansing, MI
Received for publication April 22, 1991; accepted July 5, 1991

Circa 2011
96 well data works fine too (FACSCanto, LSRII, Acuri, Cyan)

96 well plate starting at col 2
96 well data functional assay
Complex assay: 288 tubes, 7 color, 9 variables, 4 pop’n, 5 x10^6 cells

- Stimulate PBMC with IL6, IL10, and LPS. Measure phosphorylation of Stat3
- and p38 after 15 minutes of stimulation. Stain for CD4+ T cells, CD4- T cells,
- monocytes, and B cells.
- Pstat3 – ALEXA647
- Pstat1 - ALEXA488
- CD33 – PE-CY7
- CD4 – PacBlu
- CD20 – PE-Cy55
- CD3 – PE

Assay data from Dr. Peter Krutchik

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The Logic of "Logic Maps"

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Assay data from Dr. Peter Krutchik

J. Paul Robinson, Purdue University
7 color, 9 parameter Simultaneous Analysis, 80 Dose Response curves on 4 simultaneous populations

Initial Gate Criteria

CD33⁺ MN

Stat⁺-ve Width Size

CD20⁺ B Cells

Assay data from Dr. Peter Krutchik

CD4⁺ T cells

CD3⁺ T cells

CD4⁻ T cells
Assay data from Dr. Peter Krutchik

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3. The next flow cytometer

29 different variables (parameters)

14 different populations of cells

12 activation molecules

14 distinct pathways

8 point dose response curves
Initial gating 14 populations 14 sets of dose response curves

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12 x 14 x 14 = 2356
8-point dose response curves from one 96 well plate analyzed by one protocol...
In about 5 minutes!!
Summary & Conclusions

- The first 40 years took 40 years to get us to today!
- The next 10 years will expand by 10 times today’s common lab assay styles
- Automation will be efficient and cost effective and more accurate than the best analyst today
- We will stop talking about intensity values, % of change, we will talk in probability functions
- We will all have to go back to school if we are to understand these new powerful tools
- But – these are the tools that will bring systems biology into the flow cytometry laboratory
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