KILLER APPLICATIONS: SIMPLE AND AFFORDABLE IMAGING CYTOMETRY FOR TB AND MALARIA DIAGNOSIS

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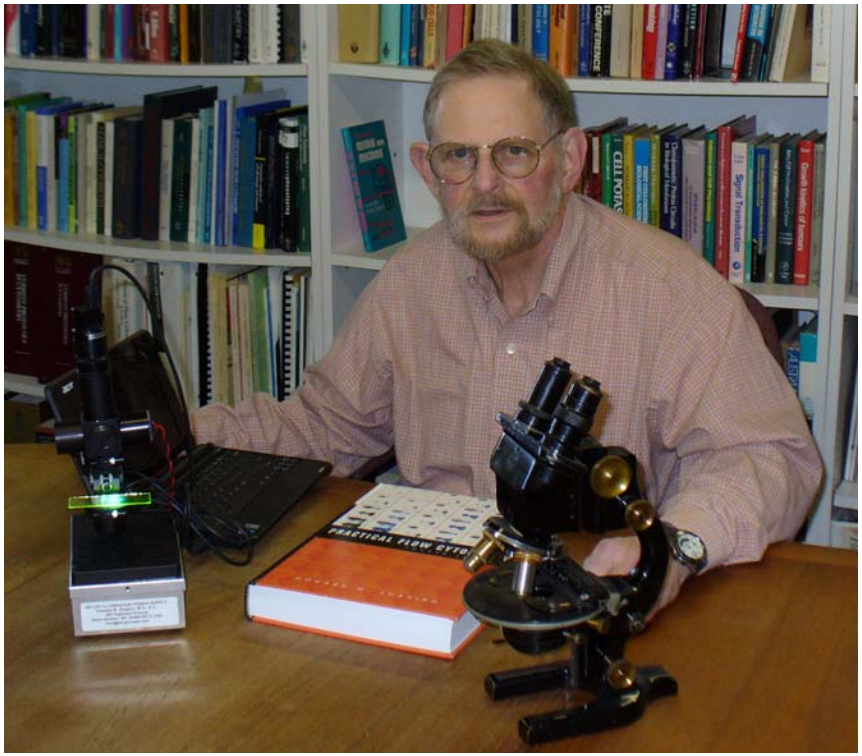
February, 2009



FOR RICHER, FOR POORER – 1960s

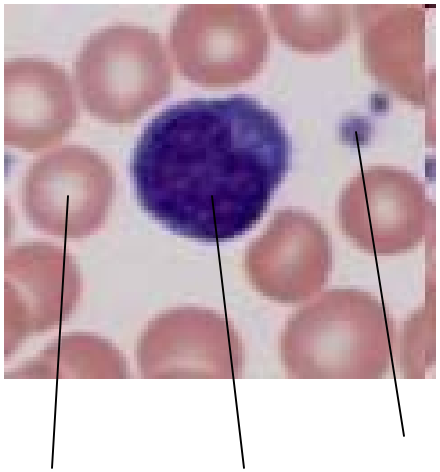
When I was in Medical School at New York University, most of the laboratory procedures requiring microscopy were performed by third-year medical students. These included:

Differential white blood cell counts ("diffs") on blood slides, performed at least once on every patient admitted to the hospital,

Ziehl-Neelsen stains of sputum slides for *Mycobacterium tuberculosis* (TB), performed on most patients with chronic cough

Giemsa stains of blood slides for malaria, performed on patients with fever who had been in areas where the disease was common.

None of these tests would have been done differently in Africa!



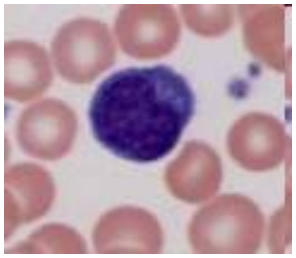
BLOOD contains three main types of cells:

Red Blood Cells (RBC; erythrocytes), the most numerous, lose both their cell nuclei, which contain the genetic material (DNA), and the RNA used for protein synthesis; they contain large amounts of the red protein hemoglobin, which carries oxygen to tissues. There are normally about 5 million RBC in 1 µL of blood.

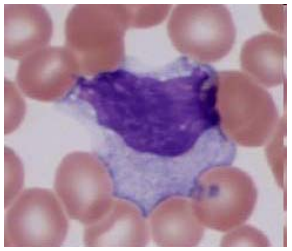
Platelets (PL) (normally about 200,000/µL) are not cells but cell fragments; they aid in blood clotting.

There are several types of **White Blood Cells** (WBC, leukocytes) in blood; different types have different functions.

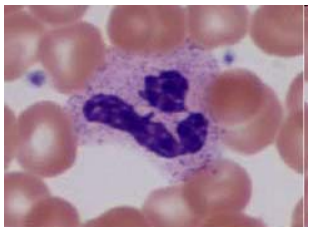
All blood cells are formed in the bone marrow by division of stem cells; as cells become mature, they are released into the circulating blood. The major types among the 5,000-10,000 **White Blood Cells** normally seen in 1 µL of blood are:



Lymphocytes, which participate in immune responses. The **B** types make antibodies, the **T** types may help in this or act on their own.

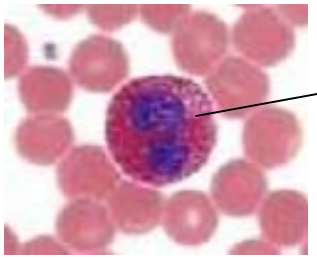


Monocytes, which are phagocytes; they can "eat" dead cells and debris. They also "present" fragments of antigen to other cells. Monocytes and lymphocytes are both "mononuclear."

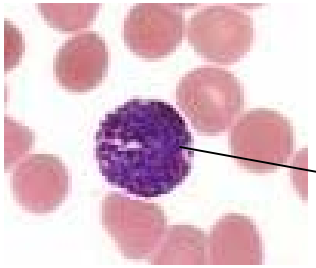


Granulocytes, which are also phagocytes; they contain granules and have lobed nuclei, making them "polymorphonuclear."

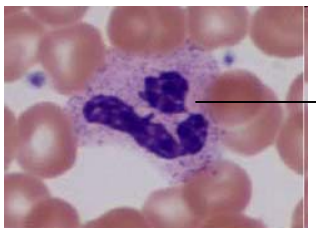
Although red blood cells appear red in untreated blood, it is difficult to visualize the internal details of white cells unless they are stained with dyes. In the 1870s, Paul Ehrlich introduced combinations of acidic and basic dyes for this purpose.



He identified three types of **granulocytes**. **Eosinophils** (or acidophils) have granules that stain predominantly with acid dyes; the red dye eosin is the acid dye now most often used. Eosinophils are present in increased amounts in allergy and in parasitic diseases.



Basophils contain granules that stain with basic dyes; they release mediators of allergy.



Eosinophils and basophils are rare; typically, **Neutrophils**, with granules that stain with both acid and basic dyes, make up about 90% of the granulocytes in peripheral blood.

In the 1890s, Romanowsky discovered that a mixture of eosin with the bluish-purple basic azure dyes formed by oxidation of methylene blue was more effective than Ehrlich's original stains because it facilitated detection of **malaria parasites** in blood. This stain was "tweaked" by others; the formula described by **Giemsa** in 1904 remains the standard for malaria diagnosis and is widely used in hematology. Staining with Romanowsky-based stains has its limitations; although it is usually possible to distinguish the five major types of mature WBCs, one cannot reliably tell small monocytes from lymphocytes or identify lymphocytes as T or B cells. Lymphocyte subtypes must be identified using antibodies to surface proteins; for example, the "helper T cells" attacked by HIV bind antibodies against the CD4 and CD3 proteins. These were unknown in the 1960s, when attempts were first made to automate WBC differential counts by microscopy.

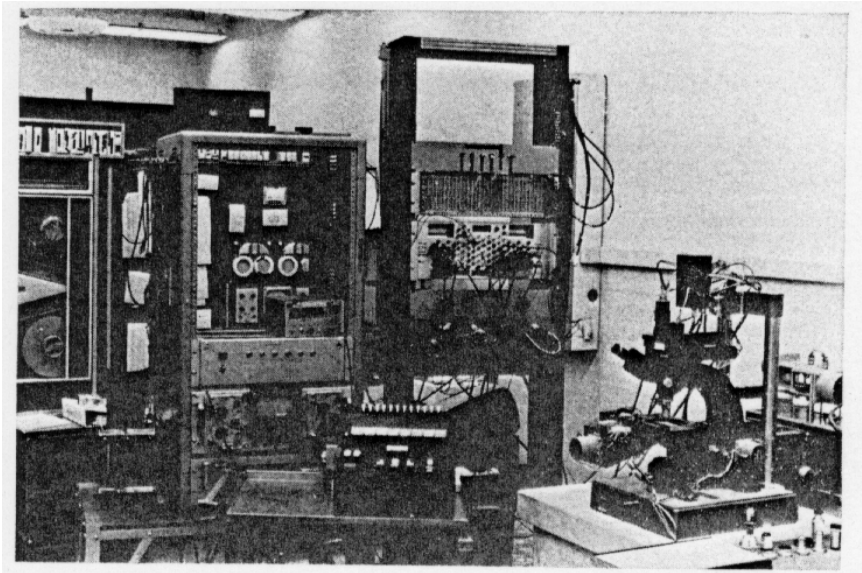


Fig. 3. The Spectre II system. The controlling computer is seen in the background. In the foreground are (from left to right) the rack housing the stepping motor controller and display oscilloscope, the operator's keyboard, the rack housing the scanner electronics, the microscope, and the monochromator. The scanner head is mounted on the microscope.

Spectre II: General-Purpose Microscope Input for a Computer

Modular design and digital control facilitate optical measurements in biology.

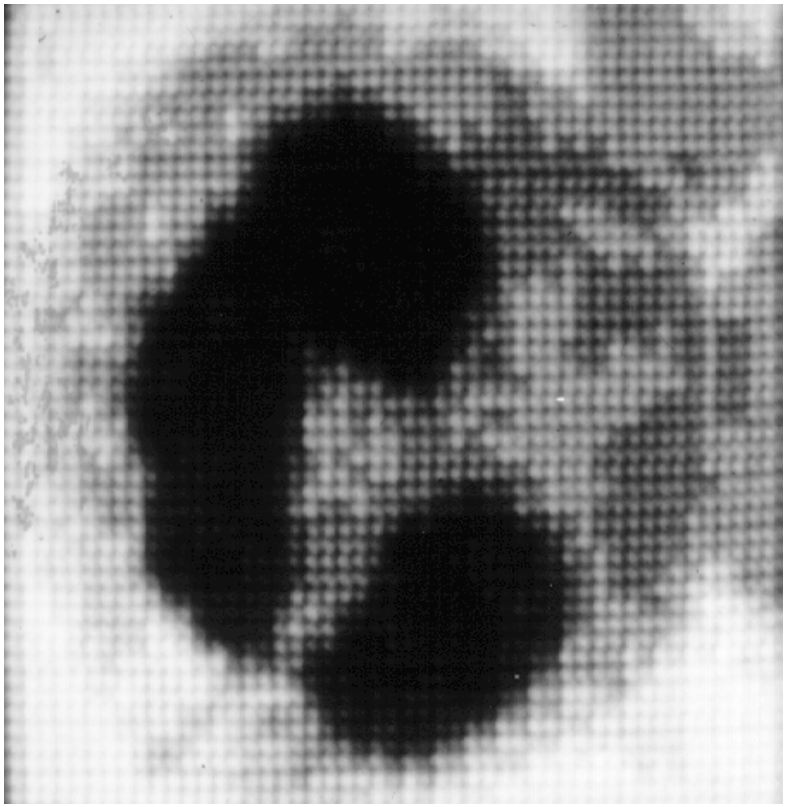
Philip G. Stein, Lewis E. Lipkin, Howard M. Shapiro

***Science* 166: 328-333, 1969**

Computerized Microscopy in the 1960s (>US$100,000): Not up to the task!

Good: arc lamp source, 0.3 µm resolution in transmitted light, computer-controlled illumination wavelength, 0.2 µm reproducible X-Y-Z motion control via joystick, unattended scanning

Bad: 256 x 256 pixel scan with 64 gray levels took **2 minutes**; real time analysis was infeasible

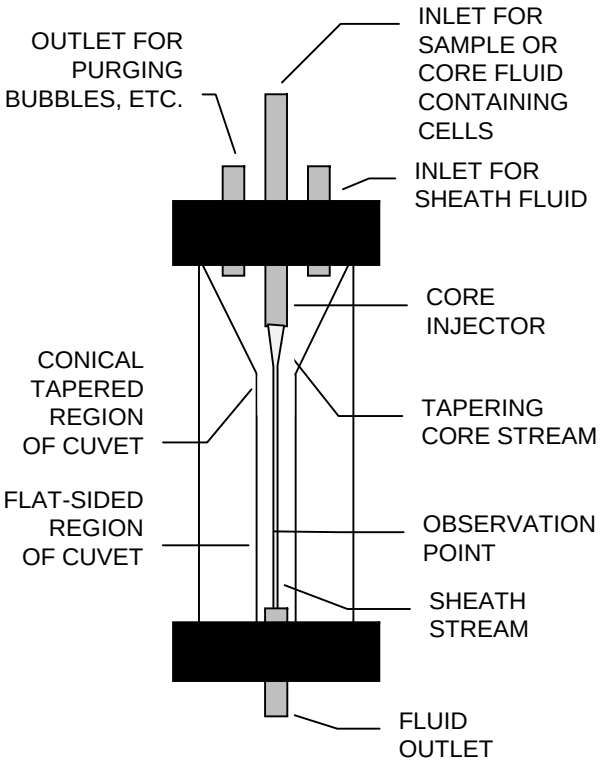


Scanned image of a neutrophil WBC stained with an eosin-methylene azure mixture similar to the Giemsa stain (J. Bacus, 1970's)

The procedure for automated differential WBC counting by microscopy was to:
1) Scan cells at one or more wavelengths at high magnification (40x/100x)
2) Find areas in high-resolution images representing nucleus, cytoplasm, etc.,
3) Extract numbers that represent size, shape, color, and texture of these, and
4) Apply a statistical procedure for identification developed from analyses of clustered data.

The instruments required precision hardware for stage motion and focusing and could only analyze about 100 cells/ min; their slow speed, large size, complexity and high cost made and make them infeasible for use in TB or malaria diagnosis, even in affluent countries.

By the late 1960s, a new technique called **flow cytometry** appeared to offer advantages over automated microscopy. In flow cytometry, measurements of physical or chemical characteristics of are made while the cells or particles pass rapidly, preferably in single file, through the measuring apparatus in a fluid stream. The entire cell is measured at once. Most modern flow cytometers measure cells' optical properties, typically light scattering and fluorescence of intrinsic molecules or externally applied reagents such as nucleic acid and protein dyes and fluorescent labeled antibodies.



A TYPICAL FLOW CELL DESIGN
(How the single file stream is produced)

The First Working Flow Cytometer

During World War II, Frank Gucker and colleagues (Gucker FT et al, J. Am. Chem. Soc. 1947; 69:2422-2431) built apparatus for the U. S. Army that made light scattering measurements of single bacteria as small as 0.6 μm in a flowing air stream, using a Ford headlight for dark field illumination. The instruments, placed at Camp (now Fort) Detrick and Harvard, were designed to detect and count anthrax spores and other biowarfare agents, an application in which the Army remains interested.

THE PREPARATION AND MEASUREMENT OF THE CONCENTRATION OF DILUTE BACTERIAL AEROSOLS[1, 2]

RONALD M. FERRY, LEO E. FARR, JR., AND MARY G. HARTMAN

Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts

Received October 18, 1948

Chem. Revs. 1944; 44: 389-49

P. J. CROSLAND-TAYLOR and WALLACE COULTER: OPTICAL AND ELECTRONIC FLOW CYTOMETERS FOR BLOOD CELL COUNTS (1950s)

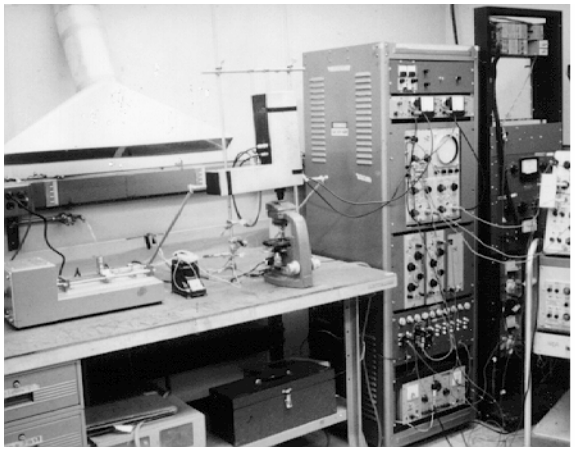
- The precision of cell counts is limited by Poisson statistics; if you count *n* cells, the standard deviation will be $\sqrt{n}$. Humans can count a few hundred cells, limiting precision to ~5 per cent; instruments can easily count thousands (~1% precision for 10,000 cells).

- Crosland-Taylor adapted the Gucker design to count blood cells in a saline stream; Coulter first attempted to build a similar optical counter and later devised a simpler electronic instrument based on the relatively low electrical conductivity of cells.

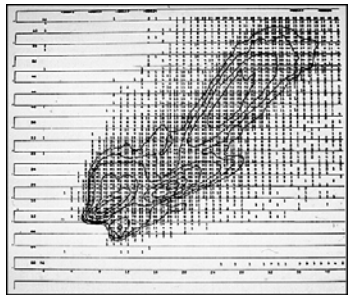
- Electronic and simple optical sensors were adequate for RBC, WBC, and platelet counting but not for differential WBC counts (diffs).

LOUIS KAMENTSKY: CREATING ANALYTICAL FLOW CYTOMETRY

• Kamentsky, who developed an optical character recognition system, was asked by IBM in the early 1960s to produce an improved scanner for cervical cancer screeing from Pap smears; recognizing scanning's limits, he learned whole cell microspectrophotometry from Torbjörn Caspersson in Sweden, and applied it to analysis of cells in flow. Initially, Kamentsky measured absorption of cell components and of dyes added to cells; he used a combination of dyes to demonstrate some clustering of different types of white cells, but the separation of different cell types was not good enough for practical differential counting. By the 1970s, Kamentsky and others found fluorescent dyes more useful.



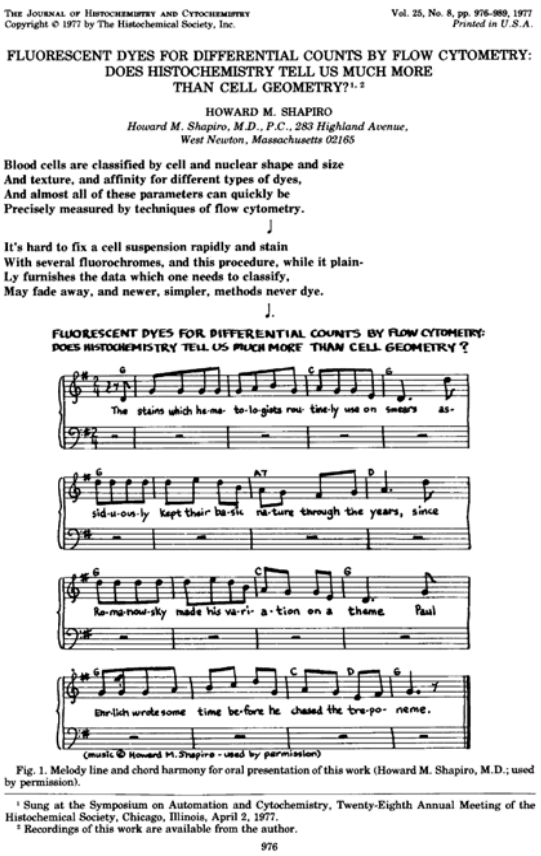
Kamentsky's Rapid Cell Spectrophotometer (IBM, 1960s): arc lamp source, scatter and absorption measurement, no sheath flow (but had a dedicated computer [IBM 1130]). WBC stained with Feulgen and naphthol yellow S (at right) show some clusters.



An early "connect-the-dots contour plot

Technicon's Hemalog D WBC differential counter (1970s): filament lamp source; absorption and scatter measurements in 3 separate flow systems detecting esterase (monocytes), peroxidase (PMN's, eos and [by their lack] lymphs), and Alcian blue (basophils).

FLOW CYTOMETRY, MORE SPECIFIC STAINING AND MULTI-PARAMETER ANALYSIS MOVED WBC IDENTIFICATION AWAY FROM A MORPHOLOGICAL BASE BEGINNING IN THE 1970s



THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY Vol. 25, No. 8, pp. 976-980, 1977
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FLUORESCENT DYES FOR DIFFERENTIAL COUNTS BY FLOW CYTOMETRY: DOES HISTOCHEMISTRY TELL US MUCH MORE THAN CELL GEOMETRY?[1, 2]

HOWARD M. SHAPIRO
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**Blood cells are classified by cell and nuclear shape and size
And texture, and affinity for different types of dyes,
And almost all of these parameters can quickly be
Precisely measured by techniques of flow cytometry.**

♩

**It's hard to fix a cell suspension rapidly and stain
With several fluorochromes, and this procedure, while it plain-
Ly furnishes the data which one needs to classify,
May fade away, and newer, simpler, methods never dye.**

♩.

FLUORESCENT DYES FOR DIFFERENTIAL COUNTS BY FLOW CYTOMETRY: DOES HISTOCHEMISTRY TELL US MUCH MORE THAN CELL GEOMETRY ?

Fig. 1. Melody line and chord harmony for oral presentation of this work (Howard M. Shapiro, M.D.; used by permission).

[1] Sung at the Symposium on Automation and Cytochemistry, Twenty-Eighth Annual Meeting of the Histochemical Society, Chicago, Illinois, April 2, 1977.
[2] Recordings of this work are available from the author.

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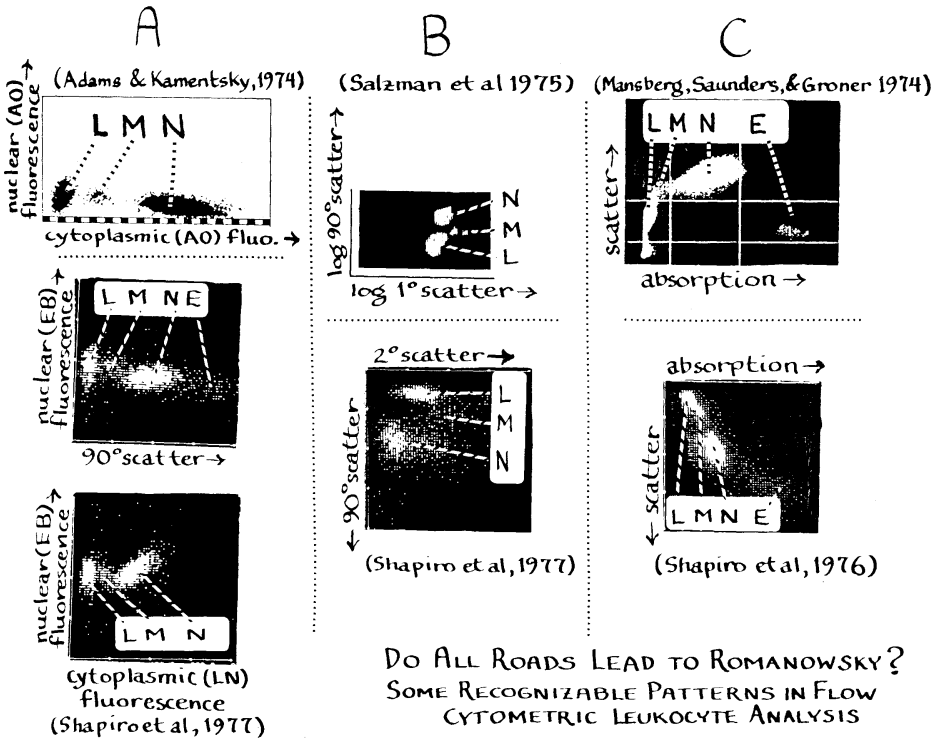
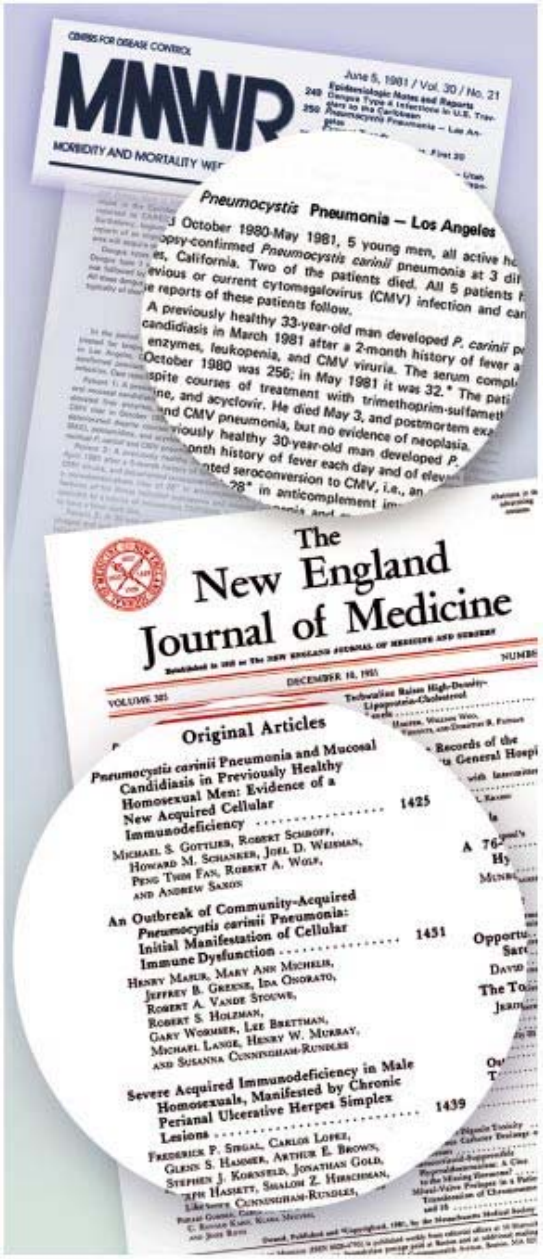


L = lymphocyte, M = monocyte, N = neutrophil, E = eosinophil

By the mid-1980s, it was clear that automated diffs could be done using various parameters, including polarized scatter (Abbott), which need "never dye." Although the stained smear is still the official "gold standard", it is about to be replaced by one that is based on fluorescence measurements using a combination of dyes and monoclonal antibodies to CD antigens; over 300 such antigens have been identified since the late 1970s. Automated flow cytometric differential counters are used by every lab that can afford them (typical cost ≥ US$10,000); fluorescence flow cytometers, usually ≥ $50,000, are used to immunophenotype leukemias and lymphomas and count stem cells, but their best known application is counting CD4+ T cells to monitor treatment of HIV/AIDS.



"Monoclonal-antibody analysis of peripheral-blood T-cell subpopulations revealed virtual elimination of the Leu-3+ *[now CD4+]*helper/inducer subset, an increased percentage of the Leu-2+ *[now CD8+]* suppressor/cytoxic subset, and an increased percentage of cells bearing the thymocyte-associated antigen T10."

--Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA, Saxon A: *Pneumocystis carinii* pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. New Engl J Med. 1981; 305:1425-1431.

AIDS, first defined in 1981 using then newly available monoclonal antibodies, provided a literal "killer application" for fluorescence flow cytometry, but basic cytometric principles might be equally applicable to combating the associated epidemics of **tuberculosis** (**TB)** and **malaria** if affordable apparatus suitable for use in resource-poor countries could be devised.

HIV/AIDS, malaria, and TB combine to kill an estimated six million people each year!

NOT ALL LABS ARE RESOURCE-POOR, BUT ALL LABS ARE RESOURCE-LIMITED

Reagents, manpower, energy, instrument time, and data storage media all cost money.

More complex procedures are more difficult to export to other laboratories, and more difficult to quality control. EXAMPLE: It took until 1988 to get several dozen US AIDS labs to produce consistent CD4 counts from a single specimen.

A "minimalist" approach to cytometry does not imply a fallback to "second-class" procedures, but rather an appropriate response to the realities of 21st century life in both poor and rich countries.

TRYING TO MAKE FLOW CYTOMETRY SIMPLER

1983: He-Ne lasers cost less and save energy

Cytometry 4:276–279 (1983)

BRIEF COMMUNICATION

Immunofluorescence Measurement in a Flow Cytometer Using Low-Power Helium–Neon Laser Excitation[1]

Howard M. Shapiro, Alexander N. Glazer, Lisa Christenson, John M. Williams, and Terry B. Strom

Center for Blood Research, Boston, Massachusetts 02115 (H.M.S., L.C.); Department of Microbiology and Immunology, University of California, Berkeley, California (A.N.G.); and Renal Division, Department of Medicine, Beth Israel and Brigham and Women's Hospitals, Boston, Massachusetts 02215 (J.M.W., T.B.S.)

Received for publication July 19, 1983; accepted August 30, 1983.

Helium–neon lasers are economical and efficient light sources; their utility in flow cytometry to date has been limited by the lack of fluorescent probes that can be excited at 633 nm. Allophycocyanin (APC), a highly fluorescent phycobiliprotein, can be used as an antibody label and has spectral characteristics suitable for use with He–Ne lasers; we undertook to resolve whether a low-power (7 mW) He–Ne laser could provide sufficient excitation to permit flow cytometric detection of APC-labeled antibodies on cell surfaces. We made an APC conjugate of monoclonal antibody 4F2, which reacts with an antigen abundant on the surfaces of activated human T-lymphocytes; APC-4F2 was used to stain blood mononuclear cells that had been cultured with and without phytohemagglutinin (PHA). Cells so stained were examined in a flow cytometer with orthogonal illumination at 633 nm from a 7 mW He–Ne laser; antibody-bearing cells were detectable by fluorescence emission above 665 nm. Cells from the same cultures were stained with fluorescein-labeled 4F2 antibody and examined in a flow cytometer with argon ion laser excitation at 488 nm. Percentages of antibody-bearing cells determined from APC fluorescence and from fluorescein fluorescence were in good agreement. It thus appears that He–Ne lasers and APC-antibodies are usable for immunofluorescence measurements; the sensitivity attainable with this technique remains to be determined.

Key terms: Allophycocyanin, flow cytometry, He–Ne laser, immunofluorescence, phycobiliproteins

"Free-standing instruments of the type we used can be assembled for less than the price of most fluorescence microscopes; alternatively, one might add a 7-15 mW He-Ne laser to a commercial flow cytometer already equipped with an argon ion laser. This should enable detection of triply (fluorescein, phycoerythrin, and APC) labeled cells in a simpler and less expensive instrument than those now in use for this purpose. Both approaches offer the potential of making the benefits of immunofluorescence flow cytometry available to a larger community of clinicians and researchers; we are hopeful that this potential will soon be realized."

IMPROVING FLOW CYTOMETRY: THINKING INSIDE THE BOX…

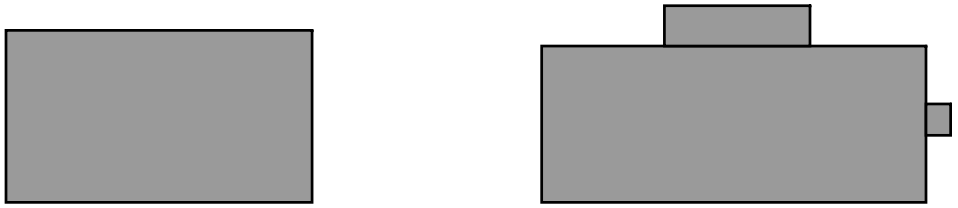
Smaller, more efficient, less costly lasers; better light collection, new detectors

Replacing analog and hybrid circuitry with digital signal processing (DSP) circuitry for pulse processing, fluorescence compensation, and logarithmic transformation

Miniaturizing electronic, optical and mechanical components as much as is practical.

RELATIVE SIZE, ELECTRICAL POWER REQUIREMENTS, AND COSTS OF AIR-COOLED LASERS

POWER SUPPLY **LASER HEAD**



488 nm, 10-25 mW AIR-COOLED ARGON LASER (1970-~2000)
(1 kW; $6,000)

12”

633 nm, 10 mW HELIUM-NEON LASER (1978-)
(<200 W; $1,000)

532 nm, 10-25 mW FREQUENCY-DOUBLED YAG LASER (1990-)
(40 W; $6,000, more power for more $; less reliable lasers are much cheaper. A 490 nm semiconductor laser (2000-) is slightly more expensive)

635, 670, or 780 nm, 5-10 mW DIODE LASER (1990)
(< 1W; $200-$600; 370 nm or 407 nm (2000-) costs thousands)

THE INCREDIBLE SHRINKING COMPUTER

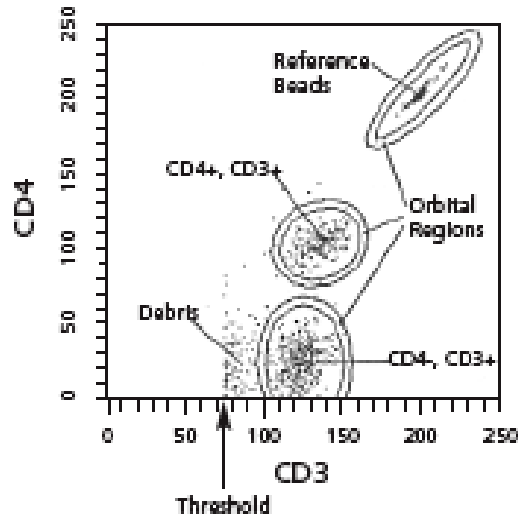
=

≤

	IBM 7094 (1961)	IBM PC (1981)	Lenovo Laptop (2008)
COST ($)	millions	thousands	hundreds
POWER	kW	hundreds of Watts	tens of Watts

The OLPC Project's XO laptop () designed for use in resource-poor areas, uses only a few watts of power at most; sufficient processing and storage capacity to run most analytical flow cytometers is now available in even smaller and equally efficient cellular telephones and music players, some of which have highly sophisticated touchscreen interfaces.

TRYING TO MAKE FLOW CYTOMETRIC CD4 SIMPLER



BD Biosciences FACSCount

No lysis or washing necessary

2-parameter fluorescence

CD3-PECy5/CD4-PE

Beads for absolute count

Partec CyFlow

2-parameter fluorescence with

CD4-PE and SSC or another antibody

(CD4 alone didn't work)

Volumetric absolute count

PointCare - Gold label CD4 (scatter), CBC from tube

LESSONS LEARNED FROM ATTEMPTS AT FLOW CYTOMETER DESIGN FOR CD4 COUNTING IN RESOURCE-POOR AREAS:

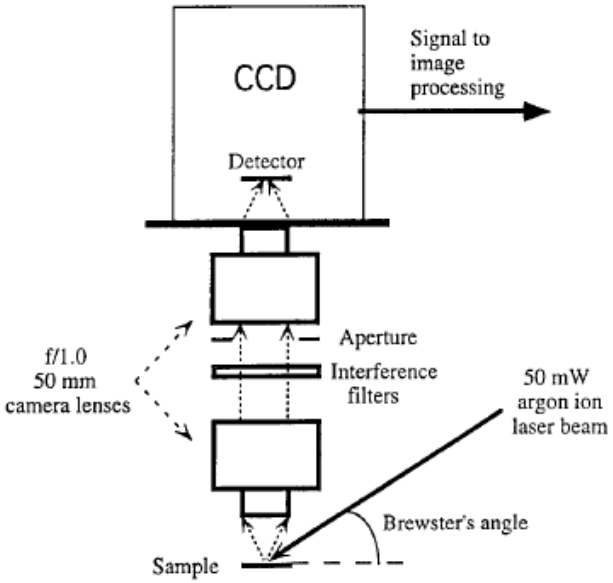
- The simplest flow cytometer (http://www.cytometryforlife.org) still needs fluidic and electro-optic components that make it relatively complex.
- For applications in resource-poor areas, instead of depopulating a box full of relatively expensive components, one should consider starting with an empty box and putting in as little as is needed to do the job.
- One should use as little sample, and do as little to it, as possible. Scatter and extinction measurements require neither reagent nor fixation/permeabilization. Vital dyes (e.g., HO33342 for DNA, pyronin Y for RNA, R640 for protein, fluorogenic substrates, antibodies to surface markers) need no fixation. DNA/RNA probes and antibodies to intracellular antigens require fixation/permeabilization; DNA/RNA probes also require hybridization.

WHAT ABOUT ALTERNATIVES TO FLOW CYTOMETRY?

- Cell identification in modern flow cytometry is based almost entirely on measurements of intensities of fluorescence emitted (and/or light scattered) by cells at various wavelengths. Morphological information need not be collected. Most common tasks require 4 or fewer colors. Could the same cytometric measurements be made in a simpler, less expensive apparatus?

ALTERNATIVES TO MICROSCOPY AND FLOW CYTOMETRY

LASER SCANNING CYTOMETRY (1988) is neither simpler nor less expensive than flow cytometry. The CompuCyte LSC (at left) uses expensive laser sources and PMT detectors, like a flow cytometer, but adds imaging capability. Chemunex sells a similar laser scanning system for detection of bacteria on filters.



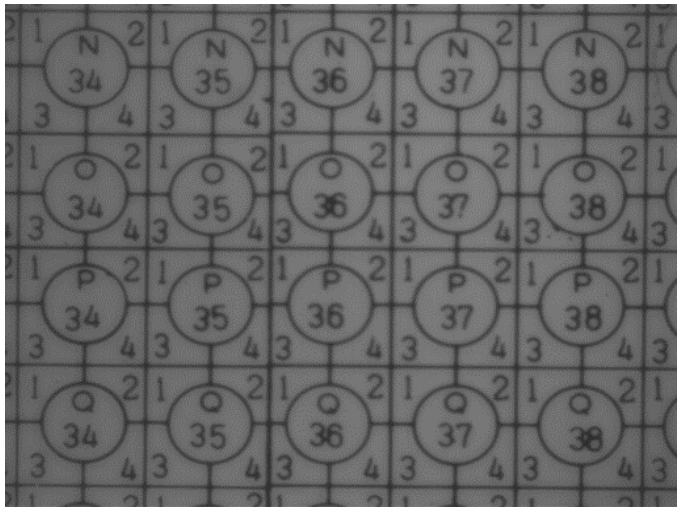
LOW-RESOLUTION IMAGING (1994)

Wittrup et al (Cytometry 16:206-13) described an instrument in which a CCD was used to collect an 1x image of a 1 x 1 cm field illuminated by an expanded laser beam, making pixels bigger than cells. Sensitivity was good (a few hundred fluorescein MESF); precision was not (CV > 12%), due to nonuniform illumination. The CCD and the laser together cost >$20,000, so the system, although simple, was not a low cost alternative to automated microscopy, flow cytometry, or laser scanning.

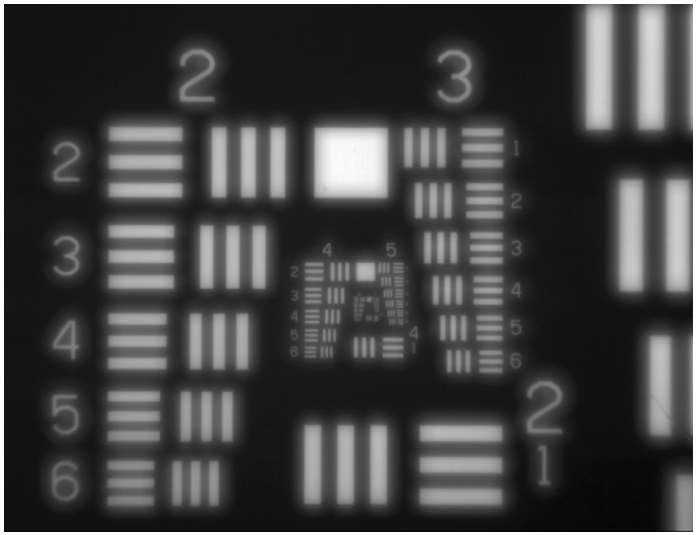
A "PERSONAL" CYTOMETER

Since 2000, high-intensity LEDs and better and cheaper camera chips have made lo-res imaging feasible. In the system at left, a 10-bit monochrome, 1.3 MP CMOS camera makes a 1x image of a 4.5 by 5.6 mm area of a slide using two 2.5x microscope objectives. Illumination comes from a 3W LED with an efficient collecting lens; over 100 mW/cm^2 of light at 420-470 nm can be delivered to the slide. Power comes from four D batteries. A system can be assembled for under $1,500, even using relatively expensive components from optical supply houses; production instruments could sell for under $2,000. The system has no moving parts. Camera power comes from a notebook computer via the USB port. Multiwavelength instruments can use multiple or color cameras and/or multiple LEDs. Several organizations are developing CD4 counters using this approach.

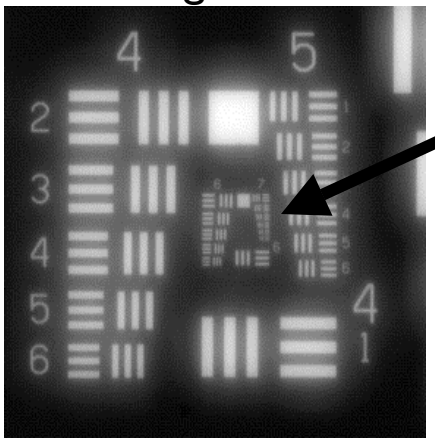
CHARACTERIZATION OF OPTICAL QUALITY OF THE SYSTEM



"England Finder" overlaid on uranium glass slide; large squares are 1 mm on a side. The illumination from a 60 mm focusing lens would be adequate for one well of a 96 well plate; a field as large as 10 x 12.5 mm could be accommodated.



USAF negative resolution target overlaid on uranium glass slide.



Enlarged portion shows resolution is >100 line pairs/mm.

WHY THIS ISN'T A FREE LUNCH

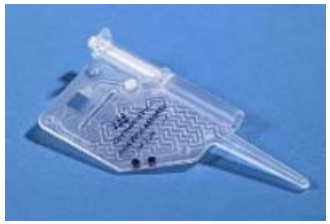
In a benchtop flow cytometer, cells are exposed to 1-2 mW excitation light for a few microseconds; light collection is efficient, but, more than 95% of the time, the laser is not illuminating cells.

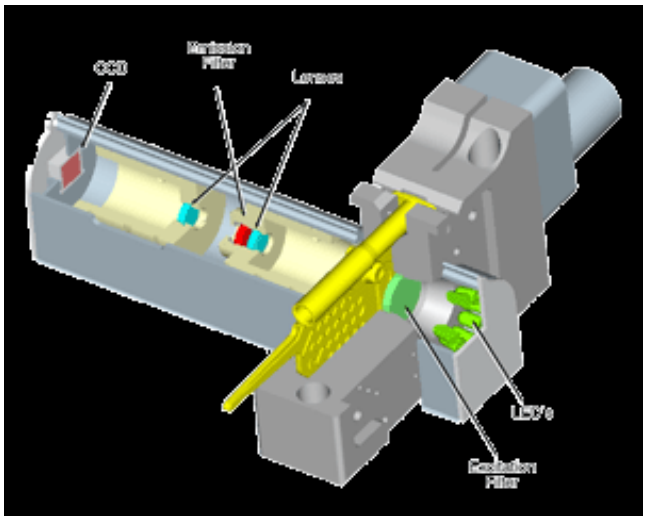
A 3W blue LED can deliver >100 mW over a 1 cm^2 area. Each cell receives only ~1/1000 as much excitation as in a flow cytometer, and light collection in a low-resolution imaging system may be only about 1/50 as efficient. However, the quantum efficiency of the CCD is about 3 times as high as that of a PMT, so exposing the entire sample for periods of ~10 milliseconds to a few seconds allows collection of as many photoelectrons per cell as in flow cytometry.

Higher-resolution, higher-magnification imaging systems using LED illumination have detected single dye molecules; LEDs can now replace arc lamps as excitation sources for fluorescence microscopy, providing longer life and lower power consumption at lower cost.

The **QX3** "toy" microscope, developed by Intel and Mattel in 1998-9, attached to a USB port and cost $99. It could be adapted for fluorescence but was not very sensitive.

The **NucleoCounter**, introduced by the Danish company ChemoMetec in 2002, uses propidium with and without detergent to obtain a total and nonviable cell count from cells in disposable plastic cartridges. Multiple green LEDs provide illumination. Higher intensity LEDs, which did not become available until 2003, allowed CD4 immnuofluorescence measurement to be envisioned.





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A Microchip CD4 Counting Method for HIV Monitoring in Resource-Poor Settings

William R. Rodriguez[1,2,3*], Nicolaos Christodoulides[4], Pierre N. Floriano[4], Susan Graham[3], Sanghamitra Mohanty[4], Meredith Dixon[1], Mina Hsiang[1], Trevor Peter[5], Shabnam Zavahir[5], Ibou Thior[5], Dwight Romanovicz[4], Bruce Bernard[4], Adrian P. Goodey[4], Bruce D. Walker[1,2], John T. McDevitt[4*]

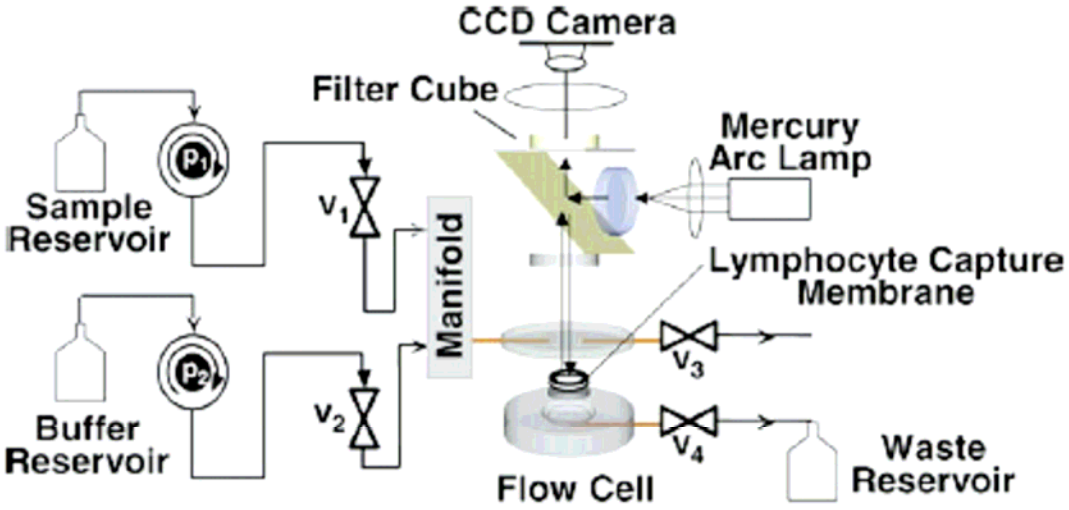

Figure 1. Components of the ETC System

The 2005 paper by Rodriguez et al generated considerable buzz, but the work was done with a conventional fluorescence microscope. The descendant LabNow instrument, above right, measures CD3 and CD4 using 8 LEDs and relatively complex optics and electronics; it also incorporates a red cell removal system which we believe is unnecessary and adds to consumable costs.

Cytometry Part A 71A:132–142 (2007)

A Single Platform Image Cytometer for Resource-Poor Settings to Monitor Disease Progression in HIV Infection

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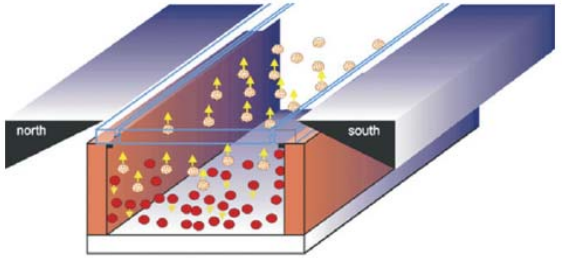


FIG. 1. Schematic representation of the immunomagnetic separation principle: immunomagnetically labeled cells (yellow balls) move to the upper surface of the chamber, while other cells (red balls) move to the bottom under influence of the gravity force.

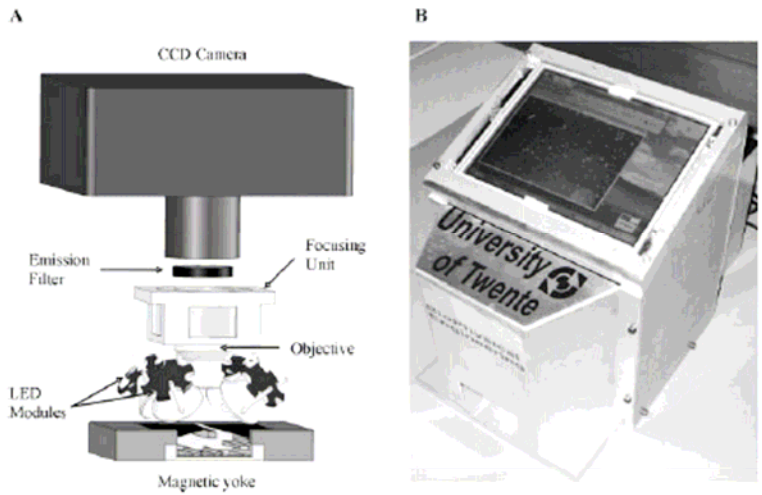
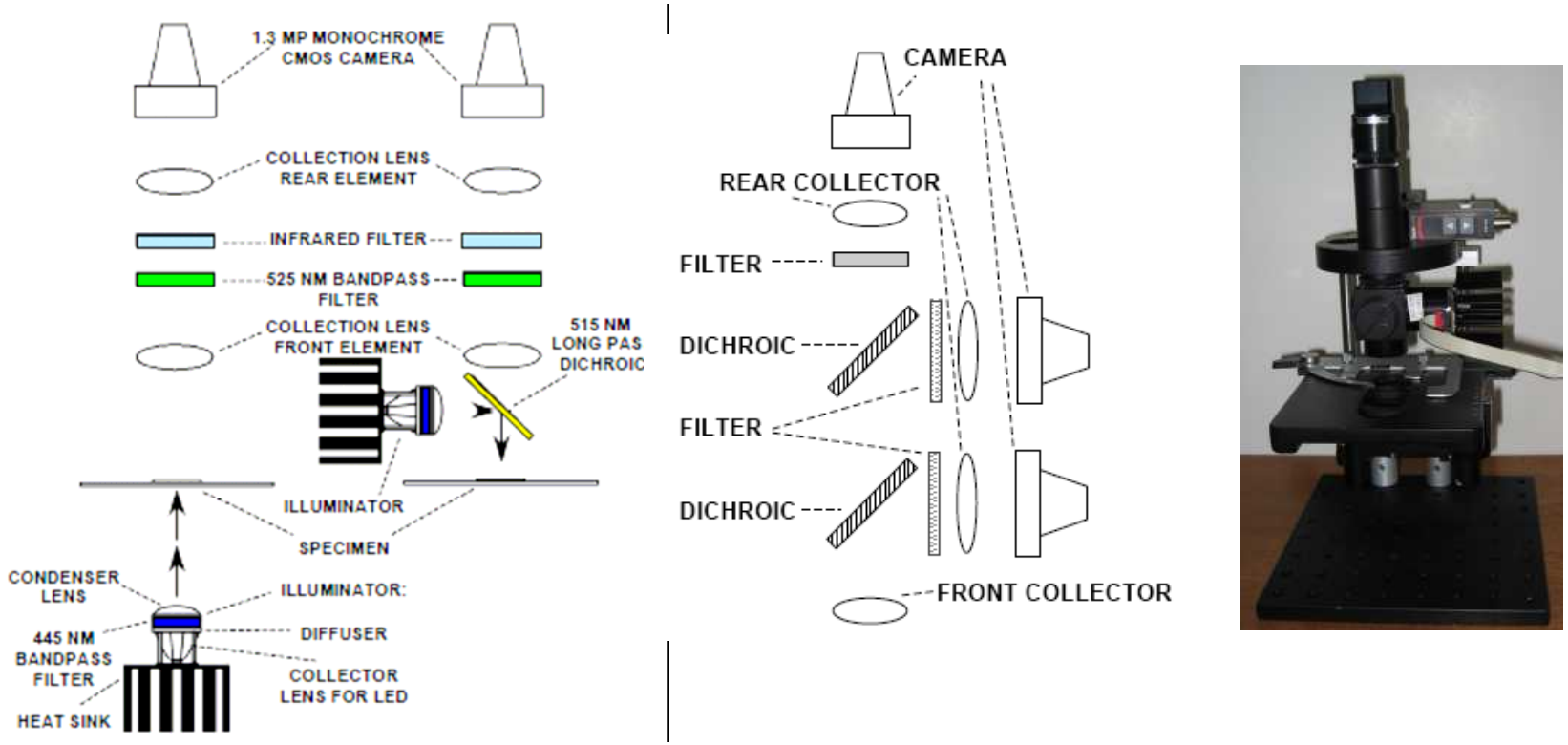


FIG. 5. (A) A schematic layout of the optical part of the image cytometer. See text for more details. (B) A photograph of the instrument prototype.

The Immunicon/U. of Twente system, at left, uses a magnetically tagged CD3 antibody, which allows T cells to be pulled to the surface of the observation chamber (top). Several LEDs and associated optics are needed for excitation of a PE-labeled CD4 antibody.

Inverness has also recently introduced an imaging CD4 counter (http://www.pimatest.com).

a **b** **c** **d**



Substage fluorescence illumination, shown at **a**, is adequate for signals well above background, e.g., from nucleic acid dyes; epiillumination configurations such as the one shown at **b**, are needed for measurements of weaker signals, as from immunofluorescence. The arrangement shown at **c** allows multicolor imaging without moving parts, but necessitates image registration; our CD4 prototype, at **d**, uses a filter wheel and a single very high intensity LED that tolerates current pulses of 10A. We can also use two LEDs and one camera.

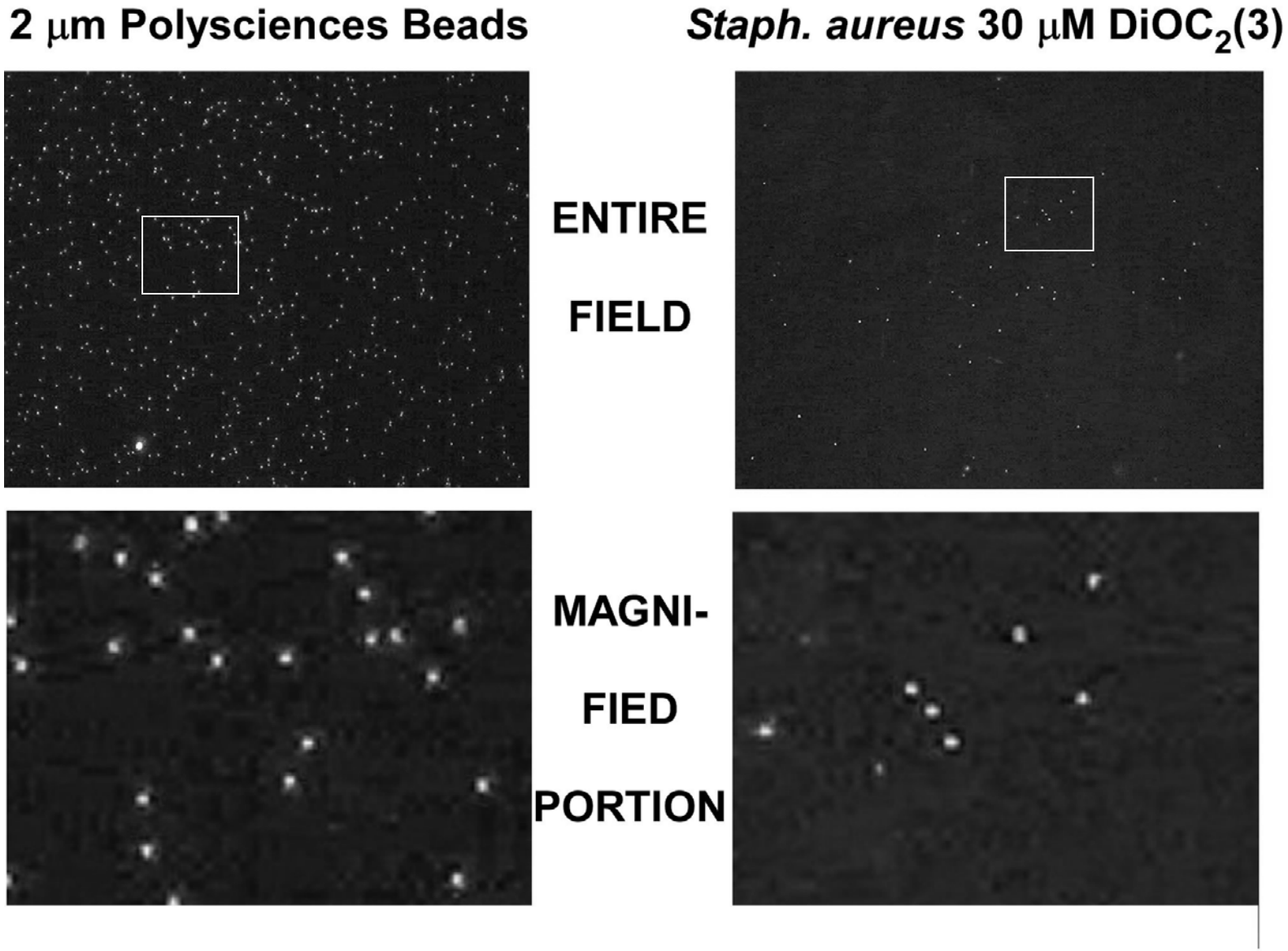
TUBERCULOSIS AND MALARIA: NEGLECTED "KILLER APPLICATIONS"

Although development of inexpensive CD4 counters for HIV/AIDS has attracted much interest, treating the disease is expensive, and a cure is unlikely to be found soon. By contrast, most victims of TB and malaria do not have HIV/AIDS, and could be cured by inexpensive drugs in weeks to months if diagnosed promptly and properly. Nonetheless, we are unaware of any work by others on low-cost cytometric approaches to either TB or malaria.

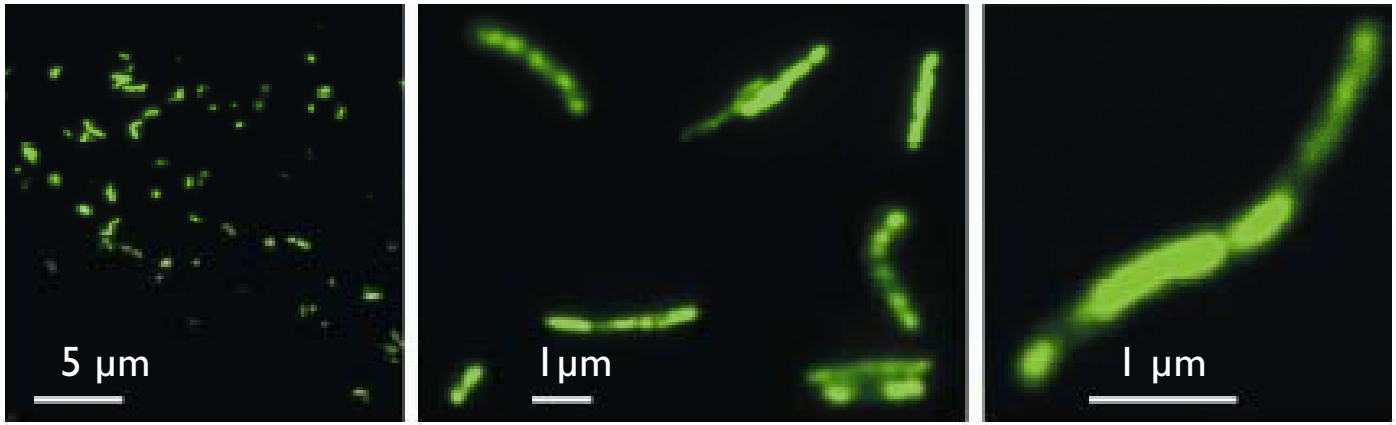
The diagnosis of both TB and malaria and is cell-based, typically made by microscopy of stained smears of sputum (TB) and blood (malaria); although cytometry has been shown to be effective for diagnosis of both conditions, conventional cytometers have been too complex and costly to be widely applied. The Ziehl-Neelsen stain used for TB diagnosis by transmitted light dates from 1883, and the auramine O fluorescent stain from 1938; the Giemsa stain used for malaria diagnosis dates from 1904.

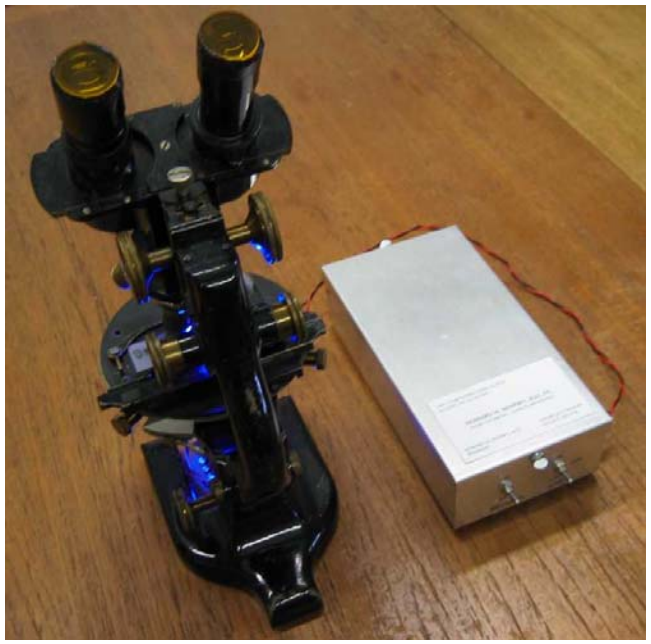
We have investigated the possibility of using simple fluorescence imaging cytometers for diagnosis and drug sensitivity testing of both TB and malaria, beginning by reexamining the foundations of slide-based diagnosis of both conditions.

BACTERIAL DETECTION BY THE IMAGING CYTOMETER



Mycobacterium tuberculosis (MTB) was first described as acid-fast, i.e., retaining dye after acid-alcohol washing, by Paul Ehrlich in the 1880s. **Mycolic acid**, a component of mycobacterial cell walls, prevents acid alcohol from extracting dyes such as fuchsin (used in the classical Ziehl-Neelsen transmitted light stain [1883]) and auramine O, the standard fluorescent stain for mycobacteria [1938]). Fluorescence spectroscopy and confocal microscopy (with Dr. Thomas Hänscheid, Lisbon [Lancet Infect Dis 2007; 7:236-237.]) suggest that most of the fluorescence retained after acid washing comes from complexes of dye with nucleic acids rather than from dye bound to mycolic acid, as is widely believed. Fluorescence microscopy is generally agreed to be more sensitive than transmitted light microscopy for diagnosis of TB.



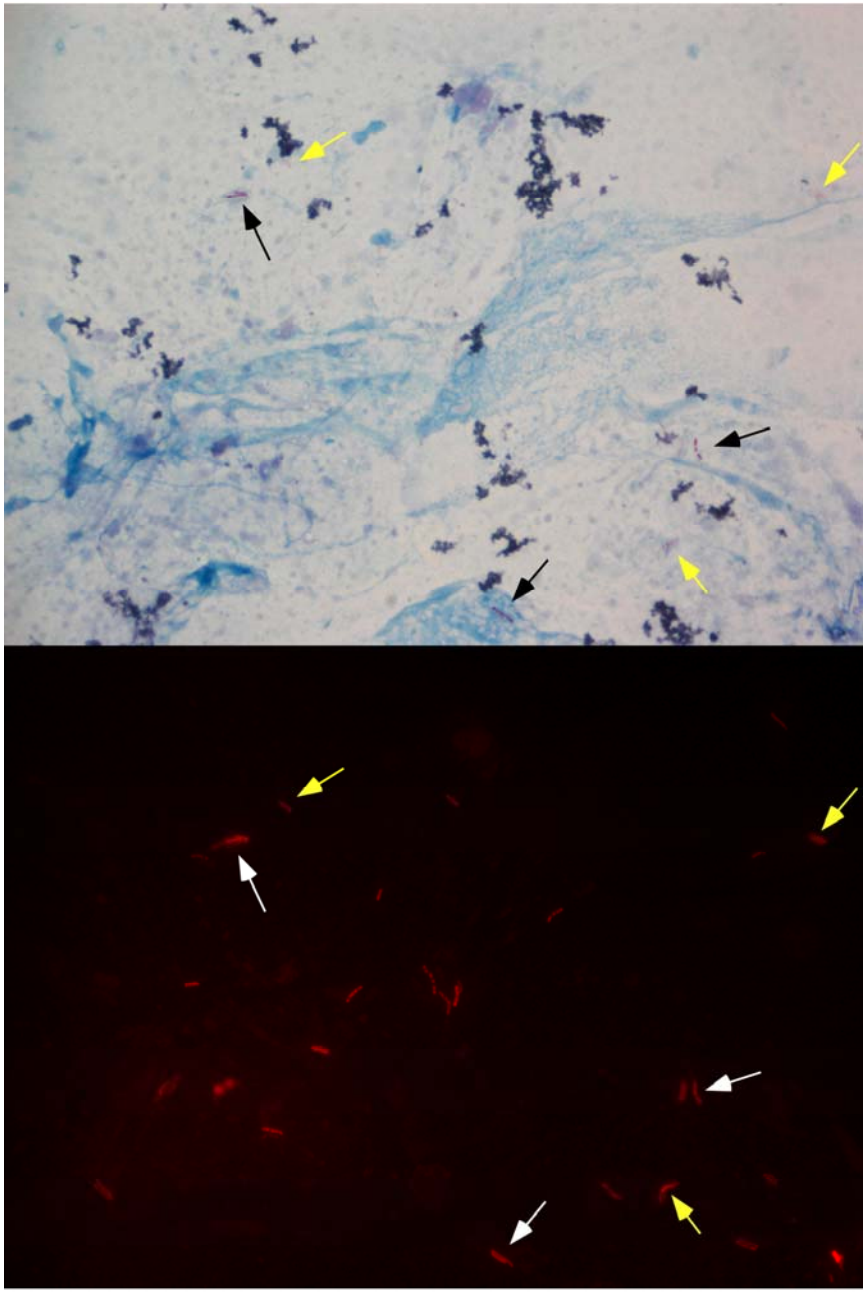


A transmitted light microscope can be adapted for fluorescence for <US$200, BUT - A human observer cannot spend the time required to scan enough fields to detect organisms present in low levels in sputum! TB diagnosis is a "rare event analysis" problem. A typical sputum smear contains 10 µL sputum spread over 10,000 high power (100x oil immersion objective) fields. No more than 500 fields are typically observed; if the concentration of organisms and microcolonies in the sputum is 5,000/mL, the entire slide will contain 50; the average number detected will be <3, and there is a reasonable probability that no organisms will be seen. Concentration of sputum by filtration or centrifugation will increase the odds of detection, as will scanning at lower magnification, allowing more fields to be examined. This is practical when fluorescence microscopy is used, but not with brightfield microscopy. An automated microscope, working at 250x-450x magnification, is too expensive and too large to be used for TB diagnosis in a resource-poor area; a laser scanning cytometer, although shown effective for detection of TB organisms in sputum (C. Pina-Vaz et al, J Clin Microbiol 2004; 42:906–908), is even larger and more expensive.

CONTROL SLIDE **TB POSITIVE SPUTUM**

DETECTION OF *M. TUBERCULOSIS* BY LO-RES IMAGING

The bright spots in the positive slide represent clumps; individual organisms can be seen clearly on a magnified image (monochrome camera, 450 nm excitation, 525 nm bandpass filter, which transmits less nonspecific fluorescence than the yellow long pass filter used in microscopy). Whether optimized low-background stains such as the auramine O formulation used here will work in the field remains to be determined.



THE ZIEHL-NEELSEN (Z-N) STAIN IN A NEW LIGHT

(H. Shapiro and T. Hänscheid, J. Microbiol Methods, 2008; 74:119-120.)

The black (top panel, transmitted light, 1000x) and white (bottom panel, 546 nm excitation, fluorescence >590 nm) arrows point to the objects most easily identified as *M. tuberculosis* (MTB); yellow arrows point to objects suggestive of MTB in the transmitted light image. The fluorescence image reveals more than a dozen other brightly stained objects also exhibiting the morphology of MTB.

We are surprised that we appear to have been the first people in 125 years to look at Z-N stained sputum slides using a fluorescence microscope, but, in our limited experience, the pictures are entirely typical; we have never seen more MTB in a field using transmitted light than in fluorescence. Z-N may be a better fluorescent stain than auramine O!

ANTIMICROBIAL SUSCEPTIBILITY TESTS IN TB

The generation time of *Mycobacterium tuberculosis* (MTB) is 18 hours; conventional susceptibility tests take 5 days-4 weeks after isolation of organisms. Rapid testing is particularly essential in dealing with drug-resistant MDR and XDR TB.

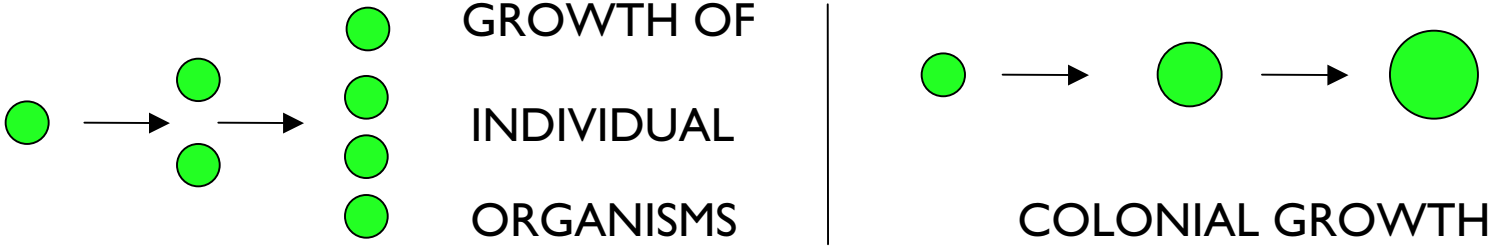
Cytometric susceptibility tests can work with a smaller inoculum (10^4-10^5 cells/mL) and detect effects on population growth in between one and two generation times, meaning 24-36 hr in the case of TB.

"Safe" flow cytometric assays can be done by counting organisms in fixed cultures. Ronald Schell et al at the University of Wisconsin have shown that rapid safe flow cytometric assays can detect susceptibility of MTB to all first-line and many second-line drugs; the Schell group's work has been confirmed by Reis et al in Brazil, who noted that their study was done entirely in a developing country. Cidália Pina-Vaz et al have also developed a rapid safe flow cytometric assay using a nucleic acid stain.

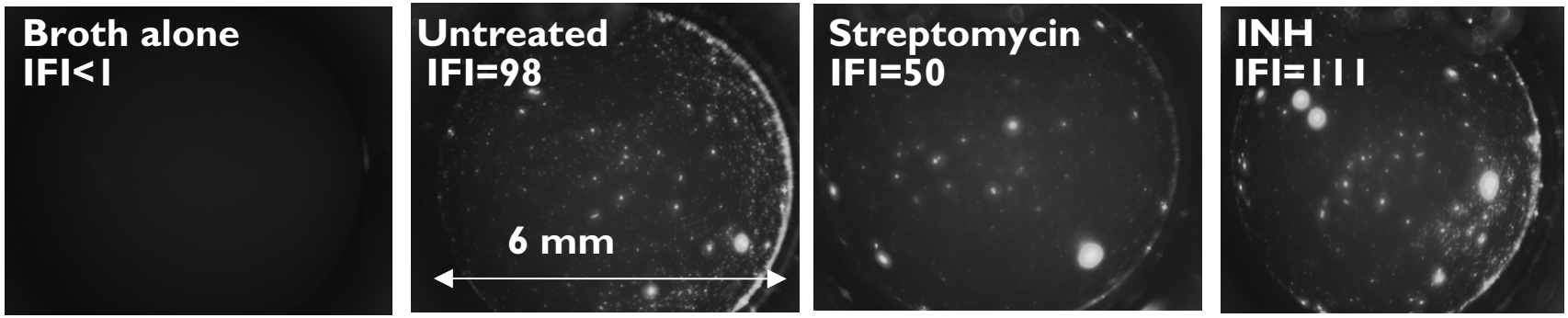
The Microscopic Observation Direct Susceptibility (MODS) test (N Eng J Med 2006; 355:1539-1550), requiring observation of colonial growth of unstained MTB in multiwell plates, provides information more rapidly (median 7 days) than more expensive automated methods. Eliminating the need for a trained microscopist is highly desirable.

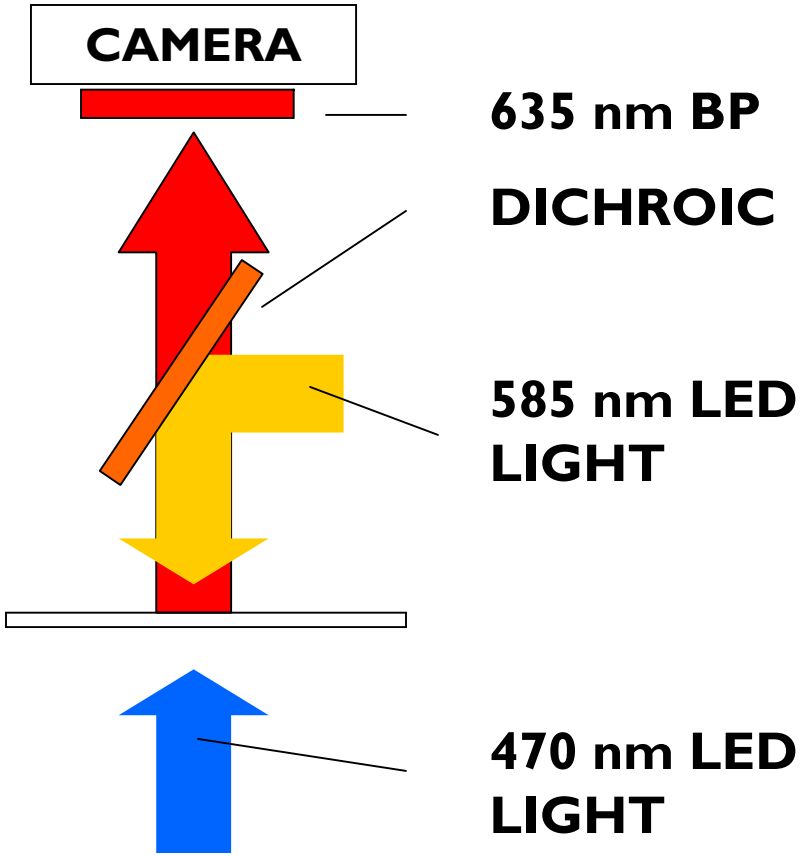
DNA DYES FOR ANTIMICROBIAL SUSCEPTIBILITY TESTS

Dyes such as Pico Green, which is used for DNA quantification on gels, are advantageous for susceptibility testing because the integrated fluorescence intensity (IFI), which "counts genomes," detects two types of growth:



Measuring fluorescence from whole wells without imaging of colonies needs too high a concentration of bacteria to be usable for rapid testing of MTB. Images below of *M. smegmatis* (generation time ~3 hr) after 5 hr exposure to streptomycin (sensitive) and INH (resistant) clearly show response; the method should work for MTB in <48 hr.





The system shown at left may be usable to discriminate virulent MTB from nonvirulent mycobacteria based on changes in color of a simple dye after interaction with mycolic acids in the cell wall. This could facilitate automation of the rapid MODS (Microscopic Observation Direct Susceptibility) test for TB diagnosis and drug resistance determination. MODS, which requires only relatively inexpensive equipment and reagents, can detect TB at levels of 6 CFU/mL sputum; the practical level of sensitivity of PCR is no higher.

FOR RICHER, FOR POORER (2009)

The antimicrobial susceptibility testing schema just described for TB will work for other pathogens, including bacteria, fungi, and parasites.

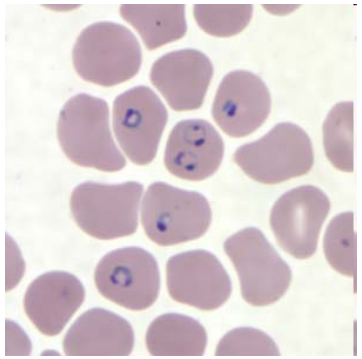
We established over 20 years ago that flow cytometry and fluorescent dyes could detect a urinary tract infection (defined by the presence of 10,000-100,000 organisms/mL) and determine whether the cause was a Gram-positive (e.g., *Staphylococcus saprophyticus*), or Gram-negative (e.g., *E. coli*) bacterium or a fungus (e.g., Candida albicans) within 10-15 minutes, and determine susceptibilities for bacterial infections in a 60-90 minute (2-3 generation) time frame. In the 1980s, the cost and complexity of the flow cytometer made the concept infeasible.

A minimalist fluorescence image cytometer could now easily be placed at the point of care (hospital, clinic, private practice, nursing home) and be operated by personnel with minimal training.

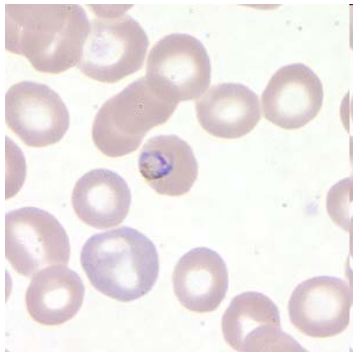
MALARIA DIAGNOSIS - MORE THAN MEETS THE EYE

Malaria deaths are estimated at 800,000/year; the victims are predominantly children and pregnant women. Most deaths are due to *Plasmodium* falciparum; other human malaria parasites (*P. vivax, P. malariae, P. ovale,* and *P. knowlesi*) are typically less deadly. As is the case with TB, a human observer cannot scan enough fields to detect pathogens present at low levels (5-50 parasites /μL) in the specimen! The observer typically examines a slide until no more than 500 white cells (WBC) are observed. With a typical WBC count of 8,000/ /μL, no more than 3 parasites will be detected under typical conditions; the Poisson statistical probability that none will be seen is about 8 per cent. Although fluorescence microscopy permits scanning at lower magnification, allowing more fields to be examined, the observer must still rely on morphology to detect parasites. An automated microscope, working at 250x-450x magnification, is too expensive and too large to be used for malaria diagnosis in a resource-poor area, as are flow and laser scanning cytometers, which have been shown otherwise effective. A low-resolution fluorescence imaging cytometer, capable of collecting information from an area of the slide at least 1 x 1 cm, corresponding to a volume of several microliters of blood, should be usable for malaria diagnosis if suitably specific reagents can be found. This could provide accurate and precise information about **parasite density** (the number of parasites/μL blood) relevant to monitoring treatment and to determining the efficacy of antibody-based and molecular diagnostic tests, drugs, and vaccines.

Plasmodium falciparum

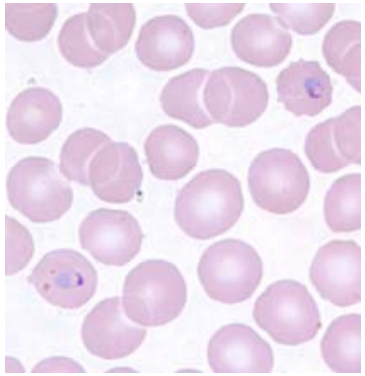


ring forms



trophozoite

Plasmodium vivax



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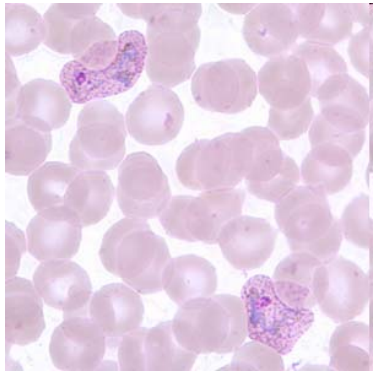
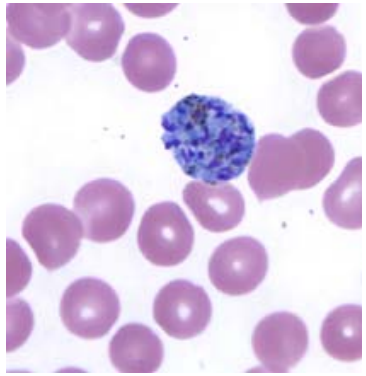
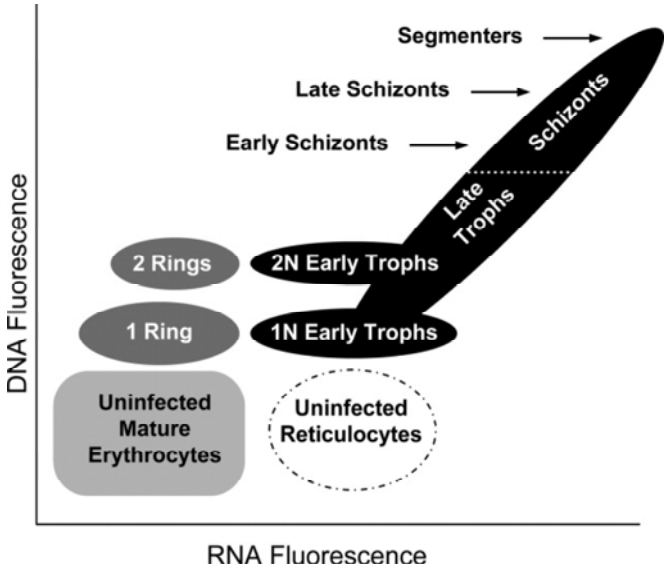


trophozoites



schizont

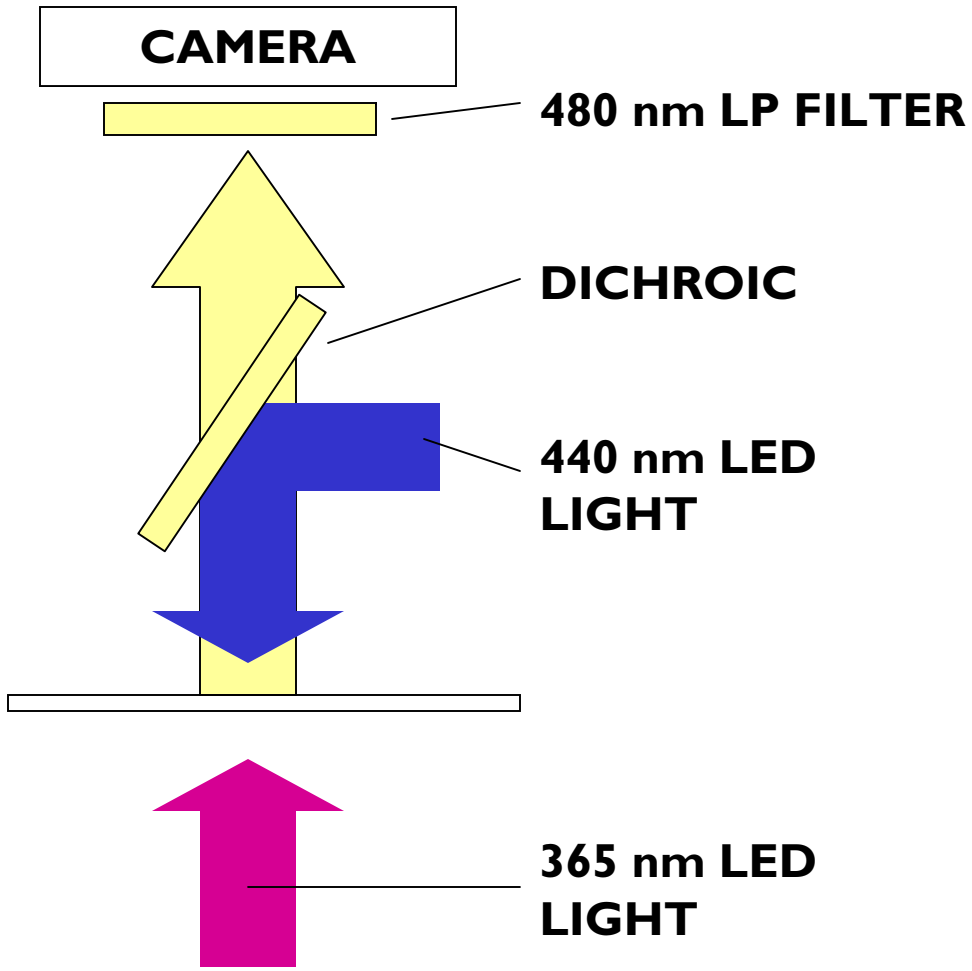
Different stages of development of malaria parasites in blood (earliest at left) are shown here in Giemsa-stained thin smears. Later stages of *P. falciparum* typically are not found in blood, whereas later stages of other species are. Because the early stages are so small, it is hard to automate their detection and identification by microscopy, even at high magnification.



CYTOMETRIC APPROACHES TO MALARIA DIAGNOSIS

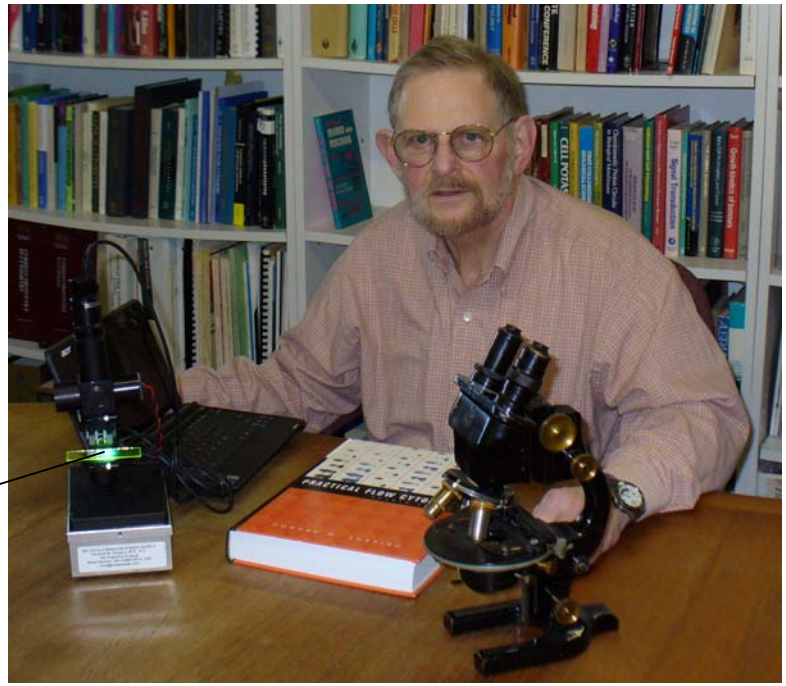
The Giemsa stain (1904), stains DNA, but not stoichiometrically; however, quantification of DNA and RNA was shown in 1986 (Hare, J Histochem Cytochem 34: 1651) to identify species and stages of parasites without morphologic information and could be done using lo-res fluorescence imaging.The image at left, (from Grimberg BT, EricksonJJ, Sramkoski RM, Jacobberger JW, Zimmerman PA; Cytometry Part A, 2008; 73A: 546-554), shows different stages of parasite development identifiable by Hoechst 33342/ thiazole orange DNA/RNA staining. It should also be possible to distinguish infection by *Plasmodium falciparum*, the most deadly of the human malaria parasites, from infection by other species, using combinations of nucleic acid dyes, and it is likely that addition of protein and/or lipid stains would allow discrimination among other species. WBCs in the specimen can also be counted in either case, allowing more accurate and precise determination of parasite density than can be accomplished by microscopy.

The configuration shown schematically at right should be optimal for detecting malaria parasites in blood films and for discriminating *P. falciparum* from other species (*P. vivax, P. ovale, P. malariae, P. knowlesi*) that infect man. It could also be used for cytometric determination of growth inhibition of parasites by drugs and antibodies, offering the demonstrated advantages of flow cytometry in a much cheaper, simpler, sturdier instrument usable even in many poor countries.



THIS IS A TOY!

THIS IS NOT!



OTHER LO-RES IMAGING APPLICATIONS

In addition to cell-based assays, the instruments can be used for multiplex bead-based chemical analyses. They should be cost-effective for such tasks as food and water microbiology and biowarfare agent detection as well as for clinical applications. Making the capacities of a benchtop flow cytometer available for less than a tenth of the price will also open other markets worldwide.

In hematology, WBC and RBC counting, differentials, and possibly reticulocyte counts could be approached using low-resolution imaging.