BMS 631 "Why the flow cytometer was developed"

Flow Cytometry: Theory: Lecture 1

J. Paul Robinson

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Purdue University

All materials used in this course are available for download on the web at

Link to Lecture page http://tinyurl.com/2wkpp

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WEB http://www.cyto.purdue.edu

- Introduction
- Course Requirements

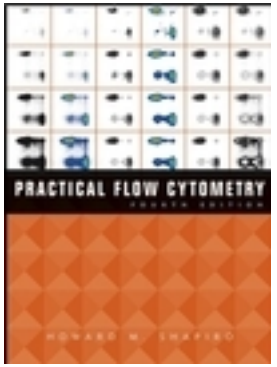
Lecture Series: 2020

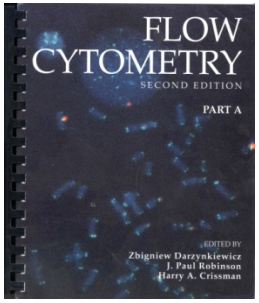
Structure of this course

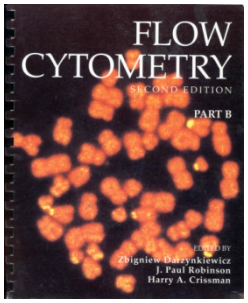
- Lectures: The class consists primarily of lectures and lecture discussions with mini tutorials as necessary.
- Practicals: There are no practical components to the 631 course. We will however, look at some instruments and instrument components to gain some perspectives.
- Quizes: There is an end of term quiz take home quiz (65%).
- Homework reading & attendance, and student Seminar: 35% of grade
- Seminar: Each student must present a seminar at the conclusion of the course. This seminar must be discussed with the course director prior to preparation. This is worth 25% of the final grade and will be composed of attendance and presentation. You must give me an outline and discuss the seminar with me at least one week prior to presentation. You must provide me with a copy of the original PPT file. Only one student can present on any particular topic.

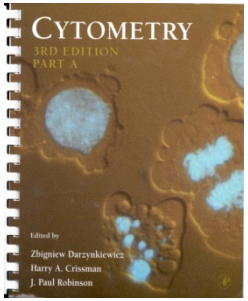
Sources of information

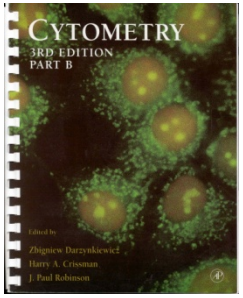
- **Flow Cytometry and Sorting, 2nd ed.** (M.R. Melamed, T. Lindmo, M.L. Mendelsohn, eds.), Wiley-Liss, New York, 1990 - referred to here as **MLM**
- **Flow Cytometry: Instrumentation and Data Analysis** (M.A. Van Dilla, P.N. Dean, O.D. Laerum, M.R. Melamed, eds.), Academic Press, London, 1985 – referred to as **VDLM**
- **Practical Flow Cytometry** 3nd edition (1994),4th Ed (2003) H. Shapiro: Alan R. Liss, New York - referred to as **PFC**
- **Introduction to Flow Cytometry**. J. Watson, Cambridge Press, 1991 referred to as **IFC**
- **Methods in Cell Biology:** v.40,41, 63, 64 Darzynkiewicz, Robinson & Crissman, Academic Press, 1994, 2000 **MCB**
- **Data Analysis in Flow Cytometry:A Dynamic Approach-Book on CDROM** M. Ormerod referred to as **DAFC**
- **Flow Cytometry: First Principles**. (2nd Ed) Alice Longobardi Givan, Wiley-Liss, 2001 referred to as **AFCFP**

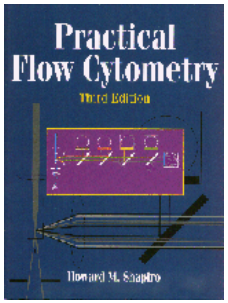


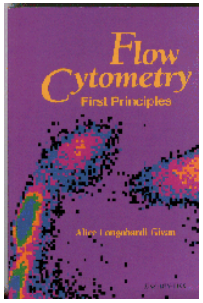


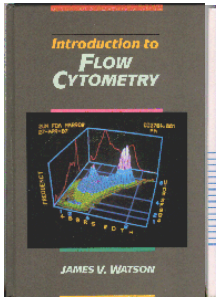


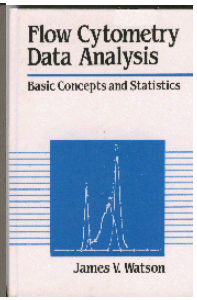












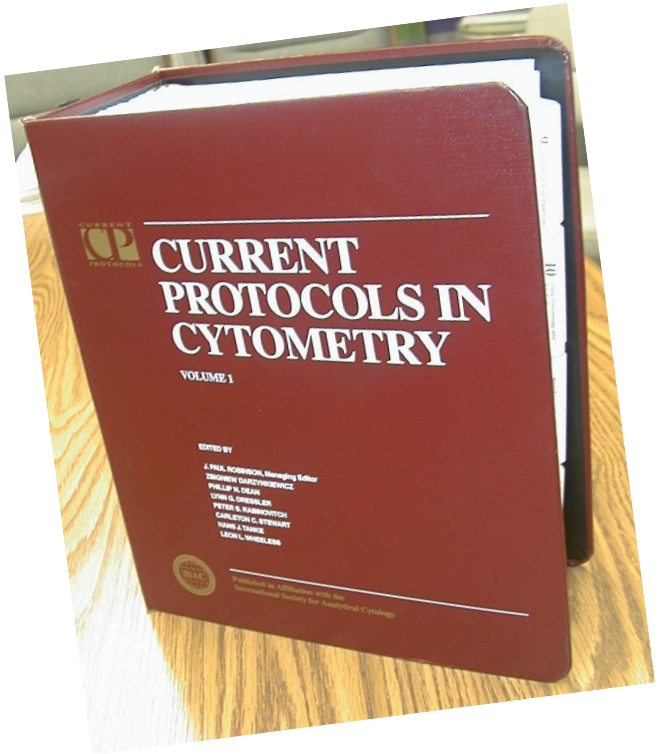
More information on flow cytometry books can be found on our website at:
http://www.cyto.purdue.edu/flowcyt/books/bookindx.htm

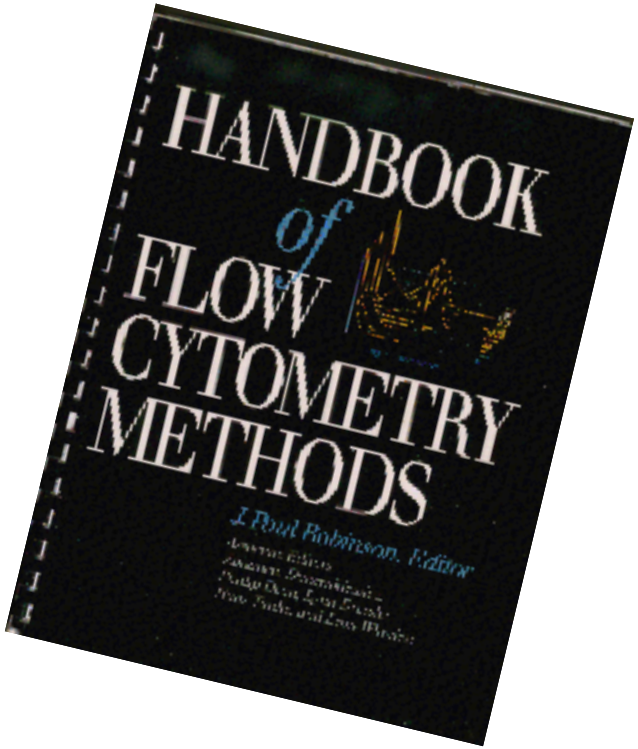
Note: All of these books are in Prof. Robinson's library in Lynn Hall, Room B140 and may be checked out for 24 hour periods.

Methods and Practical Assistance

- For help with protocols there are several choices including the MCB references on the previous slide (Methods in Cell Biology)
- The Handbook of Flow Cytometry Methods morphed into

Current Protocols in Cytometry which is the definitive work on cytometry protocls





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Reference Material

The course will use Shapiro:

***Practical Flow Cytometry*,** 4nd edition (2003), Howard Shapiro, Wiley-Liss, New York, as the main reference text.

You can get a free copy of the text here http://ls.beckmancoulter.com/en-us/flow-cytometry/sidebar/research-tools/learning-tools/practical-flow-cytometry

http://www.beckmancoulter.de/Practical%20Flow%20Cytometry.html WORKS

Supplementary books with good background materials

Introduction to Flow Cytometry. J. Watson, Cambridge Press, 1991 (hard to find, but excellent)

Flow Cytometry: First Principles. Alice Longobardi Givan, Wiley-Liss, 1992 (several editions)

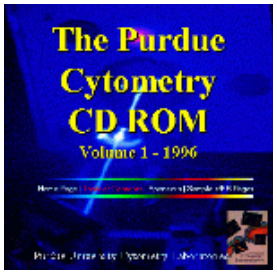
Flow Cytometry: A Practical Approach. M.G. Omerod, IRL Press, 1990 (good background)

Methods in Cell Biology: vols 40,41. Darzynkiewicz, Robinson & Crissman, Academic Press, 1994 (several more recent volumes

Additional Background Sources

Go here to download: http://www.cyto.purdue.edu/flowcyt/educate/pptslide.htm

- Powerpoint presentations references as J.Paul Robinson (**JPR**); Robert Murphry (**RFM**), Carleton Stewart (**CS**)
- Web sources of these presentation are:
- http://www.cyto.purdue.edu/flowcyt/educate/pptslide.htm
- http://www.cyto.purdue.edu/flowcyt/educate1.htm



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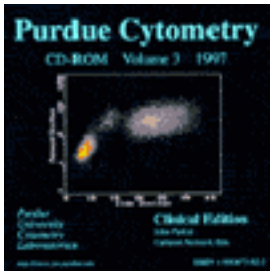

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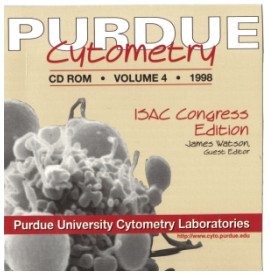

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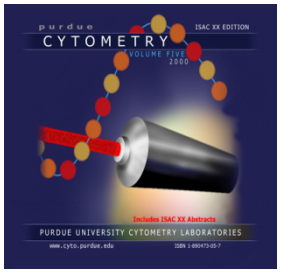
Additional Sources include the Purdue Cytometry CD-ROM series



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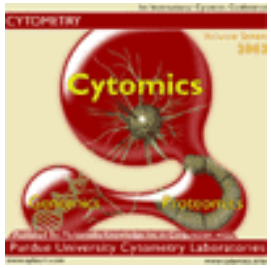

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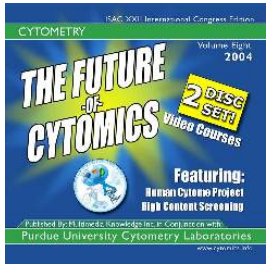

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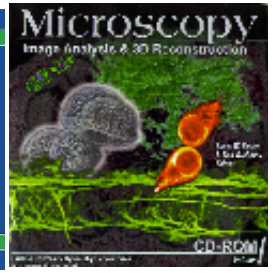

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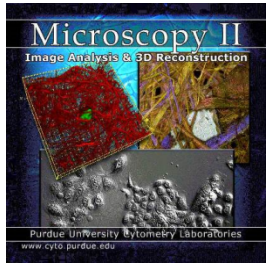

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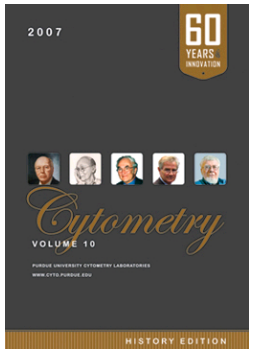

DVD V10

http://www.cyto.purdue.edu/flowcyt/cdseries.htm

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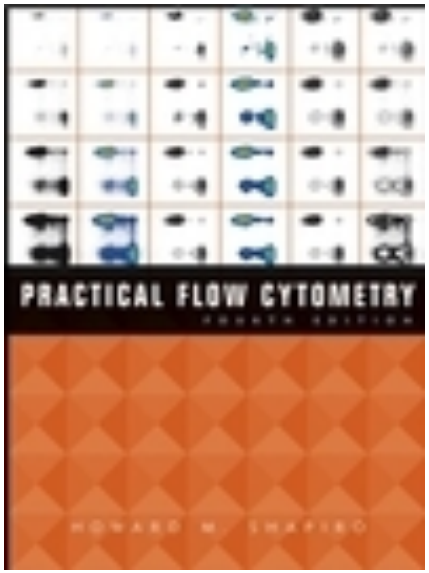
- Introduction to the course.
- Discussion of texts and associated reading materials.
- Discussion of expectations of students and special concerns.
- Methods of evaluation and testing for the course.
- Allocation of special review areas and discussion of areas for presentation of laboratory seminar.
- Introduction to flow cytometry principles

References: (Shapiro pp 1-5; Watson pp 1-4; Givan pp 1-9)

Course Text



Shapiro, H. Practical Flow Cytometry, Wiley-Liss, New York, NY 4th Edition

NOTE: ONLY GET THE 4th EDITION

Note: Amazon.com and look for 2nd hand books for around $10-$50 (new price $125 but may not be available) Despite being quite old, the core information in this books is tremendously useful and you will find it an excellent reference book.

OR

Download Free (you must register first) at
http://www.coulterflow.com/bciflow/practical.php

Student Seminars

Allowable Topics for Seminars

The topic for the student seminar must be based upon an understanding of a component of the technology. It must demonstrate a complete understanding of the principle involved and the application to biology.

Evaluation: The seminar counts for 25% of the course. See requirements below.

EXAMPLES OF PREVIOUS SEMINARS

- Evaluation of Small Particles using Flow Cytometry
- Kinetic Measurements using Flow Cytometry
- Monoclonal Antibodies, Avidin-Biotin Technology using Fluorescent Conjugates in Flow
- Fluorescent Molecules used in Flow Cytometry
- Optical Filters used in Flow Cytometry
- The Optical System in a Flow Cytometer
- The Fluidic System of a Flow Cytometer
- The Principles of Sorting in Flow Cytometry
- Parameters used in Flow Cytometry
- Parameters & Probes for Evaluation of DNA & RNA in Flow Cytometry
- Any *other topic can be suggested*

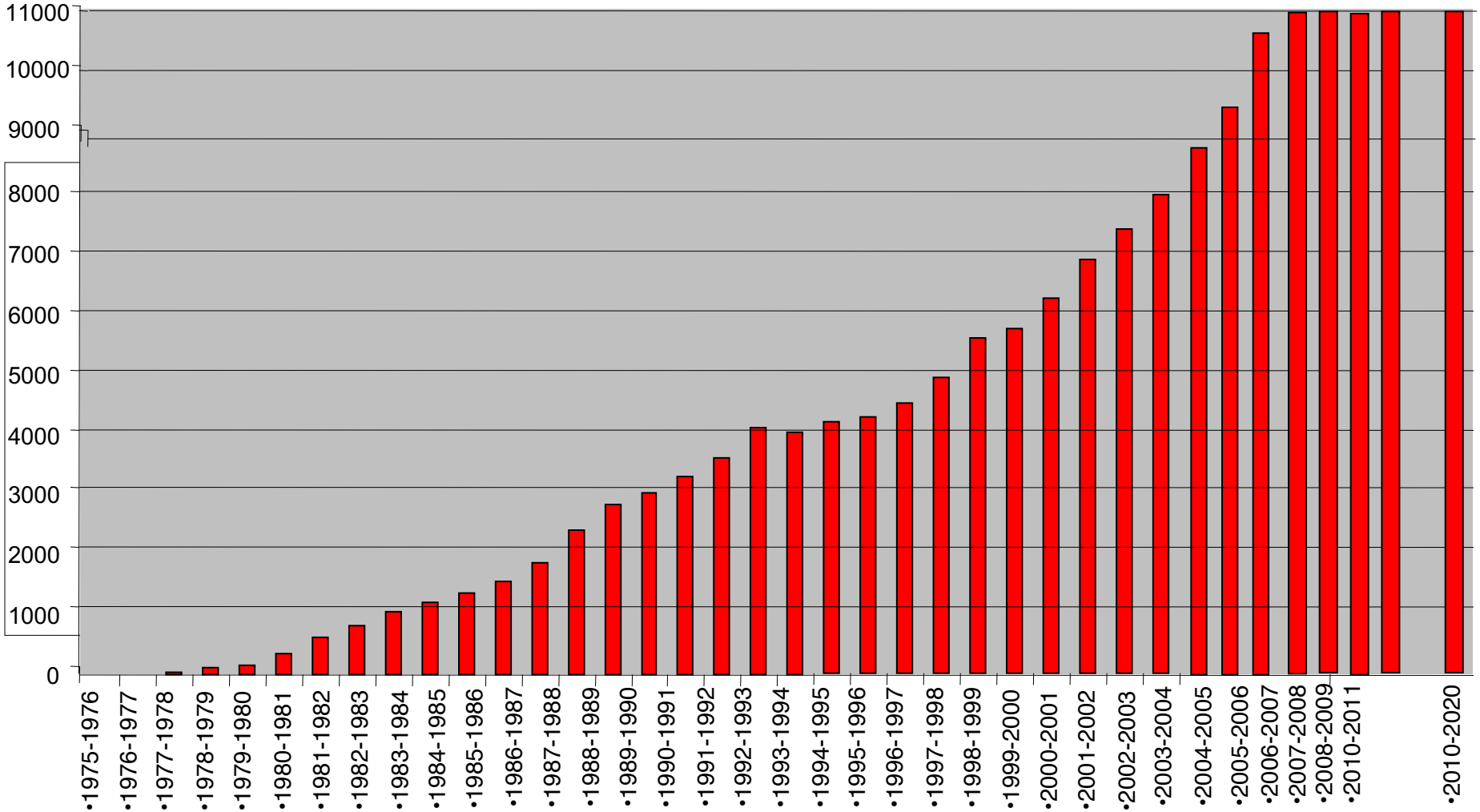
RULES: Presentations on research projects **WILL NOT BE ALLOWED**. The purpose of this seminar is to demonstrate your technical knowledge in a particular area of flow cytometry. The seminar may be recorded and must not exceed 15 minutes. All presentations must be made using Powerpoint. Electronic must be provided in advance for evaluation. All material must be approved by the course instructor before presentation.

Introduction to the terminology, types of measurements, capabilities of flow cytometry, uses & applications

- Comparison between flow cytometry and fluorescence microscopy
- Transmitted light
- Scatter
- Sensitivity, precision of measurements, statistics, populations
- Flow cytometry generally does not provide spatial information (exception would be Amnis™ that does flow and imaging)

Publications using the keyword "flow cytometry" from PubMed

115,000 – refs 2010
125,411 - refs 2011
212,069 - refs 2020



The field of flow cytometry continually grows

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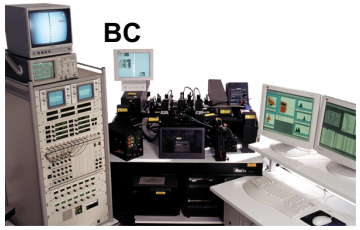
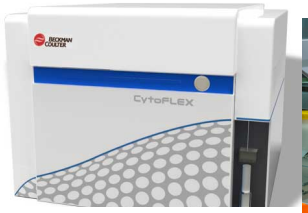
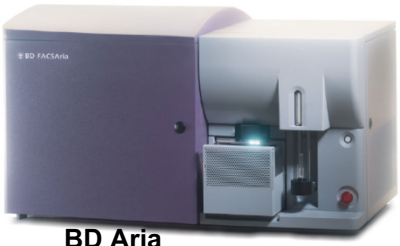
Flow Cytometry

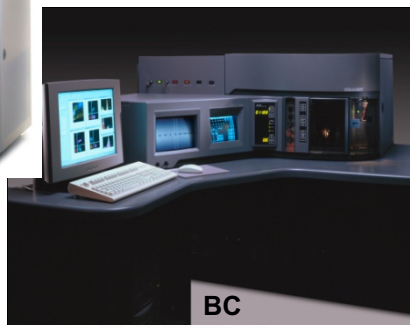
- Technology that measures properties of single cells
- Measures fluorescence, light scatter, and other properties of cells and particles
- Can provide correlated data that links different population profiles

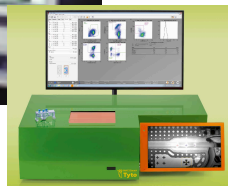
Commercial Instruments

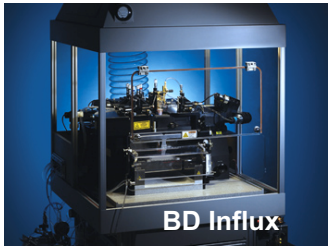












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What can Flow Cytometry Do?

- Enumerate particles in suspension
- Determine "biologicals" from "non-biologicals"
- Separate "live" from "dead" particles
- Evaluate 10^5 to $5x10^6$ particles in less than 1 min
- Measure particle-scatter as well as innate fluorescence or 2^o fluorescence
- Physically sort single particles for subsequent analysis

What are the principles?

- Light scattered by a laser or other light source
- Specific fluorescence detection
- Hydrodynamically-focused stream of particles
- Electrostatic particle separation for sorting or chip-based sorting
- Multivariate data analysis capability

Definitions

- **Flow Cytometry**
  - Measuring properties of cells in flow
- **Flow Sorting**
  - Sorting (separating) cells based on properties measured in flow
  - Also called **Fluorescence-Activated Cell Sorting (FACS) – this is a proprietary term from BD -** this term can refer to sorting, but it is not usual to use it for analysis. FACS is thus frequently misused and likely by folks who learn flow on BD instruments. It is far better to use the term flow cytometry which is generic and covers both sorting and analysis.

Technical Components

Detection Systems

Photomultiplier Tubes (PMTs)

Historically 1-2 (old instruments)

Current benchtop instruments 3-14

Complex instruments mostly 10-20 now up to 50

Array PMTs 32-40 (spectral system mostly)

Diodes

Light scatter detectors

Cytoflex (Beckman Coutler is all APDs)

APD arrays (spectral systems)

SiPMs – next generation systems

Illumination Systems

Lasers 325, **350-363, 380, 405, 407, 420, 457, 488, 514, 532, 543, 568, 600, 633, 660nm +… 680nm, 801nm + others as developed**

Argon ion, Krypton ion, HeNe, HeCd, Yag, solid state, diode

Arc Lamps

Mercury, Mercury-Xenon (most lines) (rarely used currently)

Stains...

Up to 1850's - *only natural stains were available such as Saffron (which was what Leeuwenhoek used to stain muscle cells)*

Ehrlich - *used acidic and basic dyes to identify acidophilic, eosinophilic, basophilic and neutrophilic leukocytes 1880's to study the dynamics of ocular fluids- used fluorescein*

Fluorescence UV Microscope - August Köhler - 1904

Pappenheim & Unna (early 1900's) - *combined methyl green and pyronin to stain nuclei green and cytoplasm red*

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*

Andrew Moldavan

It is unclear if Moldavan ever built his cell counter. His short article (less than half a page) describes a number of problems but no results.

"The purpose of the experiment is to have each microscopical cell passing through the capillary tube, register itself automatically on the photoelectric apparatus, thus creating a micro-current which can be amplified and recorded."

Manuscript:

Photo-Electric Technique for the Counting of Microscopical Cells

Andrew Moldavan
Montreal, Canada
Science 80:188-189, 1934

Moldavan (1934) demonstrates use of a suspending fluid in which were blood cells - the measurements were made in a capillary tube using a photoelectric sensor to make extinction measurements

Moldavan's Paper

SCIENTIFIC APPARATUS AND LABORATORY METHODS

PHOTO-ELECTRIC TECHNIQUE FOR THE COUNTING OF MICROSCOPICAL CELLS

THE technique of progressive dilutions used by early bacteriologists to isolate species and to estimate the probable number of cells is still applied in the determination of quanta of viruses, bacteriophages and enzymes.

The isolation and counting of bacterial and fungic species have been so simplified by the introduction of solid culture media, differential stains and micromanipulation that the dilution technique, which in part is embodied in the standard plate count method, is no longer carried to its final point: *e.g.*, one organism per dilution bottle.

The following is a brief description of an attempt to adapt photoelectric methods to the direct counting of microscopical cells in suspension in water.

A capillary glass tube, made from a small tube elongated over a gas flame, is placed under the high magnification field of a microscope. The microscopical cells in suspension in water (red blood cells or neutral-red stained yeast cells) are forced under pressure to circulate through the capillary tube. A photoelectric apparatus of the smaller type is adjusted to the microscope's ocular and connected with an appropriate meter. The purpose of the experiment is to have each microscopical cell passing through the capillary tube, register itself automatically on the photoelectric apparatus, thus creating a micro-current which can be amplified and recorded.

The technical difficulties to overcome in such an experiment can be listed as follows:

(1) Difficulty to standardize capillary tubes in such way as to fill exactly the highest magnification field.

(2) Difficulty to flatten capillary tubes (as suggested by Sturges in his studies on bacterial motility) to insure proper focus.

(3) Necessity to shake dilution samples thoroughly to prevent clumping of cells in capillary tubes.

(4) Desirability of a specific photoelectric apparatus highly sensitive to microscopical objects. The ordinary commercial photoelectric apparatus is not built or intended for such purpose and shows only a faint reaction to magnified erythrocytes, neutral-red stained yeast cells or microscopical solid particles.

ANDREW MOLDAVAN

MONTREAL, CANADA

Science 24 Aug 1934:
Vol. 80, Issue 2069, pp. 188-189
DOI: 10.1126/science.80.2069.188

Torbjorn Caspersson

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."*

Manuscript:

Torbjorn O. Caspersson, History of the Development of Cytophotometry from 1935 to the present" in Analytical and Quantitative Cytology and Histology, pp2-6, 1986.

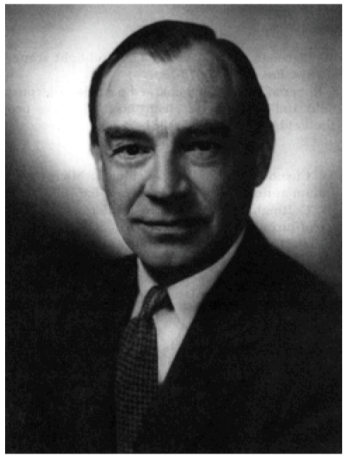
1950 - demonstrated that both DNA and RNA increase in actively growing cells famous monograph in 1950 "*Cell Growth and Cell Function*" described nucleic acid and protein metabolism during normal and abnormal growth. - These studies were made using a Cadmium spark source for a UV light, and primitive electronic circuits for detection of signals.

Used Feulgen stain to stain nuclei

Fluorescence Labeling Technique

Coons et al 1941 - developed the fluorescence antibody technique - they labeled antipneumococcal antibodies with anthracene allowing them to detect both the organism and the antibody in tissue using UV excited blue fluorescence

"Moreover, when Type II and III organisms were dried on different parts of the same slide, exposed to the conjugate for 30 minutes, washed in saline and distilled water, and mounted in glycerol, individual Type III organisms could be seen with the fluorescence microscope……"



Courtesy of the Harvard Medical School Countway Library

ALBERT HEWETT COONS

1912—1978

A Biographical Memoir by
HUGH O. MCDEVITT

Manuscript:

Immunological Properties of an Antibody Containing a Fluorescent Group

Albert H. Coons, Hugh J. Creech and R. Norman Jones
Department of Bacteriology and Immunology, Harvard Medical School, and the Chemical Laboratory, Harvard University
Proc. Soc. Exp.Biol.Med. 47:200-202, 1941

Coons and Kaplan (1950) - conjugated fluorescein with isocyanate - better blue green fluorescent signal - further away from tissue autofluorescence. This method used a very dangerous preparative step using phosgene gas

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Albert H Coons 1961 memory of his discovery

He agreed to try to couple anthracene isocyanate to some antipneumococcal antiserum with which I provided him, and promptly did so. This antibody solution agglutinated specific pneumococci.

one. Fluorescein was chosen as the label because of the brilliance of its fluorescence and because no green-fluorescing materials had been reported in mammalian tissue.

In order to do this, I needed a fluorescence microscope. Here I had another stroke of luck. Dr. Allan Grafflin,[10] at that time an Assistant Professor of Anatomy, was engaged in the assembly of an apparatus for fluoresence microscopy. He had been my laboratory instructor in histology and now he enthusiastically put his resources at my service and indeed hastened the construction of the fluorescence microscope so that I could use it. This microscope was designed to be a powerful one. It had a 15-ampere direct current carbon arc light source and was mounted on a Zeiss photomicrographic optical bench.

end, the free hand. Poincaré (7) wrote that the way to solve a difficult problem was first to work hard at it; then, when solutions fail to appear, to drop it. Do something else, or take a vacation, and give the subconscious a chance. First, the hard stocking of the mind with the facts, and the struggle over them; then the latent period, and finally, the sudden insight into the solution.

O. N., Cancer Res., **7:** 297, 1947.
5. Coons, A. H., Creech, H. J. and Jones, R. N., Proc. Soc. Exper. Biol. & Med., **47:** 200, 1941.
6. Coons, A. H., Creech, H. J., Jones, R. N., and Berliner, E., J. Immunol., **45:** 159, 1942.
7. Poincaré,. Henri. *Mathematical Creation, The Foundations of Science*, translated by George Bruce Halsted, The Science Press, 1913.

"The Beginnings of Immunofluorescence"
Albert H. Coons, J. Immunol. 87: 499-503, 1961

3 pages of methods just to make a fluorescent conjugate….in 1950

1. Synthesis of Fluorescein Isocyanate

The synthesis was carried out by the methods of Bogert and Wright (19), and of Coons, Creech, Jones, and Berliner (1). The procedure to be described is an improvement of these in that the two isomers of nitrofluorescein theoretically possible from the fusion of 4-nitrophthalic acid and resorcinol have been separated and purified by fractional crystallization of their diacetates.

The course of the synthesis was as follows: 4-nitrophthalic acid was heated with two equivalents of resorcinol, with the production of nitrofluorescein. The crude product was refluxed with acetic anhydride, and the resulting nitrofluorescein diacetate subjected to fractional crystallization. Two isomeric diacetates were separated. Each was saponified and the pure nitrofluorescein isomer recovered. Catalytic hydrogenation produced the corresponding aminofluorescein, which was converted as needed to the isocyanate by treatment with phosgene. No attempt was made to isolate the isocyanates.

(*a*) *Preparation of Nitrofluorescein.*—100 gm. of 4-nitrophthalic acid[1] and 100 gm. of resorcinol[1] were intimately mixed in a beaker and heated on an oil bath at 195–200°C. until the mass was dry (12 hours). When cool, the melt was chipped from the beaker, ground in a mortar, and boiled with 1600 ml. 0.6 N HCl for 1 hour. After washing by decantation with three 300 ml. amounts of hot HCl solution, it was collected hot on a large Buchner funnel. The resulting brown paste was washed on the funnel with 3 liters of water, and dried in the oven at 110°C. Yield 176 gm. (98 per cent). *Crude nitrofluorescein.*

(*b*) *Preparation of Nitrofluorescein Diacetate and Separation of Isomers.*—100 gm. of this crude nitrofluorescein was refluxed with 400 gm. of acetic anhydride for 2 hours, and set aside to cool. Crystallization was induced by seeding from a small preliminary run. The whitish yellow crystals of diacetate were collected the next day on a Buchner funnel, and washed with two 10 ml. portions of acetic anhydride, and 20 ml. ethanol (excluded from the filtrate). Yield 21 gm. (17 per cent) (fraction 1).

The filtrate was concentrated by boiling to a volume of about 300 ml. and seeded as before. Yield 11.2 gm. (9.5 per cent) (fraction 2).

Fractions 1 and 2 were combined, dissolved with heat in about 110 ml. acetic anhydride, filtered hot, and set aside to crystallize. Yield 26 gm. (20 per cent). M.p. 213–219°C. After repeated recrystallizations from benzene and ethanol, m.p. 221–222.5°C.[2] Calculated for $C_4H_{19}O_9N$: C 62.47; H 3.28; N 3.04. Found: C 62.62; H 3.05; N 3.06.[3] *Nitrofluorescein diacetate I.*

The filtrate from fraction 2 was concentrated to a thick paste *in vacuo* on the boiling water bath. On cooling, the dark brown mass became a viscous gum. To it was added 90 ml. benzene; the solution warmed to about 60°C., and stirred. The gum slowly mixed with the benzene, and crystallization began at once. After several hours the yellow-white asbestos-like needles

[1] Eastman Kodak Company.
[2] All melting points are uncorrected.
[3] Micro analyses by Miss Shirley Katz.

were collected and washed quickly on the funnel with 30 ml. of cold benzene. Yield 33 gm. (26 per cent).

These needles were dissolved in 200 ml. benzene, filtered hot, and allowed to stand overnight. The resulting long white feathery needles were collected, washed quickly with about 40 ml. benzene, and dried in air. Yield 15 gm. (12 per cent). M.p. 189–190°C. After repeated recrystallization from benzene and ethanol, m.p. 215–216°C. Calculated for $C_4H_{19}O_9N$: C 62.47; H 3.28; N 3.04. Found: C 62.61; H 3.19; N 3.03. *Nitrofluorescein diacetate II.*

(*c*) *Recovery of Nitrofluorescein Isomers.*—5 gm. of diacetate I was added to 100 ml. hot filtered saturated alcoholic sodium hydroxide, warmed gently, and shaken for a few minutes. An immediate red color resulted. The solution was filtered and poured into 4 volumes of water, acidified with stirring with 2 ml. concentrated HCl, and allowed to stand for several hours. The nitrofluorescein isomer precipitated as a yellow powder which was separated with suction, washed with 500 ml. water, and dried. Yield 4.05 gm. (99 per cent). For analysis, a portion was crystallized from isopropanol. The orange crystals gradually darken on heating but do not melt up to 350°C. Calculated for $C_{20}H_{11}O_5N$: C 63.66; H 2.93; N 3.71. Found: C 63.47; H 3.04; N 3.59. *Nitrofluorescein I.*

To obtain the other nitrofluorescein isomer, 7 gm. of once recrystallized diacetate II was added to 100 ml. hot filtered saturated alcoholic NaOH with stirring. In this case, crystallization of the sodium salt began promptly, and after 2 hours 5 gm. of orange-red crystals were obtained (75 per cent). They were very soluble in water with a red color and faint green fluorescence. To obtain the nitrofluorescein itself, 3 gm. of these crystals was dissolved in 300 ml. water, and acidified with stirring by the addition of 2 ml. concentrated HCl. A prompt yellow precipitate formed which turned red on standing in its mother liquor overnight. Collected, washed, and dried. Yield: 2.1 gm. (74 per cent). A small portion was crystallized from isopropanol. It failed to melt up to 350°C. Calculated for $C_{20}H_{11}O_5N$: C 63.66; H 2.93; N 3.71. Found: C 63.41; H 3.16; N 3.43. *Nitrofluorescein II.*

(*d*) *Reduction of Nitro Compounds.*—2 gm. of nitrofluorescein I was suspended in 100 ml. absolute ethanol and shaken with about 1.5 gm. Raney nickel in an atmosphere of hydrogen at room temperature and pressure. The reaction began promptly and at the end of 90 minutes the theoretical amount of H_2 had been taken up. The nickel was removed by centrifugation, washed with 15 ml. of absolute alcohol, and the washings added to the main lot. The alcohol-amine solution was diluted with an equal amount (115 ml.) of water and allowed to stand. Colorless needles slowly formed which were separated by filtration after standing overnight. Yield: 580 mg. of colorless matted needles which turned red slowly in air and instantly in the presence of water. Kept in the desiccator they slowly darkened to yellow and then to brownish red. M.p. 215–220°C. (decomposed). (Addition of 50 ml. water to the mother liquor yielded another 510 mg.) Yield 61 per cent. *Amino-fluorescein I.* Micro analyses on two different samples of this compound gave low values for C, H, and N. Therefore, the hydrochloride was prepared for analysis. 400 mg. of this amine was dissolved with heat in 15 ml. 2 N HCl, filtered hot, and the dark red crystals collected and dried in the desiccator over H_2SO_4 and solid NaOH. Yield 330 mg. (81 per cent). This compound does not melt. Calculated for $C_{20}H_{12}O_5N{\cdot}HCl$: C 62.58; H 3.68; N 3.65; Cl 9.24. Found: C 62.71; H 3.66; N 3.61; Cl 9.26. *Amino-fluorescein HCl I.*

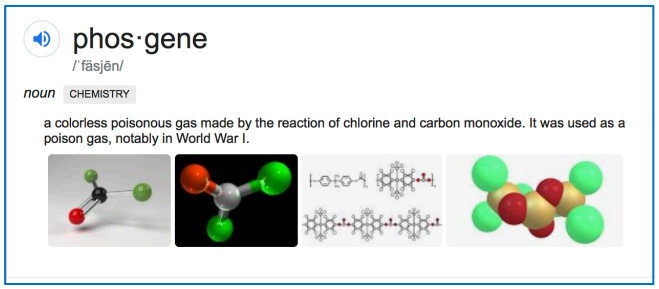
2 gm. of nitrofluorescein II was reduced and crystallized as above except that 4 volumes of water was added to the alcohol-amine solution. Canary yellow crystals which did not darken in air and which were stable on storage resulted. Yield: 1.12 gm. (67 per cent). M.p. 315–316°C. (decomposed) (put in bath at 285°). *Amino-fluorescein II.* Micro analyses on two different samples of this compound gave low values for C and N, a high value for H. For analysis, therefore, 60 mg. of amine II was dissolved in 1.5 ml. 2 N HCl with heat, filtered, redissolved by warming and the addition of a few drops of 2 N HCl, and allowed to stand. The red crystals were collected, dried in the desiccator with NaOH, and weighed. Yield:

50 mg. (82 per cent). This compound does not melt. Calculated for $C_{20}H_{14}O_5N{\cdot}HCl$: C 62.58; H 3.68; N 3.65; Cl 9.24. Found: C 62.66; H 3.85; N 3.67; Cl 9.56. *Amino-fluorescein HCl II.*

In common with fluorescein itself (20), each isomer of both nitro- and amino-fluorescein exists in a red and yellow form; the red form of amino-fluorescein II has been observed only on heating to about 150°C.

We have not attempted to determine which of the two possible positions in the molecule is occupied by the N atom in either of the two isomeric series described above.

(*e*) *Preparation of Fluorescein Isocyanate.*—The required amount of fluorescein amine (10 to 60 mg.) was added to 5 ml. of dry acetone,[4] and added dropwise from a dropping funnel to 15 ml. acetone saturated with phosgene, and through which phosgene was constantly bubbled.[5] As each drop of amine solution entered the reaction flask a yellow precipitate formed which rapidly dissolved. The solution in the flask became slightly warm. The reaction was allowed to continue for 30 minutes, by which time the flask had cooled again. The reaction flask was removed from the phosgene-train, three small anthracite chips added as an anti-bumping device, and the solution taken to dryness *in vacuo* over a water bath at 45°C. (10 to 15 minutes). This step served to remove the excess phosgene and acetone. The greenish brown gum was immediately dissolved in 2 volumes (1 to 2 ml.) of acetone and 1 volume of dioxane,[6] and this solution of fluorescein isocyanate added dropwise to the stirred chilled protein solution described below. Care must be taken to exclude water from the isocyanate solution until the moment of use, as it decomposes rapidly at room temperature in the presence of water.



15 minutes). This step served to remove the excess phosgene and acetone. The greenish brown gum was immediately dissolved in 2 volumes (1 to 2 ml.) of acetone and 1 volume of dioxane,[6] and this solution of fluorescein isocyanate added dropwise to the stirred chilled protein solution described below. Care must be taken to exclude water from the isocyanate solution until the moment of use, as it decomposes rapidly at room temperature in the presence of water.

"Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody" Coons, A. H. & Kaplan, M. H, J. Exp Med 91:1013, 1950

Melvin Kaplan reflects on origins of immunofluorescence

"Immunohistochemistry has gradually become useful in many areas of biology. Till now its weakness as a scientific method has been the difficulty of quantitating it.

"Recently however, computer activated light microscopes have made possible the rapid measurement of $I\mu^2$ areas of fluorescent cells. Such microscopes, attached to a computer, print out measurements of fluorescence intensity, allowing rapid comparison of the amount of antigen per unit area in various cells and parts of them. So far this ability is only beginning to be exploited.

CITATION CLASSIC - DISK-ELECTROPHORESIS OF BASIC-PROTEINS AND PEPTIDES ON POLYACRYLAMIDE GELS

CC/LIFE SCI, (6): 19-19 1981

Original Paper : Reisfeld R A, Lewis U J & Williams D E. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195:281-3, 1962.

A1981KZ58200002

Ref#4488

CC/NUMBER 6
FEBRUARY 9, 1

This Week's Citation Classic

Coons A H & Kaplan M H. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. *J Exp Med.* 91:1-13, 1950. [Dept. Bacteriology and Immunology, Harvard Med. Sch., Boston, MA]

A method employing the specificity of antibody labeled with fluorescein for the localization of antigen under the fluorescence microscope is presented. Included in the paper are a description of the synthesis of fluorescein isocyanate, the labeling material, and a method for removing over labeled proteins which bind indiscriminately to tissue elements and obscure specific reactions. [The *SCI*® indicates that this paper has been cited over 1,465 times since 1961.]

Albert H. Coons
Department of Pathology
Harvard Medical School
Boston, MA 02115

January 14, 1981
(revised)

"This paper described improvements in the method published earlier for the specific localization of foreign antigenic materials in tissue cells.[1] It was a general method tor the histological localization of any antigen because it utilized specific antibody labeled with fluorescein as a histochemical reagent. Diluted specific antibody solutions so labeled were flooded over tissue sections. Any antigen present bound the antibody and fixed it in place. Excess reagent could be washed away leaving the bound antibody in place and it in turn could be localized by bombardment by light of appropriate wave length and visualized under the fluorescence microscope. Naturally, the critical step was the binding of the fluorochrome-labeled antibody by the antigen and the ability to wash away any excess fluorescent reagent. This principle joined the specificity of the antibody molecule to the resolving power of the light microscope; such a union provided a general method now called immunohistochemistry for the investigation of native and foreign antigenic molecules in many locations and under many circumstances. Since then the same principle has been extended for use with the electron microscope by using antibody labeled with ferritin or with enzymes like horse radish peroxidase.

"Of course such reagents localizing and identifying antigens rapidly came to be used for the specific identification of various infectious agents: bacteria, rickettsiae, and viruses. It has also been applied to the study of autoimmune disease, e.g., nephritis, and in the detection of autoantibodies against tissue components. Immunofluorescence so-called therefore became a feature of the diagnostic, as well as the research, laboratory.

"Immunohistochemistry has gradually become useful in many areas of biology. Till now its weakness as a scientific method has been the difficulty of quantitating it.

"Recently however, computer activated light microscopes have made possible the rapid measurement of $I\mu^2$ areas of fluorescent cells. Such microscopes, attached to a computer, print out measurements of fluorescence intensity, allowing rapid comparison of the amount of antigen per unit area in various cells and parts of them. So far this ability is only beginning to be exploited.

"Surprisingly enough, and to the good fortune of anyone who wants to apply such a method, it has turned out that the antibody molecule is quite stable to many chemical manipulations and does not lose its specificity unless the label attaches itself close to the actual combining site.

"Addendum. Albert H. Coons died suddenly September 30, 1978. His commentary above is remarkably understated. The impact of this work on research in many biological disciplines, and, in particular, on studies of immunobiologic and pathologic processes is universally recognized. We remember him for the charm of his company, his penetrating wisdom, and his admonition: 'good research work stands on its own legs' " —Melvin H. Kaplan

1. Coons A H, Creech H J, Jones R N & Berliner G. The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. J. *Immunology* 45:159-70, 1942. [The *SCI*® indicates that this paper has been cited over 285 times since 1961.]

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PUCL cytometry laboratories

Oswald T. Avery

- **(1944) Oswald T. Avery (1887-1955)** - demonstrated that DNA was the carrier of genetic information

The Discovery of the "**Transforming Principle**"

Avery's key discovery was that the transforming substance, which produced permanent, heritable change in an organism (pneumococci), was deoxyribonucleic acid.

The phenomenon of transformation, Avery wrote, "*has been interpreted from a genetic point of view. The inducing substance has been likened to a gene, and the capsular antigen which is produced in response to it has been regarded as a gene product*." ….

"…*If the results of the present study on the chemical nature of the transforming principle are confirmed, then nucleic acids must be regarded as possessing biological specificity....*"
Journal of Experimental Medicine, 1944

https://doi.org/10.1074/jbc.R200002200

This article "Oswald T. Avery and the Nobel Prize in Medicine" is really worth reading

Gucker - 1947

- Developed a flow cytometer for detection of bacteria in aerosols
- Published paper in 1947 (work was done during WWII and was classified).
- Goal was rapid identification of airborne bacteria and spores used in biological warfare
- Instrument: Sheath of filtered air flowing through a dark-field flow illuminated chamber. Light source was a Ford headlamp, PMT detector (very early use of PMT)

Gucker's Apparatus

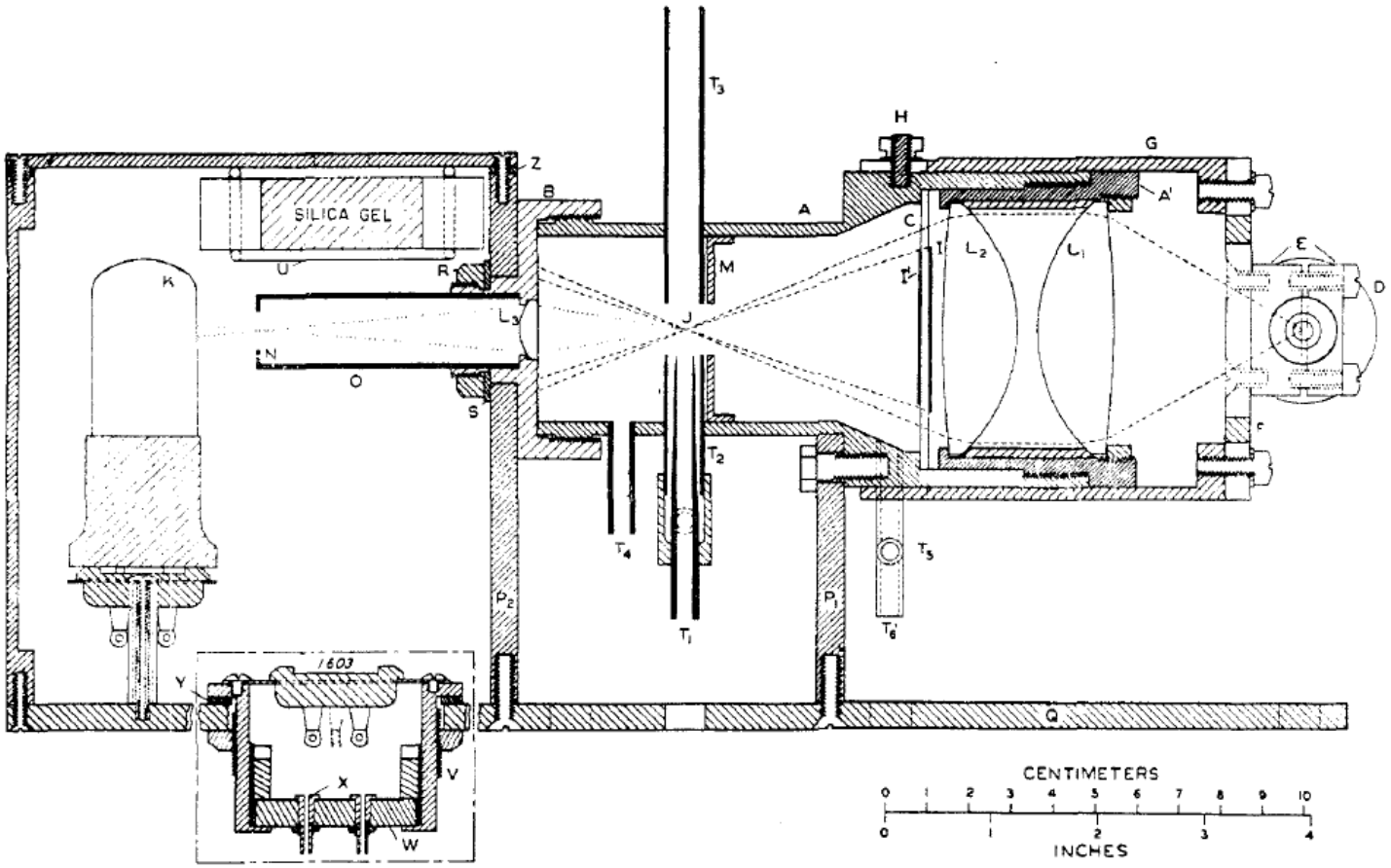


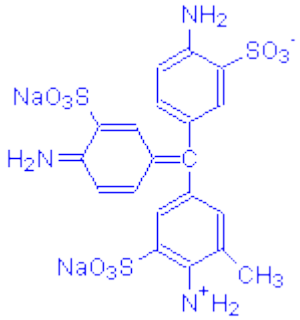
Fig. 1.—Smoke cell unit.

https://pubs.acs.org/doi/pdf/10.1021/cr60138a009

A Photoelectronic Counter for Colloidal Particles1
BY FRANK T. GUCKER, J R . , ~ CHESTER T. O'KONSKI,~ HUGH B. PICKARD3 AND JAMES N. PITTS, **JR.4**

H.P. Friedman

Friedman (1950) - combined acid fuchsin, acridine yellow and berberine for uterine cancer detection using fluorescence microscopy



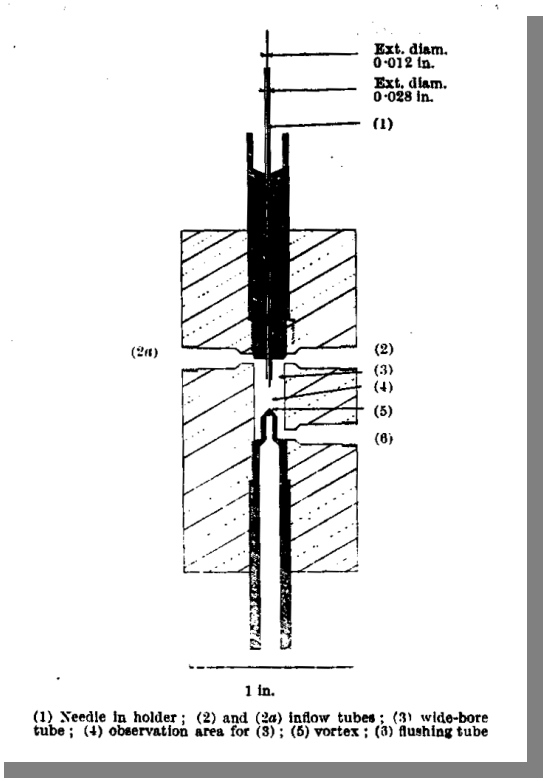
Acid Fuchsin
Other Names
Acid magenta
Acid rubin
Acid roseine

Absorption Max 540-545

Reference: Friedman HPJr: The use of ultraviolet light and fluorescent dyes in the detection of uterine cancer by vaginal smear. Am J Obst Gynec 59:852, 1950 (ref 4482)

P.J. Crosland-Taylor

Sheath Flow Principle – 1952-3



(1) Needle in holder; (2) and (2a) inflow tubes; (3) wide-bore tube; (4) observation area for (3); (5) vortex; (6) flushing tube

"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."

The whole apparatus is filled with gas-free distilled water which is allowed to flow via tubes 2 and 2*a* into the wide tube 4 and into the vortex 5. Tube 6 is normally closed. The suspension of cells is then passed into the needle (1). As the stream of cells emerges from the tapered tip of the needle it is narrowed by the faster peripheral stream in 3, and as the vortex is approached the stream of cells narrows further as the velocity increases.

A Device for Counting Small Particles Suspended in a Fluid through a Tube P.J. Crosland-Taylor
Bland-Sutton Institute of Pathology Middlesex Hospital, London, W.1. June 17, 1952 Nature 171: 37-38, 1953

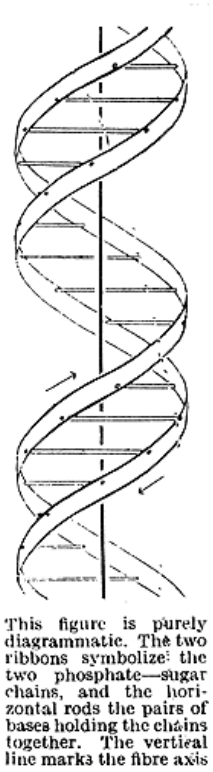
Watson & Crick-1953

The work which began with Avery's identification of DNA as the "transforming principle" thus led to research that overturned the old conception of DNA as a repetitive and simple molecule, confirmed DNA's role in genetic transmission, and, with James Watson and Francis Crick's 1953 paper, elucidated its structure.

J. D. WATSON and F. H. C. CRICK
Molecular Structure of Nucleic Acids
Nature, 25 April 1953, VOL 171,737-738 1953

This is a picture of part of the original model built by Watson and Crick at Cambridge in 1953.

One wonders what they would have done if they had 3D software like we have today…..



This figure is purely diagrammatic. The two ribbons symbolize the two phosphate—sugar chains, and the horizontal rods the pairs of bases holding the chains together. The vertical line marks the fibre axis

Molecular Structure of Nucleic Acids

equipment, and to Dr. G. E. R. Deacon and the captain and officers of R.R.S. *Discovery II* for their part in making the observations.

[1] Young, F. B., Gerrard, H., and Jevons, W., *Phil. Mag.*, **40**, 149 (1920).
[2] Longuet-Higgins, M. S., *Mon. Not. Roy. Astro. Soc., Geophys. Supp.*, **5**, 285 (1949).
[3] Von Arx, W. S., Woods Hole Papers in Phys. Oceanog. Meteor., **11** (3) (1950).
[4] Ekman, V. W., *Arkiv. Mat. Astron. Fysik.* (*Stockholm*), **2** (11) (1905).

MOLECULAR STRUCTURE OF NUCLEIC ACIDS

A Structure for Deoxyribose Nucleic Acid

WE wish to suggest a structure for the salt of deoxyribose nucleic acid (D.N.A.). This structure has novel features which are of considerable biological interest.

A structure for nucleic acid has already been proposed by Pauling and Corey[1]. They kindly made their manuscript available to us in advance of publication. Their model consists of three intertwined chains, with the phosphates near the fibre axis, and the bases on the outside. In our opinion, this structure is unsatisfactory for two reasons: (1) We believe that the material which gives the X-ray diagrams is the salt, not the free acid. Without the acidic hydrogen atoms it is not clear what forces would hold the structure together, especially as the negatively charged phosphates near the axis will repel each other. (2) Some of the van der Waals distances appear to be too small.

Another three-chain structure has also been suggested by Fraser (in the press). In his model the phosphates are on the outside and the bases on the inside, linked together by hydrogen bonds. This structure as described is rather ill-defined, and for this reason we shall not comment on it.

This figure is purely diagrammatic. The two ribbons symbolize the two phosphate–sugar chains, and the horizontal rods the pairs of bases holding the chains together. The vertical line marks the fibre axis

We wish to put forward a radically different structure for the salt of deoxyribose nucleic acid. This structure has two helical chains each coiled round the same axis (see diagram). We have made the usual chemical assumptions, namely, that each chain consists of phosphate diester groups joining β-D-deoxyribofuranose residues with 3′,5′ linkages. The two chains (but not their bases) are related by a dyad perpendicular to the fibre axis. Both chains follow right-handed helices, but owing to the dyad the sequences of the atoms in the two chains run in opposite directions. Each chain loosely resembles Furberg's[2] model No. 1; that is, the bases are on the inside of the helix and the phosphates on the outside. The configuration of the sugar and the atoms near it is close to Furberg's 'standard configuration', the sugar being roughly perpendicular to the attached base. There is a residue on each chain every 3·4 A. in the *z*-direction. We have assumed an angle of 36° between adjacent residues in the same chain, so that the structure repeats after 10 residues on each chain, that is, after 34 A. The distance of a phosphorus atom from the fibre axis is 10 A. As the phosphates are on the outside, cations have easy access to them.

The structure is an open one, and its water content is rather high. At lower water contents we would expect the bases to tilt so that the structure could become more compact.

The novel feature of the structure is the manner in which the two chains are held together by the purine and pyrimidine bases. The planes of the bases are perpendicular to the fibre axis. They are joined together in pairs, a single base from one chain being hydrogen-bonded to a single base from the other chain, so that the two lie side by side with identical *z*-co-ordinates. One of the pair must be a purine and the other a pyrimidine for bonding to occur. The hydrogen bonds are made as follows: purine position 1 to pyrimidine position 1; purine position 6 to pyrimidine position 6.

If it is assumed that the bases only occur in the structure in the most plausible tautomeric forms (that is, with the keto rather than the enol configurations) it is found that only specific pairs of bases can bond together. These pairs are: adenine (purine) with thymine (pyrimidine), and guanine (purine) with cytosine (pyrimidine).

In other words, if an adenine forms one member of a pair, on either chain, then on these assumptions the other member must be thymine; similarly for guanine and cytosine. The sequence of bases on a single chain does not appear to be restricted in any way. However, if only specific pairs of bases can be formed, it follows that if the sequence of bases on one chain is given, then the sequence on the other chain is automatically determined.

It has been found experimentally[3,4] that the ratio of the amounts of adenine to thymine, and the ratio of guanine to cytosine, are always very close to unity for deoxyribose nucleic acid.

It is probably impossible to build this structure with a ribose sugar in place of the deoxyribose, as the extra oxygen atom would make too close a van der Waals contact.

The previously published X-ray data[5,6] on deoxyribose nucleic acid are insufficient for a rigorous test of our structure. So far as we can tell, it is roughly compatible with the experimental data, but it must be regarded as unproved until it has been checked against more exact results. Some of these are given in the following communications. We were not aware of the details of the results presented there when we devised our structure, which rests mainly though not entirely on published experimental data and stereochemical arguments.

It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.

Full details of the structure, including the conditions assumed in building it, together with a set of co-ordinates for the atoms, will be published elsewhere.

We are much indebted to Dr. Jerry Donohue for constant advice and criticism, especially on interatomic distances. We have also been stimulated by a knowledge of the general nature of the unpublished experimental results and ideas of Dr. M. H. F. Wilkins, Dr. R. E. Franklin and their co-workers at King's College, London. One of us (J. D. W.) has been aided by a fellowship from the National Foundation for Infantile Paralysis.

J. D. WATSON
F. H. C. CRICK

Medical Research Council Unit for the Study of the Molecular Structure of Biological Systems,
Cavendish Laboratory, Cambridge.
April 2.

[1] Pauling, L., and Corey, R. B., *Nature*, **171**, 346 (1953); *Proc. U.S. Nat. Acad. Sci.*, **39**, 84 (1953).
[2] Furberg, S., *Acta Chem. Scand.*, **6**, 634 (1952).
[3] Chargaff, E., for references see Zamenhof, S., Brawerman, G., and Chargaff, E., *Biochim. et Biophys. Acta*, **9**, 402 (1952).
[4] Wyatt, G. R., *J. Gen. Physiol.*, **36**, 201 (1952).
[5] Astbury, W. T., Symp. Soc. Exp. Biol. 1, Nucleic Acid, 66 (Camb. Univ. Press, 1947).
[6] Wilkins, M. H. F., and Randall, J. T., *Biochim. et Biophys. Acta*, **10**, 192 (1953).

Molecular Structure of Deoxypentose Nucleic Acids

WHILE the biological properties of deoxypentose nucleic acid suggest a molecular structure containing great complexity, X-ray diffraction studies described here (cf. Astbury[1]) show the basic molecular configuration has great simplicity. The purpose of this communication is to describe, in a preliminary way, some of the experimental evidence for the polynucleotide chain configuration being helical, and existing in this form when in the natural state. A fuller account of the work will be published shortly.

The structure of deoxypentose nucleic acid is the same in all species (although the nitrogen base ratios alter considerably) in nucleoprotein, extracted or in cells, and in purified nucleate. The same linear group of polynucleotide chains may pack together parallel in different ways to give crystalline[1–3], semi-crystalline or paracrystalline material. In all cases the X-ray diffraction photograph consists of two regions, one determined largely by the regular spacing of nucleotides along the chain, and the other by the longer spacings of the chain configuration. The sequence of different nitrogen bases along the chain is not made visible.

Oriented paracrystalline deoxypentose nucleic acid ('structure *B*' in the following communication by Franklin and Gosling) gives a fibre diagram as shown in Fig. 1 (cf. ref. 4). Astbury suggested that the strong 3·4-A. reflexion corresponded to the inter-nucleotide repeat along the fibre axis. The ~ 34 A. layer lines, however, are not due to a repeat of a polynucleotide composition, but to the chain configuration repeat, which causes strong diffraction as the nucleotide chains have higher density than the interstitial water. The absence of reflexions on or near the meridian immediately suggests a helical structure with axis parallel to fibre length.

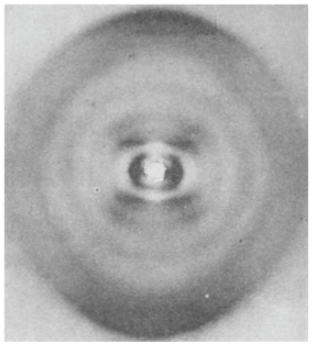


Fig. 1. Fibre diagram of deoxypentose nucleic acid from *B. coli*. Fibre axis vertical

Diffraction by Helices

It may be shown[5] (also Stokes, unpublished) that the intensity distribution in the diffraction pattern of a series of points equally spaced along a helix is given by the squares of Bessel functions. A uniform continuous helix gives a series of layer lines of spacing corresponding to the helix pitch, the intensity distribution along the *n*th layer line being proportional to the square of J_n, the *n*th order Bessel function. A straight line may be drawn approximately through the innermost maxima of each Bessel function and the origin. The angle this line makes with the equator is roughly equal to the angle between an element of the helix and the helix axis. If a unit repeats *n* times along the helix there will be a meridional reflexion (J_n^2) on the *n*th layer line. The helical configuration produces side-bands on this fundamental frequency, the effect[5] being to reproduce the intensity distribution about the origin around the new origin, on the *n*th layer line, corresponding to *C* in Fig. 2.

We will now briefly analyse in physical terms some of the effects of the shape and size of the repeat unit or nucleotide on the diffraction pattern. First, if the nucleotide consists of a unit having circular symmetry about an axis parallel to the helix axis, the whole diffraction pattern is modified by the form factor of the nucleotide. Second, if the nucleotide consists of a series of points on a radius at right-angles to the helix axis, the phases of radiation scattered by the helices of different diameter passing through each point are the same. Summation of the corresponding Bessel functions gives reinforcement for the inner-

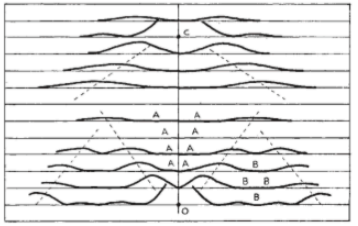
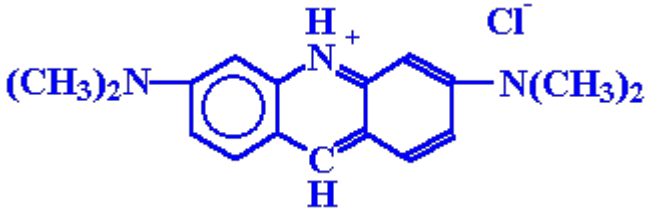


Fig. 2. Diffraction pattern of system of helices corresponding to structure of deoxypentose nucleic acid. The squares of Bessel functions are plotted about 0 on the equator and on the first, second, third and fifth layer lines for half of the nucleotide mass at 20 A. diameter and remainder distributed along a radius, the mass at a given radius being proportional to the radius. About *C* on the tenth layer line similar functions are plotted for an outer diameter of 12 A.

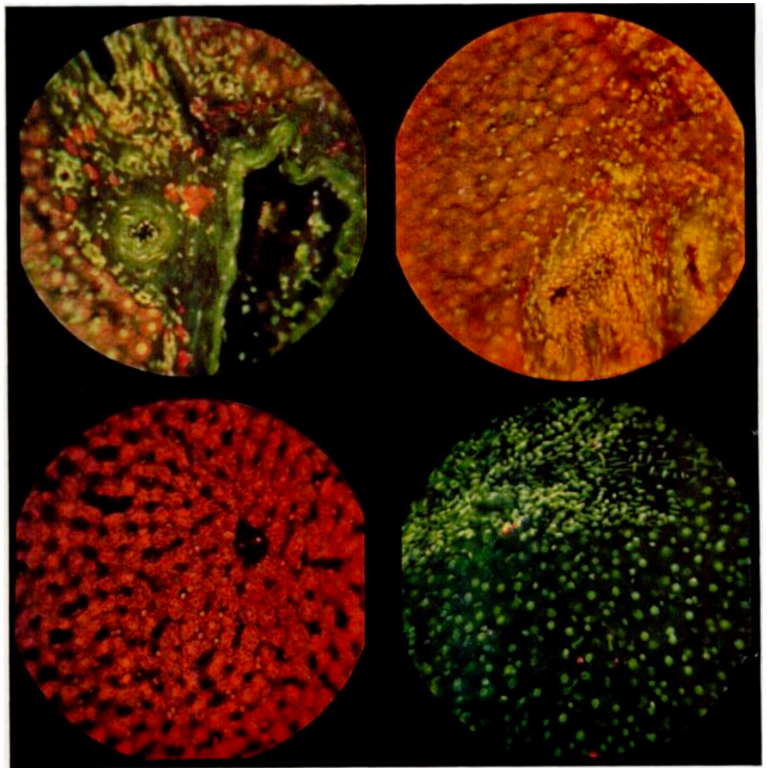
von Bertalanffy & Bickis - 1956

Ludwig von Bertalanffy (1901-1972)

von Bertalanffy & Bickis (1956)
The metachromatic fluorescence of AO was used to identify and quantitate RNA in tissues and that also that normal and malignant cells could be discriminated



AO - Absorption Max 467 nm



"Identification of cytoplasmic basophilia (ribonucleic acid) by fluorescence microscopy" von Bertalanffy, L. Bickis, I.; Journal of Histochemistry and Cytochemistry 4: 481-493, 1956

FIG. 1. Interlobular connective tissue with blood vessels and mast cells, hepatic cells at the periphery. Frozen section, acetic alcohol 1:3, AO in Krebs-Ringer 1:10,000, pH 6 (15 min.), mounted in Krebs-Ringer pH 6, exposure to U.V. (10 min.) before taking picture. × 180.

FIG. 2. Hepatic cells, connective tissue with mast cells. Procedure as in 1, but without exposure to U.V.

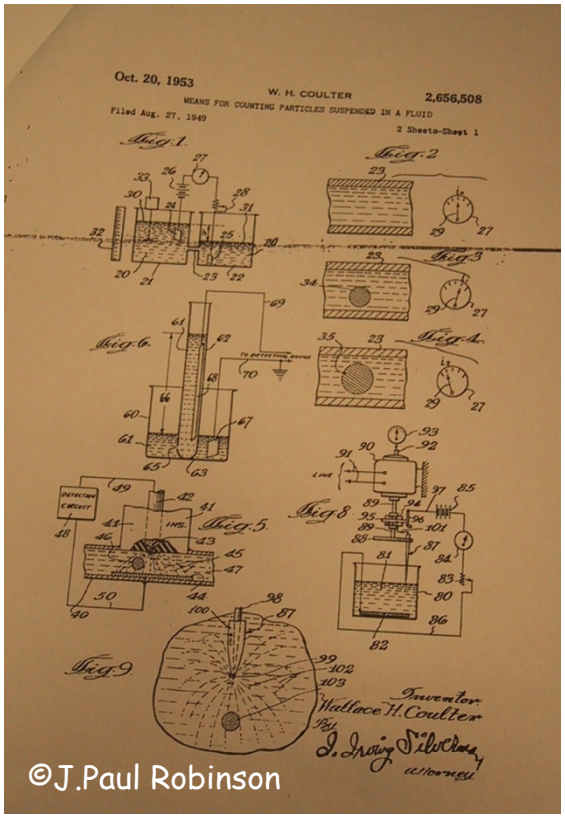
FIG. 3. Hepatic tissue. Red inclusions in cytoplasm. AO 1:5000 (10 min.), otherwise as in 1.

FIG. 4. Similar section, treated with RNase. Red fluorescence of cytoplasm and nucleoli has disappeared. Nuclei show darker green. One mast cell (upper half, left from center) visible, other red traces Kupffer cells. Acetic alcohol, dist. water (2 hrs.), AO 1:10,000 (15 min.), RNase 0.1%, 37°C. (2 hrs.), mounted in Krebs-Ringer.

Wallace Coulter

Wallace Coulter - Coulter orifice - 1956 -

(patent 1953) - measured changes in electrical conductance as cells suspended in saline passed through a small orifice



Coulter's Original 1953 Patent app'n From 1949

The first commercial version of the Coulter Counter

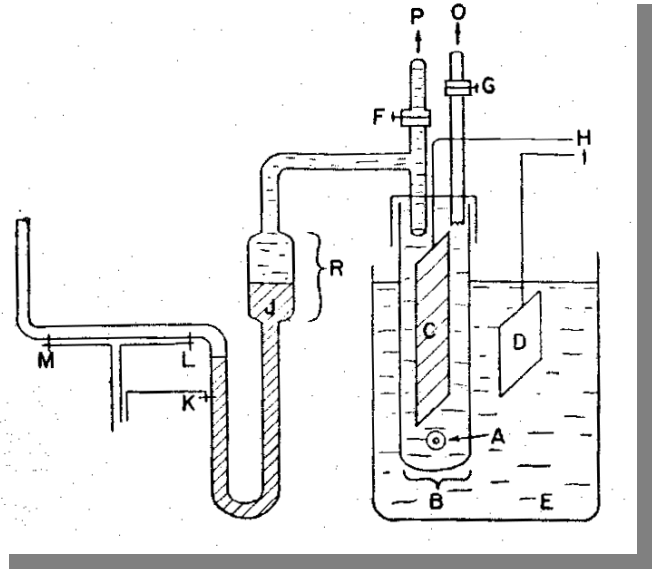
Cell Counting Theory

- The hemocytometer was the counting standard from about 1950 until the 1960's.
- the dimension of this device was 3x3x0.1 mm. Typically RBCs were counted using a 1:200 dilution from the 1 x $10^6/mm^3$ in whole blood.
- Leukocytes ($5x10^3/mm^3$) were diluted 1:10 in a lysing reagent and a dye to stain nuclei
- Statistical variation is calculated by the following:
  - -The standard deviation of a count on n items in n
  - -Considering no more than 500 cells could be possibly counted manually the standard deviation would therefore be 22
  - -the coefficient of Variation (CV) is 22/500 or 4.4%
  - -add pipetting and dilution errors and its about 10%

Early Cell Counter

Early cell counter. Katherine Williams and C.S. Sanders (Atomic Energy Research Establishment) 1948 - Unclassified in 1956. (Photo taken in Science Museum, London UK by JPR)

The first Coulter Counter



High Speed Automatic Blood Cell Counter and Cell Size Analyzer

Wallace H. Coulter

Coulter Electronics, Chicago, Illinois

:1034-1042, 1956

Preliminary draft of a talk presented before
The NATIONAL ELECTRONICS CONFERENCE
Chicago, October 3, 1956.

"HIGH SPEED AUTOMATIC BLOOD CELL COUNTER AND CELL SIZE ANALYZER"*

BY

WALLACE H. COULTER, COULTER ELECTRONICS,** CHICAGO, ILLINOIS

The instrument employs a non-optical scanning system providing a counting rate in excess of 6,000 individual cells per second with a counting interval of 15 seconds. A suspension of blood cells is passed thru a small orifice simultaneously with an electric current. The individual blood cells passing thru the orifice introduce an impedance change in the orifice determined by the size of the cell. The system counts the individual cells and provides cell size distribution. The number of cells counted per sample is approximately 100 times greater than the usual microscope count to reduce the satistical error by a factor of approximately 10 times.

Published in: *Proceedings of the National Electronics Conference, 1956.* 12:1034-1042, 1957 (Chicago: National Electronics Conference, Inc.)

*For red and white blood cells, and other microscopic particles. Special Models for counting and sizing of bacteria.

**Coulter Electronics, 2525 N. Sheffield Ave., Chicago 14, Illinois

From Coulter's only publication…

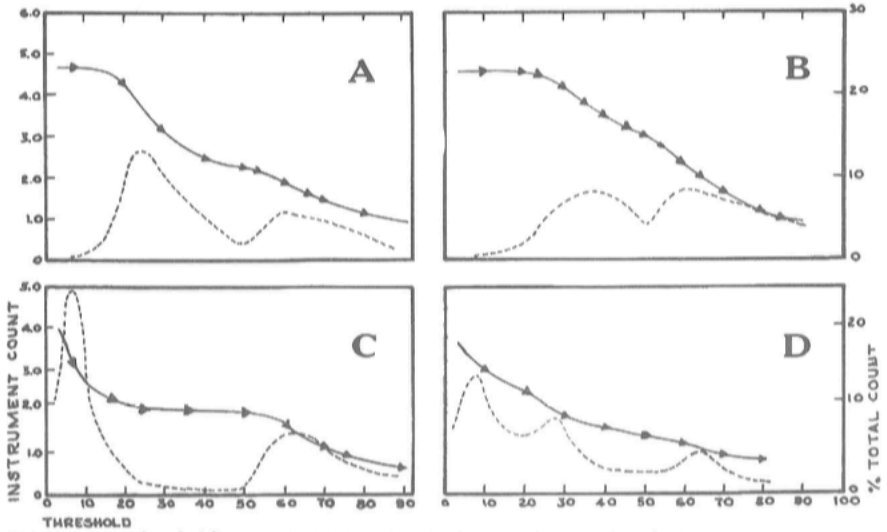


FIGURE 5. Threshold curves (solid lines) and relative cell size distribution curves (broken lines) of mixtures of different cell size population.
A) Sheep and normal human red cells.
B) Dba mouse and normal human red cells.
C) Goat and normal human red cells.
D) Goat, sheep and normal human red cells.

ACKNOWLEDGEMENT

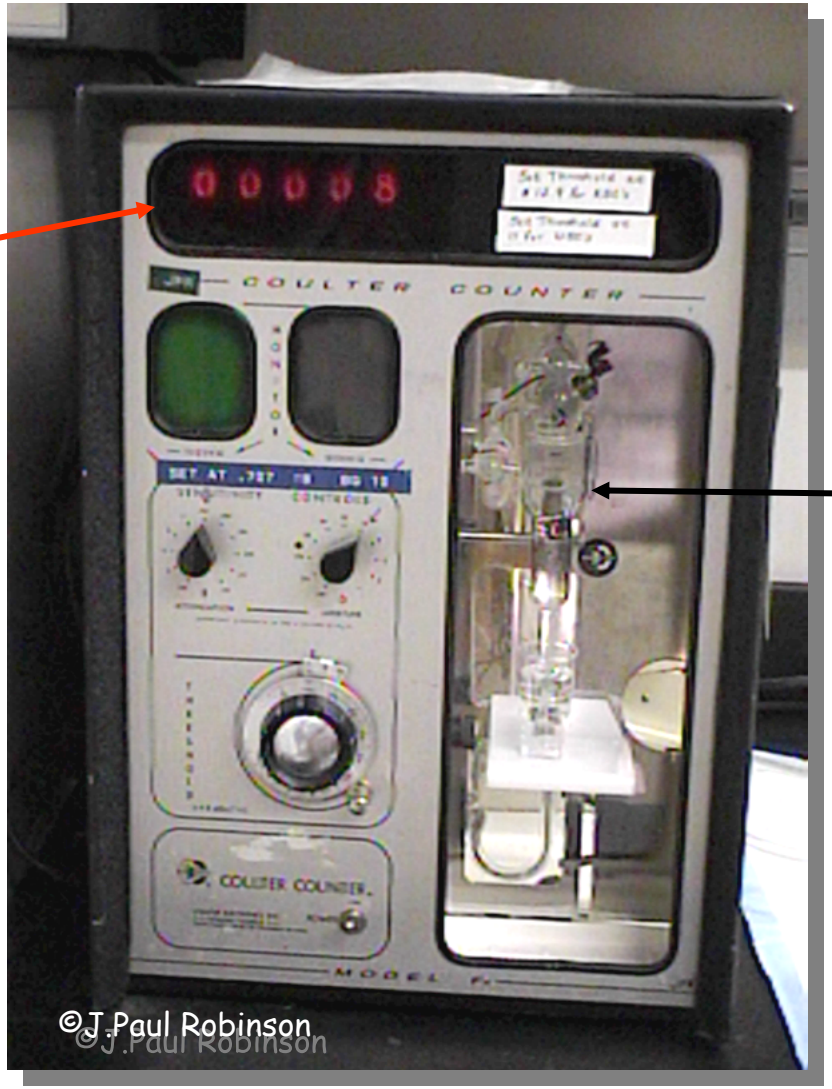
An experimental model of the counter was constructed under Office of Naval Research Contract NONR–1054(00).

Figures 1 and 5 are taken from a paper[1] by Dr. Carl F. T. Mattern, Dr. Frederick S. Brackett, and Dr. Byron J. Olson of the National Institutes Of Health, United States Public Health Service. Permission to use this material is gratefully acknowledged.

REPRINTED FROM *AMERICAN JOURNAL OF CLINICAL PATHOLOGY*
December 1956

Wallace Coulter - Coulter orifice - 1948-1956

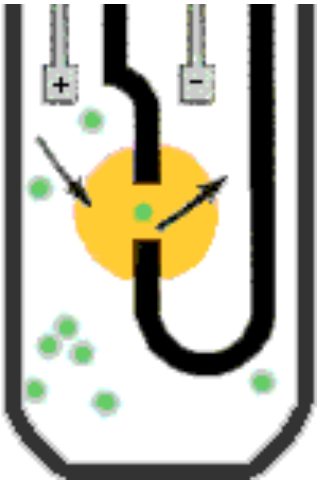
Cell counter



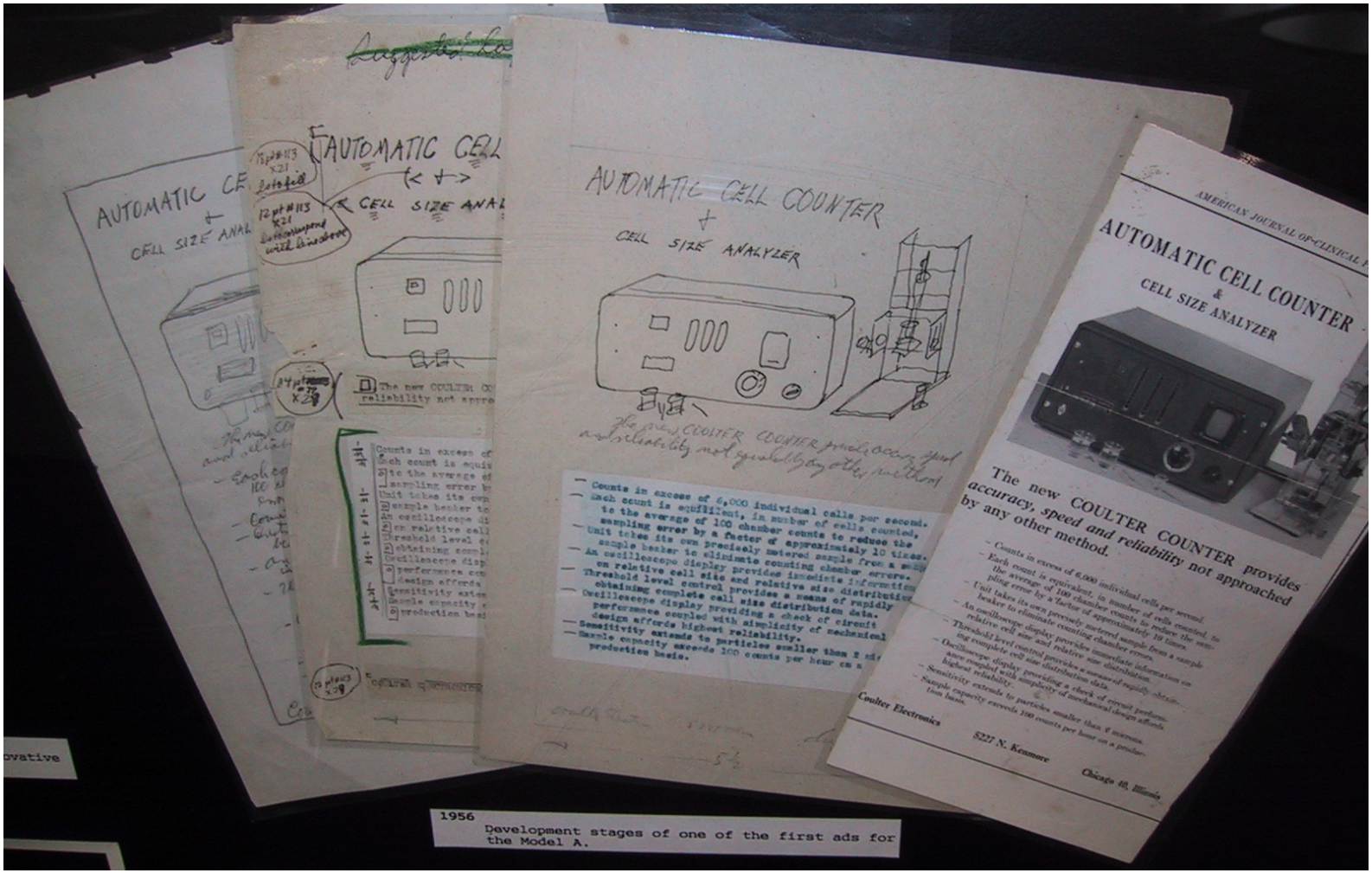
vacuum

The Coulter Model F released In 1963 – was the 4th iteration Of the Coulter Counter by Coulter Electronics, Inc.

orifice



Hand-drawn advertising drafts of the first Coulter Counter (1956)



1956
Development stages of one of the first ads for the Model A.

PUCL cytometry laboratories

History of Cellular Clinical Diagnostics

1941-43 Landmark monograph: Diagnosis of Uterine Cancer by the Vaginal Smear. Authored by George N. Papanicolaou (an anatomist) and Herbert F. Traut (a gynecologist).

Arch Pathol Lab Med. 1997 Mar;121(3):211-24.

The diagnostic value of vaginal smears in carcinoma of the uterus. 1941.

Papanicolaou GN, Traut HF.

PMID: 9111103

SUCCESS OF THE PAP SMEAR

1941 26,000 deaths per year in the United States due to cancer of the uterus as reported by Papanicolaou and Trout.

1996 4,900 estimated deaths per year in the United States due to cervical cancer with nearly a 2-fold increase in population in the intervening half century. At least half of these deaths are women who never had a Pap smear.

PAP TESTING IMPROVEMENTS

1951 Cytoanalyzer by Airborne instruments of Mineola, New York. Utilized World War II technology.1980s TICAS and CYBEST; Computer analysis and automated cytology projects. (These pioneering systems proved insufficient for general use).

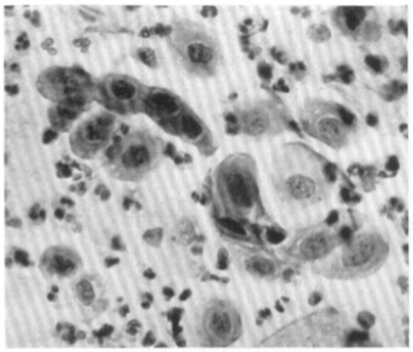


Fig. 2.—Group of normal basal cells. Menopause. ×800.

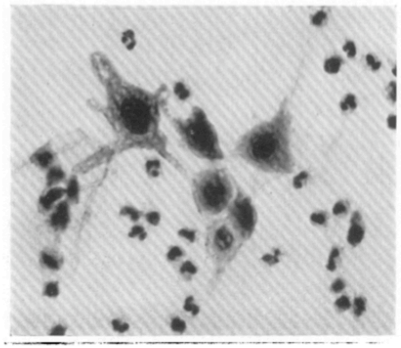


Fig. 6.—Group of aberrant basal cells. Carcinoma of cervix. ×800.

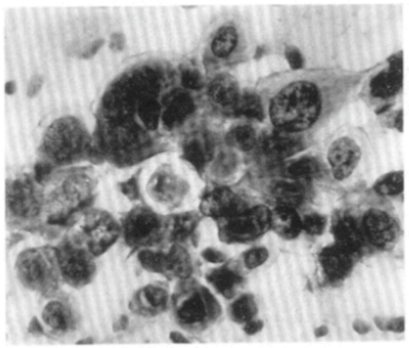


Fig. 3.—Group of abnormal cells. Carcinoma of cervix. ×800.

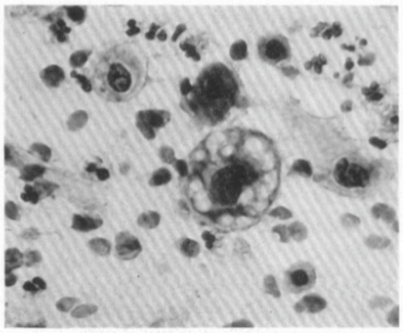


Fig. 7.—Abnormal vacuolated cell. Carcinoma of cervix. ×800.

Historical Overview

- **Marylou Ingram – 1950-60's** - identified that radiation caused increase number of binucleated lymphocytes in peripheral blood - she used a scanner to detect these rare cells (1/10000)

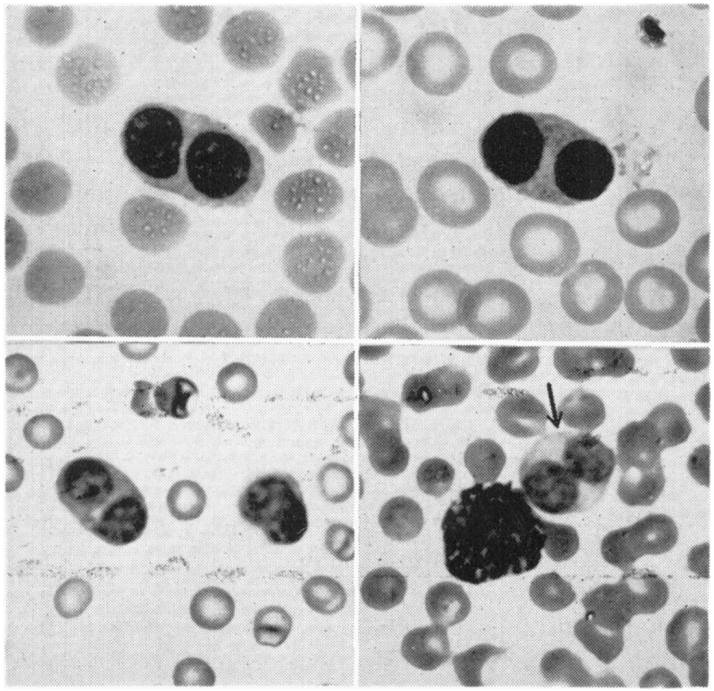


FIG. 1. Left to right: Lymphocyte with bilobed nucleus (peroxidase stain). Other cells are red blood cells. Lymphocyte with bilobed nucleus (Wright's stain. This cell contains several azurophile granules. One lymphocyte with bilobed nucleus, one normal lymphocyte (peroxidase). Normal granulocyte showing characteristic dark peroxidase positive granules and one lymphocyte with bilobed nucleus (arrow).

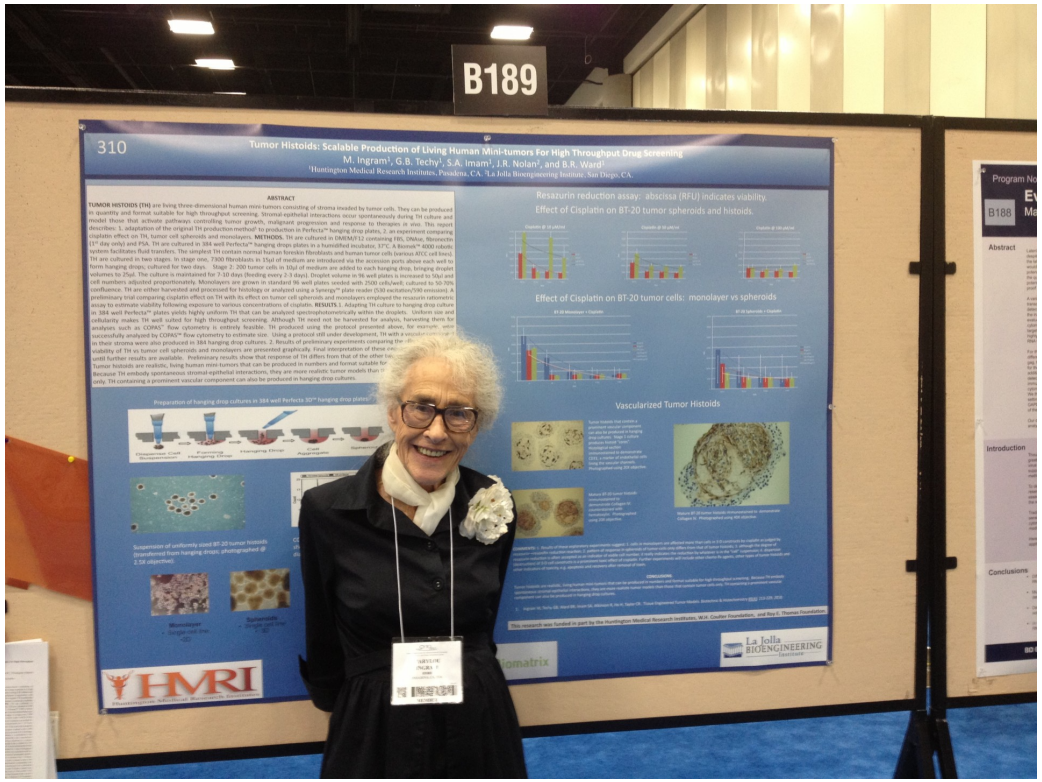
Science

Experimental Confirmation of a Previously Reported Unusual Finding in the Blood of Cyclotron Workers

M. Ingram and S. W. Barnes

Science **113** (2924), 32-34.
DOI: 10.1126/science.113.2924.32

Marylou Ingram



CYTO2010 Congress, Seattle, WA, 2010

Marylou Ingram with Brandon Price

Marylou Ingram and Automated Imaging

- **Marylou Ingram - 1960's** - identified that radiation caused increase number of binucleated lymphocytes in peripheral blood - she used a scanner to detect these rare cells (1/10000)

- **Preston 1964,** Cytoanalyzer was designed to identify Ingram's rare cells using a Vidicon based system - digitized images of lymphocytes were produced stained with eosin-methylene azure dye combinations.

A cell pattern recognition system with great intrinsic versatility, the CELL-SCAN system, has been developed by Perkin-Elmer Corporation, Norwalk, Conn., based on an original concept, the "shrink" technique, of Dr. M. Golay.[3-5] Its potentialities can be readily inferred from a general description of the system.

References: "*Preparation of Cytologic Material for Automatic Scanning Machines*" J.C.Pruitt, Ingraham, S.C.; Kaiser, R.F.; and Hilberg, A.W. – J. Nat. Cancer Inst. 22; 1105-1117, 1959
"*Field Trial of the Cytoanalyzer; 1186 Specimens Analyzed*" J.C.Pruitt, Courtney, W.B.; Hilberg, A.W.; Ingraham, S.C.; Kaiser, R.F. and Houser, M.H. J. Nat. Cancer Inst. 24; 1167-1179, 1960
"Importance of automatic pattern recognition techniques in the early detection of altered blood cell production"; Ingram, M. & Prestin, K.; Annals of the New York Academy of Sciences 113:1066-1072, 1964

Requirements of image analysis

Pattern recognition, feature extraction, parameters or descriptors, texture, color, staining properties

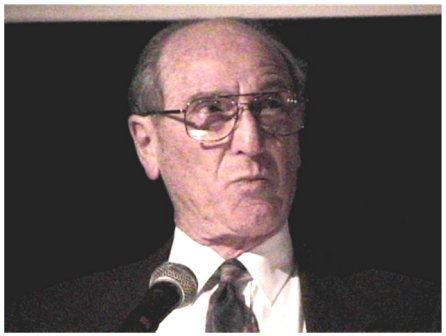
Question:
Why was it so difficult to do image analysis and image processing in the 1960's?

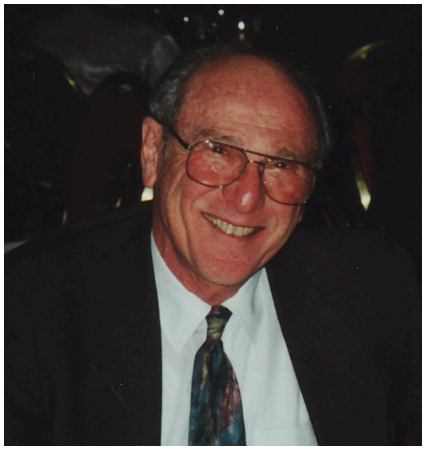
Answer: ?

(Hint: the driving force for flow cytometry was really the result of this issue)

Early Technology Developers

Mort Mendelsohn
(Died Dec 25, 2019)

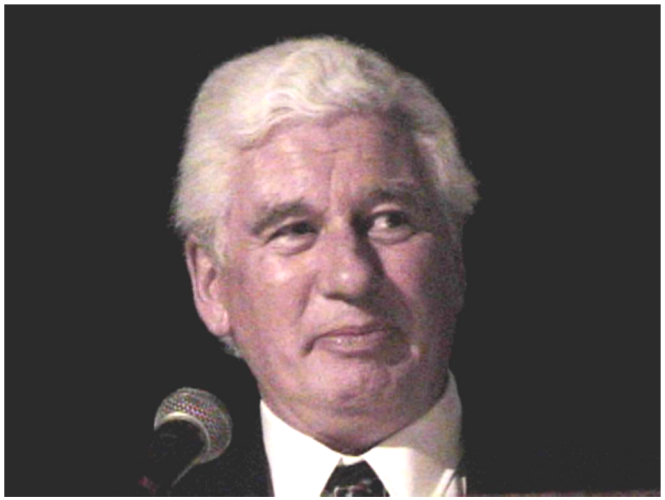




Photos taken in 1998 by JPR

CYDAC Analyzer - (1964) - Mendelsohn, Mayall, Prewitt (U.Pen)produced high resolution digital images of leukocytes - cells were stained with gallocyanin chrom alum and naphthol yellow S

Brian Mayall

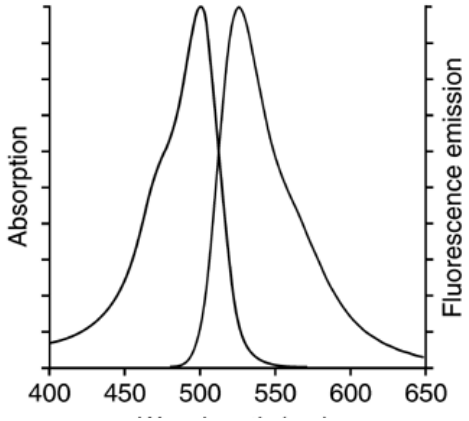


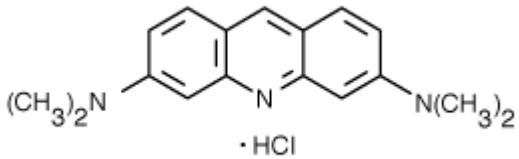
PUCL cytometry laboratories

Historical Overview

Early flow systems

Hallermann et al, Kosenow - 1964 - AO staining of leukocytes - was able to use fluorescence (in a flow-based system) to select leukocytes from red cells despite a low ratio (1/1000) because they took up lots of AO - also claimed to be able to discriminate between monocytes and PMN





Molecular Formula: $C_{17}H_{20}ClN_3$
Molecular Weight: 301.82
CAS Number/Name: 65-61-2 / 3,6-Acridinediamine, N,N,N',N'-tetramethyl-, monohydrochloride

Preview

Elektronische Differentialzählung von Granulocyten und Lymphocyten nach intravitaler Fluochromierung mit Acridinorange

Von

L. Hallermann, R. Thom und H. Gerhartz (Berlin)

Mit 1 Textabbildung

Automatisation und technische Perfektion haben auch der Routinediagnostik des klinischen Laboratoriums neue Wege eröffnet. So verwenden wir seit einigen Jahren elektronische Blutkörperchenzählgeräte; sie arbeiten entweder — wie der Coulter Counter und das Celloscop — durch Erfassung des elektrischen Widerstandes der einzelnen, eine Düse durchströmenden Zelle, wodurch sie neben der numerischen Zählung auch eine Volumenanalyse gestatten, oder optisch, indem sie die Lichtimpulse von Blutzellen, die das Dunkelfeld eines Mikroskopes in einer Reihe passieren, mittels Sekundärelektronenvervielfachern qualitativ und quantitativ messen. Da diese Vorgänge ohne mechanische Überträger nahezu trägheitslos registriert werden, lassen sich Erythrocytenzählungen schneller und genauer durchführen, als dies in einer Hämocytometerkammer möglich ist.

Vergegenwärtigt man sich, daß im Normalblut weiße und rote Blutkörperchen im Verhältnis von etwa 1:800 vorliegen, so wird nicht nur die Exaktheit der Erythrocytenzählung, sondern zugleich auch die Problematik der Leukocytenzählung verständlich, insbesondere, da wir noch kein Medium kennen, das Erythrocyten ohne Alteration der Leukocyten restlos aufzulösen ermöglicht. Diese Schwierigkeit der Trennung läßt sich nach unserer Erfahrung bei Benutzung des EEL-Zählautomaten durch die Anwendung von Vitalfarbstoffen umgehen. Einige davon erzeugen im ultravioletten Licht in kernhaltigen Zellen eine intensive Fluorescenz, während in Erythrocyten keine Anfärbung erfolgt.

B. Schlegel (ed.), *Verhandlungen der Deutschen Gesellschaft für Innere Medizin*

Scan and structure from www.probes.com

Kamentsky

He also built a fluidic cell-sorter to evaluate the cells identified in his RCS. An RCS was sent to Stanford for use by Leonard Herzenberg . The unit was also the model for the Technicon D instrument built by Technicon. (*Reference: Shapiro*)

Kamensky's first benchtop instrument the Cytograph (1970). This measured scatter using a He-Ne laser. This particular instrument was a model prior to the fluorescence detection model.

Richard Sweet

Richard Sweet developed the electrostatic ink-jet printer which was the principle used by Mack Fulwyler to create a cell-sorter.

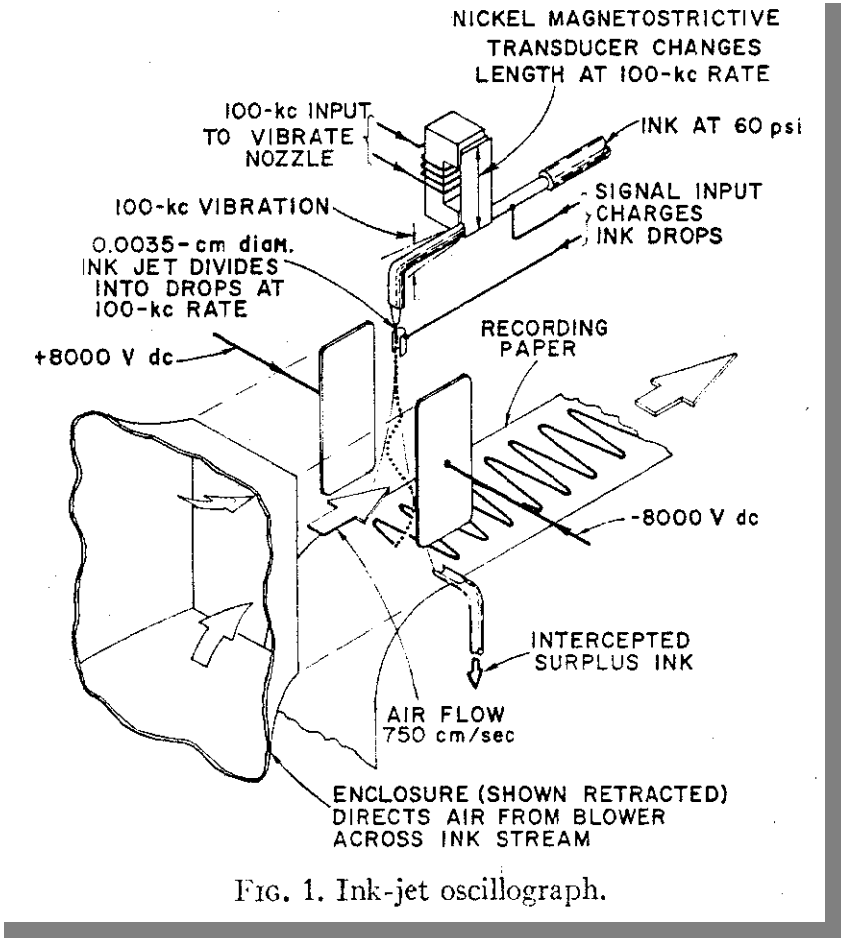


FIG. 1. Ink-jet oscillograph.

THE REVIEW OF SCIENTIFIC INSTRUMENTS

VOLUME 36, NUMBER 2 FEBRUARY 1965

High Frequency Recording with Electrostatically Deflected Ink Jets*

RICHARD G. SWEET

Systems Techniques Laboratory, Stanford Electronics Laboratories, Stanford University, Stanford, California

(Received 28 September 1964)

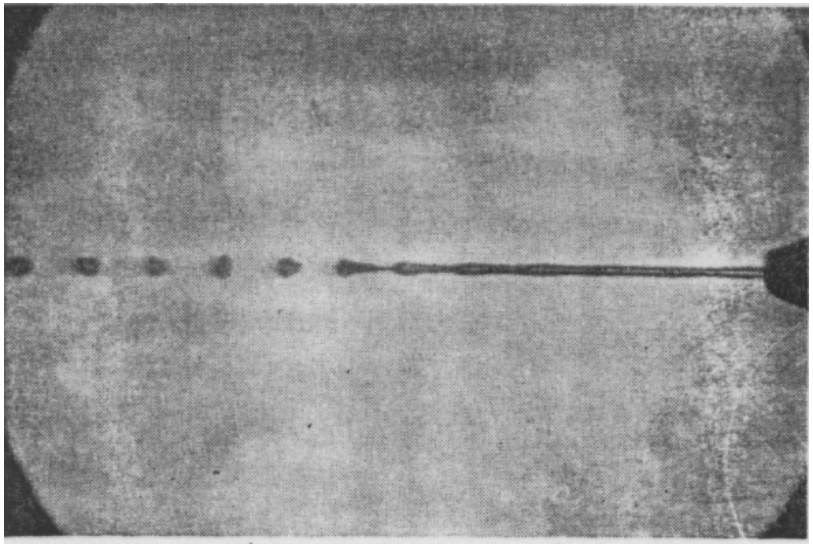
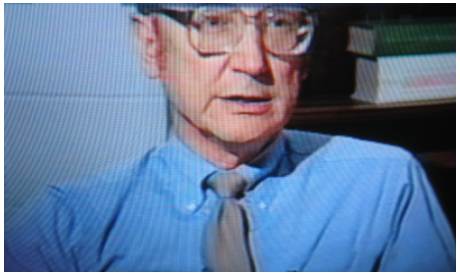


FIG. 3. Ink-drop formation.

Mack Fulwyler

Mack Fulwyler – worked in Marvin van Dilla's lab at Los Alamos. – developed the sorter in 1965 – initially used electronic cell volume - at Los Alamos National Labs - this instrument separated cells based on electronic cell volume (same principle as the Coulter counter) and used electrostatic deflection to sort. The cells sorted were RBC because they observed a bimodal distribution of cell volume when counting cells - the sorting principle was based on that developed for the inkjet printer by Richard Sweet at Stanford in 1965.



Mack Fulwyler
(Photo dated 6/91)

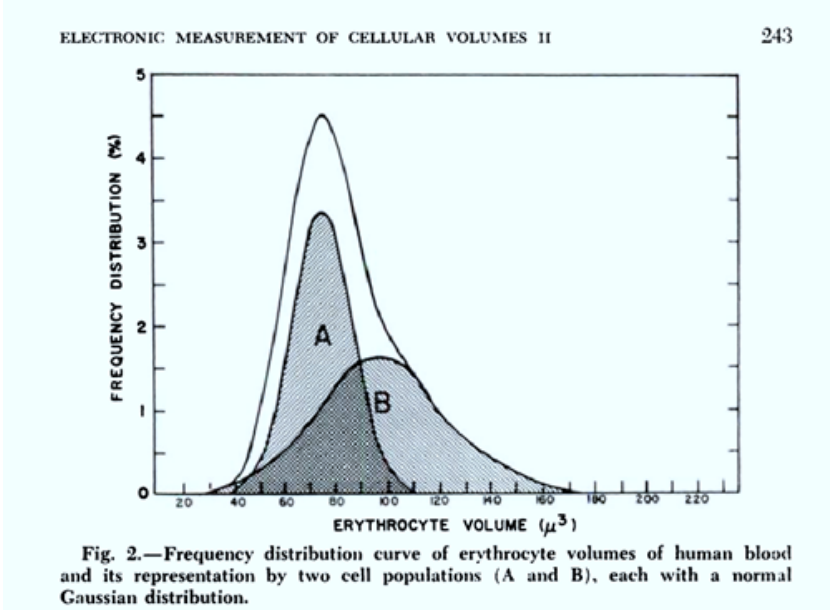
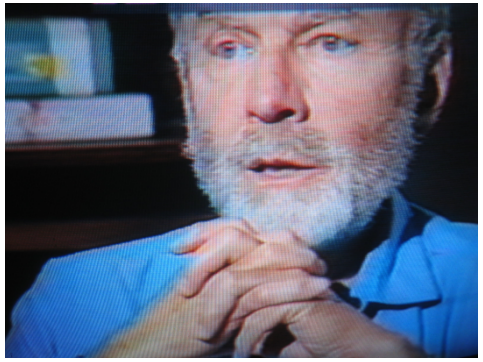


Fig. 2.—Frequency distribution curve of erythrocyte volumes of human blood and its representation by two cell populations (A and B), each with a normal Gaussian distribution.

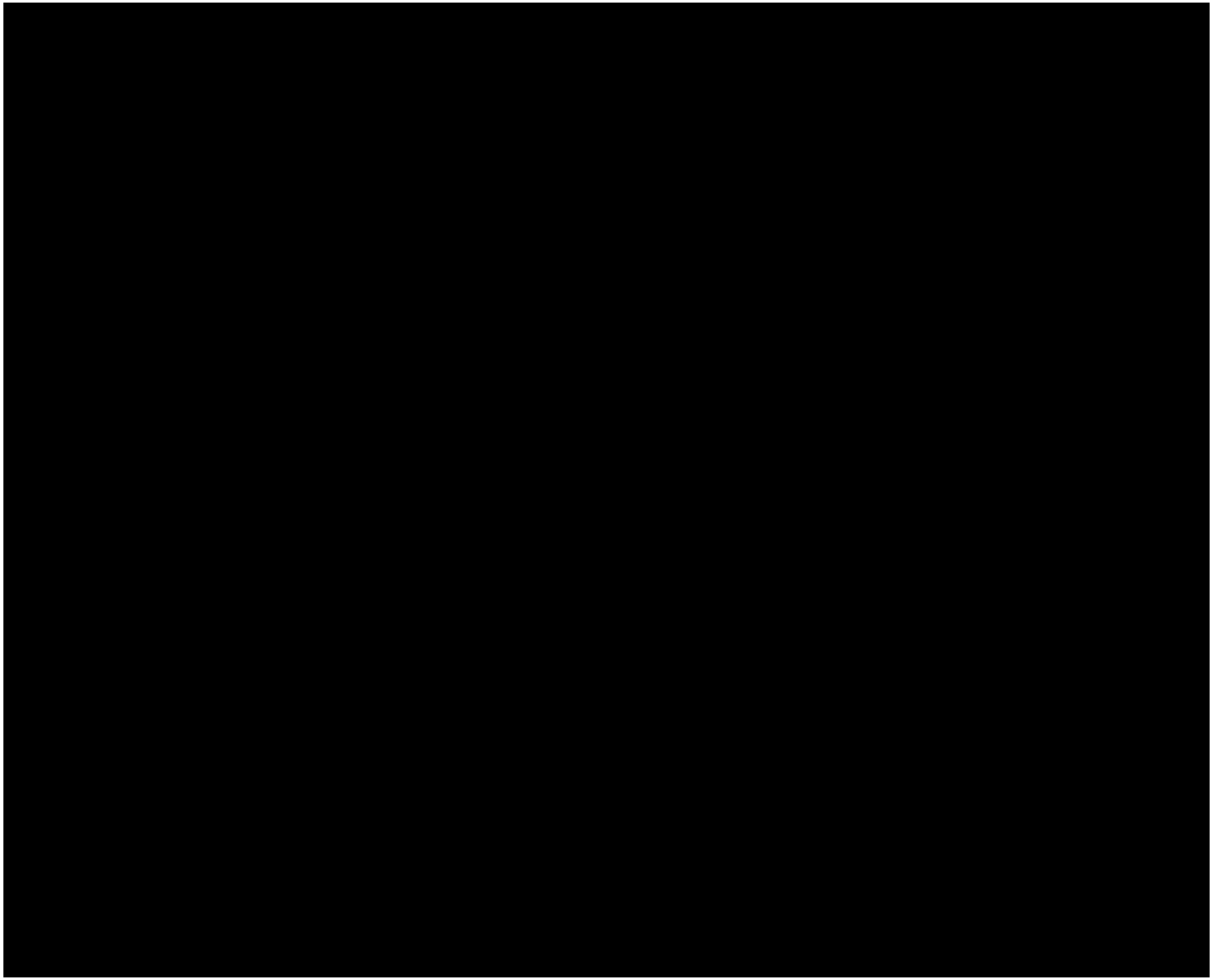


Marvin van Dilla
(Photo dated 6/91)

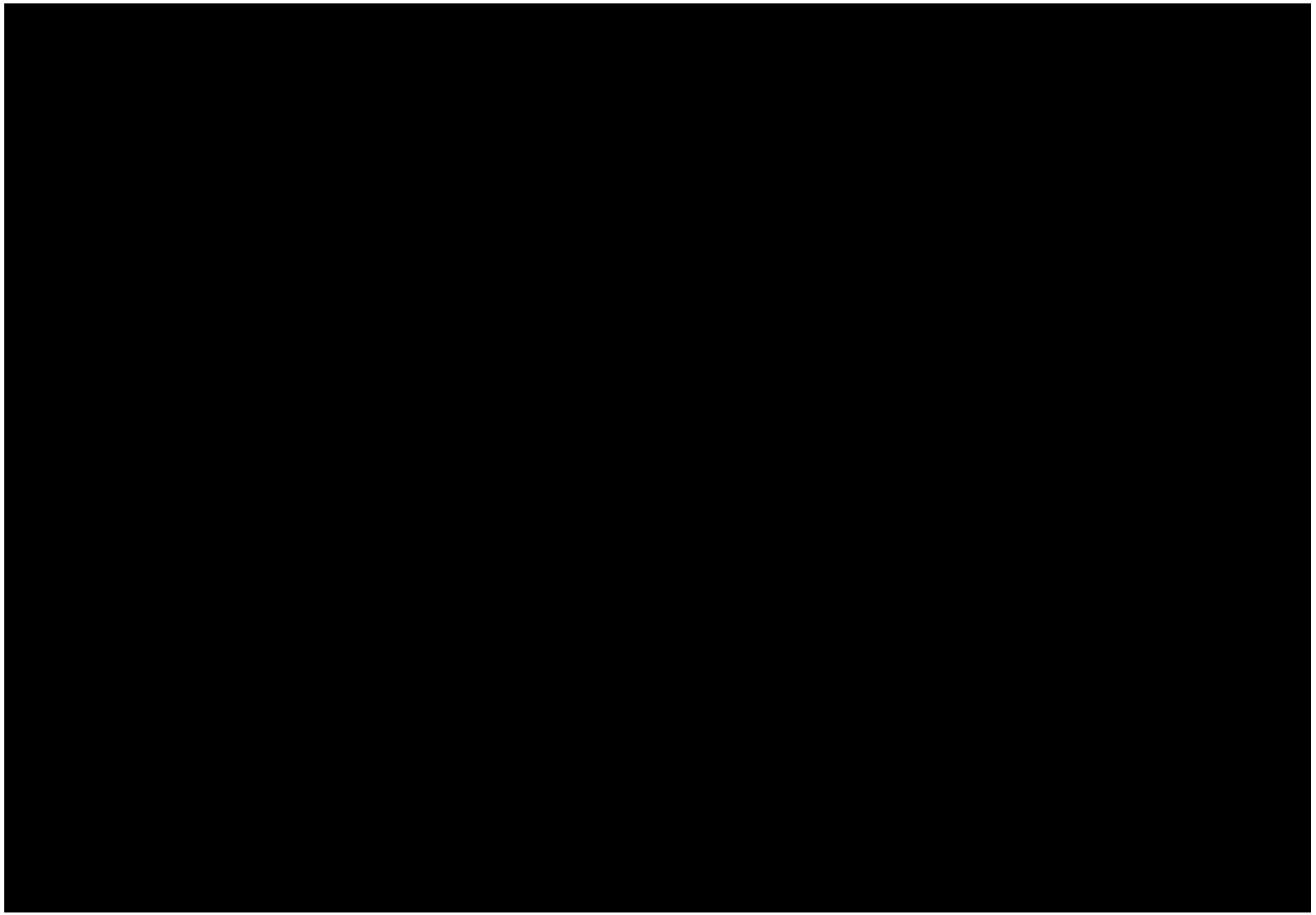
After determining that the bimodal distribution was artifactual, this group were able to sort neutrophils and lymphocytes from blood.

Lushbaugh, CC, Basmann,NJ, Glascock, B. Electronic measurement of cellular volumes. II Frequency distribution of erythrocyte volume. Blood 1962 20:241-248 Correspondence Blood 1964:23:403-405

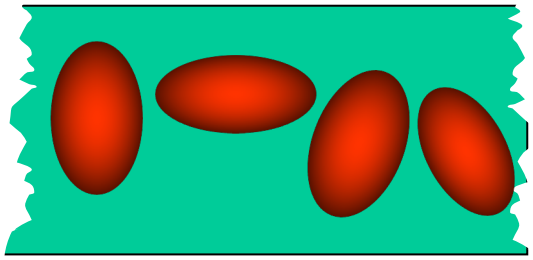
Here is Mack Fulwhyler telling the story



Here is Marvin van Dilla on the Why they were interested in fluorescence



The mysterious red cell problem

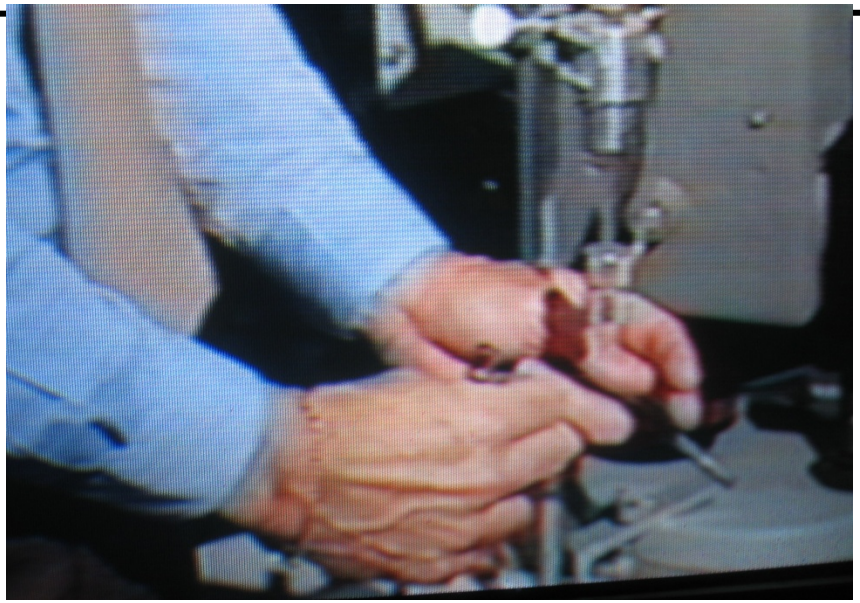


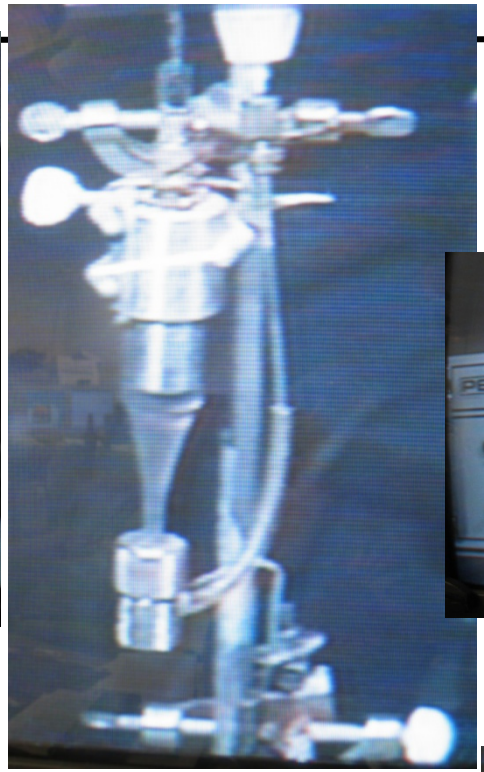
So it was determined that RBC traveling past the laser were identified as “different” only because of the artifacts developed from the current being too high at the aperture of the coulter counter.

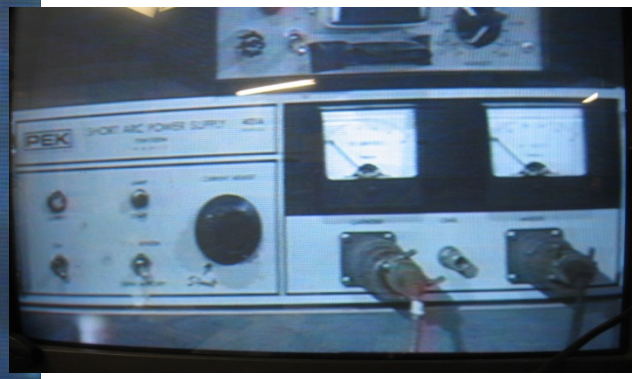
When Fulwyler sorted a single population and reran that population through the Coulter Counter, he again saw the bimodal population proving that the bimodality was an artifact.

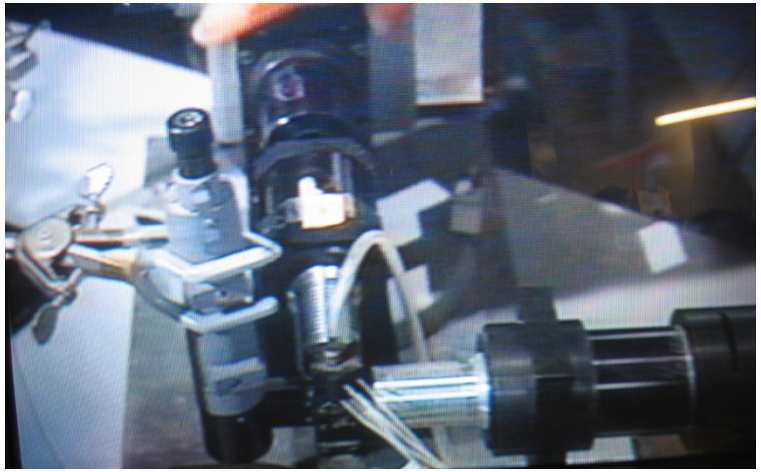
As a matter of interest, I spoke to Mack Fulwyler about this experiment, and he told me that the essentially determined the solution to this enormous problem in one afternoon, and after all that, they never actually published a paper about the problem……like good scientists, they just went on to solve a real (biological) problem!!!

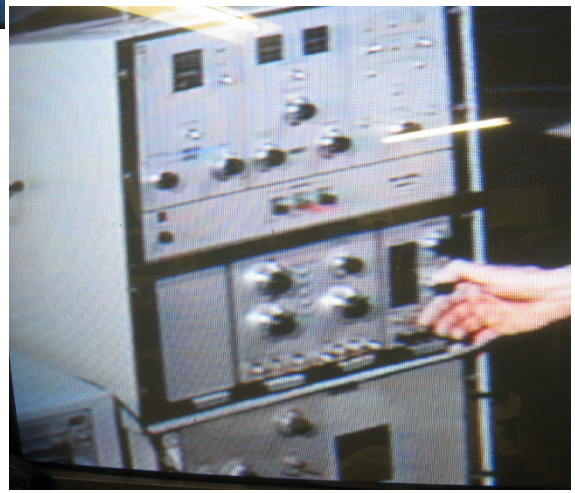
Fulwyler's Sorter











PUCL cytometry laboratories

Lecture Summary

- Introduction to course
- Reading and Support Materials
- History
- Technical Highlights

At the conclusion of this lecture you should:

1. Know what the requirements of this course are
2. Know where to track down information of importance
3. Understand a brief history of the development of flow technology
4. Be introduced into some of the fundamental principles
5. Have a perspective on why imaging was so difficult to do at the time

Note: If you use materials from this lecture series, please acknowledge the source