
Review Article

“Cellular Astronomy”—A Foreseeable Future in Cytometry

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Advances in electro-optic technology within the past 2 years, notably the development of high-intensity light-emitting diodes and highly efficient charge-coupled device cameras, have made it feasible to produce small, simple, rugged, automated fluorescence image cytometers, with selling prices well below US \$10,000, that can make measurements previously the exclusive domain of flow and scanning cytometers costing many times more. It should be feasible to apply the new cytometric technology in

scientific and geographic areas for which a previous generation of instruments was too complex and too expensive, e.g., to problems of diagnosis and management of infectious diseases prevalent at critical levels in resource-poor areas, such as the human immunodeficiency virus, malaria, and tuberculosis.

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Until the mid-20th century, determining whether cells were present in a biological specimen, and how many and what kind(s) were there, required that a human observer interpret a microscope image. Thereafter, tools developed by chemists and physicists for light measurement were adapted for use with the microscope, giving rise to cytometry, which now encompasses a collection of technologies that assist or supplant the human observer in performing numerous tasks relevant to biomedical research and clinical medicine (1–5).

Clinical problems provided much of the early motivation and funding for the development of cytometry. By the 1960s, scanning microscope systems were being used in attempts to automate Papanicolaou smear analysis for cervical cancer screening, on the one hand, and the differential white blood cell count, on the other. These tasks initially required analysis of high-resolution cell images. However, the specimen transport and imaging hardware available at the time, and the limited processing speed and storage capacity of even the largest computers of that era, hindered the development of practical instruments for clinical use.

Simpler cell detection and counting tasks were amenable to solution using flow cytometers; single-parameter optical and electronic instruments, developed in the 1940s and 1950s, proved usable for counting red and white blood cells, platelets, and microorganisms. Since

the 1970s, increasingly sophisticated multiparameter flow cytometers have been used to perform the traditional differential count and, with the aid of specific reagents such as fluorescently labeled monoclonal antibodies, to discriminate leukocyte subtypes not reliably identifiable on conventionally stained smears of blood or bone marrow.

Advances in electronics, electro-optics, and computers between the 1960s and the 1980s made it possible to develop scanning laser cytometers and imaging cytometers capable of performing many tasks previously done by flow cytometry. Until recently, these instruments, like flow cytometers, have been relatively large and expensive, restricting their use to large institutions in affluent countries.

Beginning in the 1980s, the worldwide epidemic of the acquired immunodeficiency syndrome provided flow cytometry literally and figuratively with what the computer industry calls a “killer application.” Fluorescence flow cytometry became the “gold standard” method for performing the counts of CD4⁺ T lymphocytes used to assess

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disease progression and response to therapy. Now that it is planned to make effective drug therapy available to millions of individuals with the human immunodeficiency virus in resource-poor countries in Africa, Asia, the Caribbean, and South America, there is a need for affordable cell-based diagnostic procedures. These include white cell, differential, platelet counts, and $CD4^+$ lymphocyte counts, tests for detection and antimicrobial sensitivity of tuberculosis and for detection and monitoring of malaria and other parasitic diseases. It is therefore appropriate to consider how technologies available in this new century might best be used to develop a new generation of cost-effective cytometric apparatus.

TASKS IN CLINICAL CYTOMETRY

Almost all tasks in clinical cytometry involve identification and counting of one cell type or another. The complete blood count (CBC) routinely performed in patients admitted to a hospital, which includes several examples, is now done by an automated apparatus in most developed countries. In the places that most need new technology, CBCs, if they are done at all, are often done using equipment that quickly became all too familiar to the author a little over 40 years ago, when he arrived on the wards of Bellevue Hospital as a third-year medical student. An eyewitness account may help to illustrate how we got to where we are now; some additional historical details are available elsewhere (1,5,6).

An overworked intern introduced himself, pointed me at a patient, and told me to do a CBC. After inflicting physical trauma on the patient and emotional trauma on myself, I managed to obtain an anticoagulated tube containing a few milliliters of venous blood and disappeared around the corner to the "scut lab." Some 45 min later, the intern appeared in the doorway and, shortly after asking what the expletive was taking me so long, observed that I was trying to do a red blood cell count using a hemocytometer. This had seemed entirely reasonable to me, as I had learned in the second-year clinical pathology course that a CBC included a red cell count, a white cell count, and a white cell differential count. "You idiot!" said the intern, assuming his best pedagogical demeanor. "A CBC is a hematocrit, a white count, and a differential!"

Counting Cells: The Hemocytometer and Poisson Statistics

As I was to learn over the course of the next few decades, a hemocytometer, although generally acceptable for estimating white cell counts, is not a practical tool to use for red cell counts. The problem is not so much in the gadget as in the nature of blood itself. A hemocytometer is a specially designed microscope slide on which a ruled grid defines square or rectangular areas, each with fractions of a millimeter on a side. Ridges on either side of the ruled area ensure that the thickness of the layer of diluted blood under the coverslip will be constant (typically 0.1 mm). For a white cell count, blood is typically diluted 1:20 with a solution that lyses red cells and stains white cells; the number of cells in four 1×1 -mm squares is then

counted. The total volume of diluted blood counted is therefore 0.4 mm^3 , or $0.4 \mu\text{l}$. To count the number of white cells per microliter, this number is divided by 0.4 (the volume counted, in microliters), and the result is then multiplied by 20 (the dilution factor). Because white cell counts in healthy individuals normally range from about 5,000 to about $10,000/\mu\text{l}$, one would actually count between 100 and 200 white cells in the hemocytometer.

Whether one counts cells, photons, or votes, the relation between the number of objects actually counted and the precision of the result is given by what are now known as Poisson statistics. The subject was investigated in the early 1900s by William Sealy Gosset, who happened to be using a haemocytometer (appropriate orthography for his side of the Atlantic) to count Brewer's yeast rather than blood cells. Because Gosset's employers at the Guinness Brewery felt his contributions gave them an edge in the production process, he was obliged to publish his work under the pseudonym "Student." In his 1907 paper (7), he calculated that the standard deviation of an otherwise perfect cell count in which n cells were actually counted would be $\sqrt{n}$ (i.e., $n^{1/2}$). Much earlier work, not involving cell counts (8) and not known to "Student," resulted in Poisson's name being associated with this result in a 1914 paper by Soper (9).

A white blood cell count of $5,000/\mu\text{l}$ obtained with a hemocytometer is, as noted above, based on an actual count of 100 cells. According to Poisson statistics, the minimum standard deviation associated with this count is represented by $(100)^{1/2}$ cells, or 10 cells; the coefficient of variation (CV), i.e., the standard deviation divided by the mean (which is represented by the actual count), is therefore $10/100$, or 10%. The CV in the derived count is the same as in the actual count, 10%, in this case representing 500 of 5,000 cells. If the white blood cell count were $10,000/\mu\text{l}$, 200 cells would have been counted in the hemocytometer; the associated minimum standard deviation is $(200)^{1/2}$ cells, or 14.1 cells, resulting in a minimum CV of just over 7%. To reduce the minimum CV to 5%, it is necessary to count 400 cells; to reduce it to 1%, it is necessary to count 10,000 cells.

Because blood typically contains many more red cells than white cells ($5,000,000/\mu\text{l}$ is a typical value), a blood sample is diluted 1:200 (without lysis, obviously) for red cell counts done in a hemocytometer, and a smaller area of the grid is used for counting. The standard apparatus for diluting blood in olden times was a Sahli pipette. Using a mouthpiece connected to the pipette by rubber tubing, one would suck up a small amount of blood, stopping when the level reached a mark engraved near the tip of the pipette, and then aspirate a larger amount of diluent, stopping at a second engraved mark higher up. A portion of the pipette between the two marks had a larger internal diameter to hold most of the volume of diluted sample and keep the length of the pipette manageable. Sahli pipettes are not widely used in affluent societies these days, at least as much because of their inaccuracy as because few rational individuals would consider mouth pipetting anyone's blood but their own. Even if one counted 400 cells in

performing a red cell count, dilution errors associated with pipetting would typically keep the CV of the measurement above 7%.

From a clinical point of view, it is less important to know the exact count of red blood cells per microliter than to get a fairly good idea of the oxygen-carrying capacity of the blood. This can be done by measuring the amount of hemoglobin per unit volume of blood or by measuring the hematocrit, which is the fraction of total blood volume made up of red cells. Measuring hemoglobin requires lysis and other chemical treatment of blood, followed by analysis in a colorimeter. Determining the hematocrit requires centrifuging a capillary tube of blood for a few minutes and subsequently finding the position of the top of the column of packed red cells relative to the position of the meniscus of plasma at the top of the tube. In the 1960s, hematocrit centrifuges were cheaper than hemoglobinometers, so we used the former in the scut lab. My father's generation of physicians, trained 30 years earlier, did not even have hematocrit centrifuges when they were medical students; instead, they estimated hemoglobin content by visual comparison of a diluted blood sample to a color chart.

The hematologists at Bellevue, who needed to know the number, size, and hemoglobin content of red cells to identify the causes of anemias, had already acquired an early model Coulter counter, which provided them with accurate and precise red cell counts. During the late 1940s and early 1950s, groups in Germany and England had developed cell counters in which light scattering or extinction signals from cells flowing through a small-bore channel were detected and processed; the most sophisticated of these incorporated the sheath flow principle familiar to users of modern flow cytometers. Wallace Coulter, an electrical engineer by training, was initially frustrated in attempts to build an electro-optical cell counter by his limited knowledge of optics. Later, having learned that cells are relatively poor conductors of electricity, he developed an apparatus in which cells could be detected, and their volumes measured, by the change in electrical impedance the cells produced while passing through a small saline-filled orifice. Various modern hematology analyzers use optical and/or electrical measurements to count and characterize blood cells; because the clinically relevant data are counts per unit volume of blood, these instruments are equipped to deliver known volumes of sample to the measurement system. Even the early automated blood cell counters could count hundreds of cells per second; therefore, a count of 10,000 cells could be obtained in significantly less time than was needed to count 100 or 200 cells in a hemocytometer.

Cell Discrimination and Differential Counts: Pattern Recognition

Many of the techniques for cell identification used in the scut lab and in early hematology counters are still in use today, but it remains true that different methods, visual and instrumental, define their target cell populations in different ways. To this point, I have neglected to

note that a microliter of blood typically contains several hundred thousand platelets in addition to a few thousand white cells and a few million red cells. When one counts red cells in a hemocytometer, it is easy to discriminate them visually from white cells by their hemoglobin content and from platelets by their hemoglobin content and their larger size. When one counts red cells in a Coulter counter, the only information available is an electrical signal proportional to cell volume. It is possible to set the threshold of the instrument to exclude all or almost all platelets, but the white cells, which are larger than red cells, are typically also counted. Because there are normally only one or two white cells for every thousand red cells, this does not substantially affect the accuracy of the red cell count. However, in conditions such as chronic lymphocytic leukemia, in which lymphocyte counts may reach several hundred thousand per microliter, red cell counts can be in error by a substantial amount.

When white cells are counted in a hemocytometer or a Coulter counter, red cells are lysed, although different lysing reagents are used in the two procedures. Red cells rarely resist lysis by the dilute acetic acid used for hemocytometer white cell counts; if they did, they could be identified by their pigment content. Red cells are somewhat more likely to resist lysis by saponin or detergents used with automated counters, and persistent red cells give rise to artifactual increased white cell counts. Under some circumstances, white cells may be lysed by red cell lysing reagents, thereby producing white counts lower than the actual value. In manual and automated procedures, white cells are discriminated from platelets on the basis of size.

From the late 1890s until the 1970s, white cell differential counts were based on examination of the morphology of cells in thin blood smears stained with combinations of eosin, a red acid dye that stains proteins and other basic cell constituents, and various methylene azure dyes, which, depending on concentration, impart a blue or a purple color to regions of cells containing nucleic acids and/or acid glycosaminoglycans. The white cells in normal peripheral blood include granulocytes and mononuclear cells; the latter are divided into lymphocytes and monocytes. Granulocytes typically have lobed nuclei and cytoplasmic granules; the granules in neutrophils, the predominant granulocytes, stain equally with eosin and the azure dyes, whereas the granules in the rarer eosinophils stain predominantly with eosin, and those in the even rarer basophils accumulate sufficient azure dye to assume a metachromatic purple color. Lymphocytes are typically smaller than granulocytes, with scant cytoplasm and relatively round nuclei; monocytes are typically larger than granulocytes, with larger, irregularly shaped nuclei. It was known by the early 1970s, well before blood cell taxonomy was refined with the aid of monoclonal antibodies, that morphologic characteristics discernible from conventionally stained blood smears are insufficient to distinguish even between T and B lymphocytes, let alone to identify lymphocyte subsets. In fact, it had been estab-

lished that discrimination between lymphocytes and small monocytes on smears is unreliable.

This did not much matter in the scut lab; we had no alternative but to look at smears, using high-dry or oil-immersion objectives, until we had examined a total of 100 white cells and dutifully report the percentages of each of the five major white cell types or, more accurately, six major white cell types; we distinguished immature "band" neutrophils from mature segmented neutrophils based on nuclear morphology. It took more skill than the average third-year medical student possessed to identify "abnormal" white cells in peripheral blood; these might be cell types normally restricted to the bone marrow, such as nucleated red cells and immature white cells, or leukemic blast cells, or cells involved in immune reactions, such as "atypical" lymphocytes or plasma cells.

By the mid-1960s, several research groups were attempting to develop automated differential counters that would process scanned images of cells from conventional stained smears by identifying the normal white cell types based on morphologic information and "flagging" samples containing abnormal cells or abnormal cell percentages. In 1967, I left Bellevue for the National Cancer Institute, where my colleagues and I were trying to solve the more difficult problem of identifying leukemic blast cells on stained autoradiographs and estimating their growth kinetics by counting the silver grains overlying the cell nuclei. Over the next few years, we spent a couple of hundred thousand dollars of United States taxpayers' money on building what was then the world's fanciest computerized microscope (10). It incorporated a mini-computer that controlled a microscope stage and focusing, selected wavelength and slit width in a monochromator that provided illumination from a xenon arc lamp, and collected a 256×256 pixel, 64-level gray-scale image from a single cell, scanned by a galvanometer scanner, in a blazing fast 2 min. We knew this hardware would never be fast enough for clinical use, but we hoped that we would be able to collect enough information to develop cell identification algorithms that could be used when faster hardware became available. However, even with high-resolution, multiple-wavelength, image information, we did not really have reliable ways of discriminating the blasts from everything else.

The "pattern recognition" problems that need to be solved in optical character recognition and automated cell identification from images involve two distinct phases of analysis. In the first phase, feature extraction, a set of numerical values representing various attributes of a character or cell is derived from the image. In the second phase, the identity of the cell or character is determined by calculating one or more "discriminant functions" using the numbers collected during feature extraction.

When cells are stained with relatively nonspecific dyes, such as the eosin-azure mixtures (e.g., Giemsa's and Wright's stains) used for classical differential white cell counting, the only features that can readily be extracted from images quantify morphologic characteristics such as cell and nuclear size and shape and nuclear and cytoplas-

mic color and texture or granularity. These are the same characteristics on which human observers rely for identifying cells, and the performance of discriminant functions used for cell classification is almost always evaluated on the basis of their agreement with cell identifications by hematologists, pathologists, or other expert observers.

In performing a differential white cell count, we "differentiate," or discriminate, one type of white cell from another. The white cells also "differentiate" in the embryologic sense; like all other cells in the body, they share a common ancestor in the fertilized ovum, and, like red cells and the megakaryocytes from which platelets are derived, they descend from hematopoietic stem cells, the nature and very existence of which were hotly debated before the advent of modern cytometry. The white cells in peripheral blood are almost all mature, and those of one lineage are readily distinguished from their distant cousins of another on the basis of morphologic characteristics. Morphology serves us less well in determining the lineage of the more primitive blood cell precursors normally found in bone marrow, and, as was mentioned previously, the classic stains give us little or no help in categorizing peripheral blood lymphocytes.

Thus, although my colleagues and I could not readily identify leukemic cells, the people working on automated slide-scanning differential counters had, by the early 1970s, produced instruments that could do a 100-cell white cell differential count in a minute or two, and these instruments were adopted by hospitals and clinics. By the mid-1980s, however, these slide-scanning systems had largely been replaced by flow cytometric hematology counters.

The multiparameter optical flow cytometer, as developed by Louis Kamensky in the 1960s, was conceived as representing the only practical means available at the time for automating detection of abnormal cells in cervical cytology specimens. The gold standard method, then and now, is analysis of cells on slides after staining with Papanicolaou's (Pap) stain. This, like the classic blood cell stains, is a mixture of acid and basic dyes, none of which is particularly specific; the interpretation of a "Pap smear" therefore requires assessment of morphologic characteristics of cells. Kamensky's expertise was in image analysis; he had developed optical character recognition equipment for IBM and was then asked by IBM management to look into building a device that could deal with Pap smears.

Being familiar with the computational and storage capacities of 1960s-vintage computer hardware, Kamensky was quick to realize that even a multimillion-dollar mainframe might not be able to extract relevant features from images of cells on Pap smears sufficiently rapidly to be practical for clinical use. Having been informed by pathologists that cell size and nucleic acid content were relevant features for identification of abnormal and neoplastic cells, he investigated whether these could be measured without recourse to relatively high-resolution optical microscopy and adapted procedures for whole-cell microspectrophotometry, originally developed by Torbjörn

Casperson in Stockholm, to analysis of cells flowing through a small channel on a slide. Kamensky's original instrument was a two-parameter optical flow cytometer that estimated cell size from light scattering (actually using an extinction signal) and nucleic acid content from absorption of ultraviolet light at 260 nm. Like some early hematology counters, this Rapid Cell Spectrophotometer (11) did not have sheath flow. It did, however, incorporate a dedicated minicomputer for data analysis, thereby facilitating expansion to four measurement parameters; eventually, a fluidic sorter was added (12), which allowed cells to be isolated and reexamined to confirm identifications made by the instrument.

FLOW CYTOMETRY: MAKING MORPHOLOGY IRRELEVANT?

Flow cytometry demonstrably streamlined the feature extraction process in cell analysis but did not necessarily compensate for the concomitant loss of most or all morphologic information. The successes of flow cytometry over the years have depended to a great extent on the development of multiparameter instruments and analysis methods and of highly specific fluorescent reagents (5,13). Modern flow cytometers often achieve their remarkable specificity and precision in cell identification and characterization without benefit of morphologic information, by making measurements of the intensities of light scattered by, and fluorescence emitted by, cells or other particles. The observation volume of a flow cytometer is, as often as not, substantially larger than the volume of the cells of interest; indeed, flow cytometers have been used to detect signals from single virus particles, fluorescently stained nucleic acid fragments, and other objects well below the resolution limit of optical microscopes.

The first flow cytometric differential white cell counter, developed by Leonard Ornstein and colleagues in the 1970s (6,14,15), incorporated three separate flow cytometers. In one, a cytochemical stain for peroxidase, detected by scattering and absorption measurements, was used to identify neutrophils and eosinophils. In the second, monocytes were identified by a cytochemical stain for esterase; in the third, basophils stained with Alcian blue were detected by differential light scattering at two wavelengths. Lymphocytes were identified by their scatter signature and lack of peroxidase. Other flow cytometric differential counters have used and currently use different strategies for cell identification by employing various combinations of direct- and alternate-current impedance, absorption, extinction, and light scattering measurements. Until recently, however, fluorescence measurements have not been used in differential counters.

There is some irony in this; in the 1970s, it was difficult for flow cytometric differential counters to compete with slide-scanning systems, despite the demonstrable accuracy of the flow systems and the greater precision resulting from their ability to count many more cells. Hematologists tended to trust classic staining procedures. By the mid-1980s, fluorescence flow cytometry, cell sorting, and monoclonal antibodies had made a dramatic impact on

research in hematology and immunology, and the flow-based differential counters began to be viewed as trustworthy, even though they were not measuring fluorescence. Since that time, a continually expanding repertoire of monoclonal antibodies to differentiation antigens, an ever more colorful palette of fluorescent labels, and increasingly sophisticated fluorescence flow cytometers have made it possible to identify many more lineages and sublineages of immature and mature white cells than were contemplated by the hematologists of the 1960s. Remarkably, all of this is done without the type of morphologic information for which we need imaging. The most sophisticated fluorescence flow cytometers also typically measure small-angle or forward light scatter, often erroneously referred to as a "size" measurement, and large-angle or side scatter, which provides information about internal granularity and surface roughness but does not resolve cellular detail. All the other information needed for cell identification comes from the intensity of fluorescence measurements using different excitation and emission wavelengths.

"CELLULAR ASTRONOMY": AN ALTERNATIVE CYTOMETRIC PARADIGM

Those of us who perform flow cytometry are now, in essence, doing "cellular astronomy." Astronomers cannot resolve structural details of any star except the Sun; all the information that enables them to characterize other stars comes from measurements of the intensities of emitted electromagnetic radiation at various wavelengths and from the temporal variation in emission patterns. To collect this information, astronomers most commonly use digital imaging hardware, specifically cooled charge-coupled device (CCD) cameras, but they have, appropriately, become "big pixel thinkers."

Most of us in cytometry have not. CCD cameras are widely used for analysis of cells, but almost always in the context of resolving subcellular details; give us a CCD and we instinctively try to get morphologic information we may not need. This requires working at relatively high magnification, which necessitates the incorporation into instruments of hardware and software for controlling focus and stage motion. As magnification increases, the viewable area decreases; it therefore takes longer to collect images of a given number of cells, and, as the number of pixels in a cell image increases, the software required for feature extraction becomes more complex.

The highest levels of optical resolution in image cytometry are obtained from confocal and multiphoton confocal microscopes, which are used for research applications in which high sample throughput is generally not of concern to the experimenter. Lower resolution scanning laser cytometers of various degrees of complexity (16-18) are considerably faster, although not as fast as flow cytometers, and can acquire low-resolution information about cell morphology.

Although flow cytometers are now capable of measuring fluorescence in 12 or more wavelength regions and light scattering at two or more angles, many applications

require measurement of only one or a few parameters. With an appropriate choice of reagents, one can count cells, identify white cells and various subclasses (e.g., $CD4^+$ T lymphocytes), determine cellular DNA content, or detect and count bacteria and parasites in clinical and environmental samples. However, even the simplest flow cytometers designed for such applications incorporate a certain irreducible level of complexity and an associated cost. Because imaging hardware (notably CCD cameras), efficient light sources (notably light-emitting diodes [LEDs]), and personal computers have decreased dramatically in price in recent years, it seems appropriate to ask whether a less complex, less expensive, low-resolution "cellular astronomy" imaging instrument incorporating these components could be used to perform at least some of the same cytometric tasks.

A first bold step in this direction was described by Wittrup et al. in 1994 (19). They built an apparatus, the Fluorescence Array Detector (FAD), with only one moving part (a focusing stage), in which camera lenses were used to form a 1:1 image of a 1×1 -cm field of view on a cooled 512×512 -pixel CCD detector with $20\text{-}\mu\text{m}^2$ pixels. Because each pixel collected light from an area larger than the area of a typical cell, no morphologic information was available. Low-intensity (1 mW/cm^2) illumination of the field came from the expanded beam of a 488-nm air-cooled argon ion laser. Rather than use an epi-illuminated configuration in which the fluorescence collection lens also transmitted excitation light, these investigators chose to keep the illuminating beam external to the collection lens to intercept the surface of the specimen at Brewster's angle and minimize light scattering. A software shading correction was used to compensate for the uneven illumination obtained from the laser beam. However, the CV of the fluorescence intensity distribution of $6\text{-}\mu\text{m}$ polystyrene beads was reported to be 12.9%; this relatively large variance was attributed to imperfect shading correction. Sensitivity was impressive; noise due to dark current and stray light was equivalent to only a few hundred fluorescein molecules of equivalent soluble fluorochrome per pixel, although fluorescence from conventional glass microscope slides increased background approximately 10-fold.

At the time the FAD was constructed, a cooled CCD camera cost tens of thousands of dollars, and a 50-mW air-cooled 488-nm argon laser cost at least \$8,000, thus eliminating the instrument from consideration as a low-cost replacement for a flow cytometer. Argon-ion lasers and solid-state lasers emitting at the same wavelength still cost thousands of dollars. In retrospect, the laser used in the FAD was a much less than ideal light source for the application. A laser is a relatively low-noise, high-intensity source of monochromatic light that is particularly useful in confocal and laser scanning microscopes and flow cytometers because almost all of the energy in the beam can be focused to a spot as small as a fraction of a micrometer in diameter. However, when a laser is used to illuminate an area substantially larger than its original beam diameter, as was the case in the FAD, it is difficult to obtain uniform

illumination due to the underlying intensity distribution (Gaussian in this instance) and to speckle effects resulting from the coherent nature of the light. Poor illumination uniformity was responsible for the FAD's unimpressive precision. In 1994, an arc lamp would probably have been the only feasible alternative to a laser as the light source for an instrument similar to the FAD. It has since been shown (20) that mercury arc illumination is sufficient for detection of fluorescence from single molecules by using a relatively inexpensive (about \$3,000) cooled CCD camera and conventional epi-illumination microscope optics; the integration time required is on the order of 100 ms. However, arc lamp sources and their associated optics and power supplies still cost thousands of dollars.

A less ambitious, but much less expensive and more practical low-resolution imaging cytometer was introduced as a commercial product in 2002. The NucleoCounter (ChemoMetec A/S, Allerød, Denmark) is a small benchtop cell counting instrument that incorporates a transmitted light fluorescence microscope illuminated by an array of eight green LEDs. Cell samples are introduced into the system in plastic carriers containing propidium iodide; the construction of the carrier allows cells in the sample to mix with the dye before entering a windowed area approximately 10 mm^2 in which they are imaged onto a CCD camera at low magnification and are counted with simple image analysis software. Total cell counts are obtained by adding a lysing agent to the sample before its introduction into the chamber; if the lysing agent is omitted, a count of "nonviable," i.e., membrane-damaged cells, is obtained, and subtraction of this value from the total cell count yields a "viable" cell count. The NucleoCounter can be bought for approximately \$8,000; sample carriers are a few dollars each.

The observation chamber of the NucleoCounter is essentially a large-volume hemocytometer, which, at run time, contains a volume of diluted sample corresponding to $1\text{ }\mu\text{l}$ of the original specimen. The volume of blood counted when a standard hemocytometer is used for white cell counting by visual observation is $0.02\text{ }\mu\text{l}$. The larger observation volume of the NucleoCounter facilitates precise determination of relatively low cell counts.

If one were to use a fluorescence microscope and a standard hemocytometer to count $CD4^+$ T lymphocytes in a patient with the acquired immunodeficiency syndrome whose blood contained 200 such cells per microliter, assuming the staining procedure diluted the blood sample 1:20, it would be necessary to add the results of 25 counts with a standard hemocytometer to obtain the count of 100 cells needed to attain 10% precision. The task would be simplified if one used a Nageotte hemocytometer, which provides a sample volume of $50\text{ }\mu\text{l}$ ($10 \times 10 \times 0.5\text{ mm}$) and is typically used for counting rare cell types (21). Assuming a 1:20 dilution of the blood sample, scanning the entire observation area of the Nageotte hemocytometer would yield a count of the cells from $2.5\text{ }\mu\text{l}$ of blood, or 500 cells. Although it is perfectly feasible, if somewhat tedious, for a human observer to do this, it is not feasible to do counts by visual observation at low enough magni-

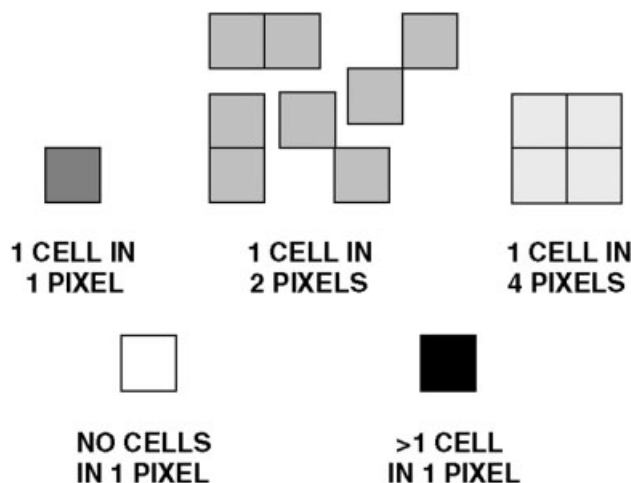


FIG. 1. Schematic illustrating comparative intensities of pixels representing none, part, or all of a cell or more than one cell in a low-resolution imaging system.

fication to eliminate the need to move the microscope stage during the procedure; and, even at the relatively low magnification typically used, it is usually necessary for the observer to adjust the focus of the microscope.

Detecting Cells in Cellular Astronomy

If a 1:1 image of the 10×10 -mm viewing area of a large-volume hemocytometer is made on a 512×512 -pixel CCD such as that used in the FAD, each of the 262,144 pixels, or "wells," of the CCD will collect light from a $20\text{-}\mu\text{m}^2$ area of the hemocytometer. This area is larger than the area of a typical cell and therefore may contain no cells, one cell, or more than one cell; it is also possible for light from a single cell to be collected in two pixels with a common edge or in four pixels with a common corner, as illustrated in Figure 1.

The first order of business in cellular astronomy, as in other types of cytometry, is defining the criteria for detecting cells. For the moment, assume we are working with an image of the intensity of fluorescence of a DNA-specific stain in resting peripheral blood lymphocytes. Thus, all of the cells are roughly the same size, smaller than $10\text{ }\mu\text{m}$ in diameter, and relatively round, and all should contain the same amount of dye. We will also assume that illumination of the entire field of view is uniform (or that the image has been corrected for non-uniform illumination). The convention used in Figure 1 is that increasing signal intensities are represented by darker shades of gray.

The wells in a CCD have a finite capacity for storing photoelectrons; if the capacity of a well is exceeded, the excess electrons may migrate to adjacent wells, producing a phenomenon known as "blooming." We will assume that our signal intensities will be kept low enough to avoid blooming in more than a small fraction of the wells. The storage capacity of the wells directly determines the dynamic range of measurements possible in a CCD. If, for

example, well capacity is 100,000 electrons and the background light level during a measurement accounts for 10 electrons being accumulated in each well, the measurement range is four decades, or 80 dB; these numbers are close to those specified for a popular CCD chip, the Kodak KAF-402ME (Eastman Kodak, Rochester, NY, USA), for which a 76-dB dynamic range is quoted. To avoid exceeding the storage capacity of wells in which signal from more than one cell accumulates, we need to adjust illumination and exposure time to keep the signal from a single cell below 50,000 electrons. This will maintain linearity if a single well captures all the signals from two cells; to accommodate signals from three cells, the signal level from a single cell would have to be kept below 33,333 electrons. Readers might recall that a similar adjustment of signal level is necessary in flow cytometry of DNA content; if G_2 - and M-phase cells are to be kept on a linear scale, it is necessary to keep the signal from G_0 - and G_1 -phase cells below half of full scale.

The "white pixel" seen in Figure 1 represents a background pixel, in which no signal from cells has been accumulated. We can define a "constellation" as a single pixel or a group of contiguous pixels with intensities above background and surrounded or "insulated" by background pixels. A constellation made up of a single black pixel results when the entire signal from at least two cells has been captured in a single well of the CCD. A constellation comprising a dark gray pixel is what would be expected if all the light from a single cell were collected in one well. The medium gray constellations of two pixels represent the situation in which light from one cell is split between two wells, and the light gray quad constellation exemplifies the case in which light from one cell is distributed among four pixels. It would be much less likely for light from a single cell to reach three pixels than one, two, or four, so the three-pixel case is not illustrated in Figure 1.

We have not yet considered how to determine what background level is. Cellular astronomy has a decided advantage over the real thing because cells are usually much easier to dilute and concentrate than are stars. Identifying individual cells in images of tissue remains a principal obstacle to automating many tasks in anatomic pathology, but the samples we are used to working with in flow cytometry are, for the most part, in what should ideally be single-cell suspensions. If we image a Nageotte hemocytometer containing a diluted sample representing $2.5\text{ }\mu\text{l}$ of blood, with a white cell count near the high end of the normal range, at $10,000/\mu\text{l}$, even in the extremely unlikely event that each of the 25,000 white cells falls on the intersection of four pixels, the remaining 162,144 pixels on the chip would remain at background level. The more dilute the sample, the less likely it becomes that two cells will fall in the same or adjacent pixels, unless they are adherent to one another.

Working with dilute samples thus provides a robust strategy for defining a threshold for cell discrimination based on a histogram of intensity levels of individual pixels. Because most pixels in the image will be at back-

ground level, these pixels will account for a large peak at the low end of the histogram, and we can set an ad hoc threshold level at the first minimum above this peak.

At the high end of the scale, there will be a peak corresponding to pixels with signal levels representing the maximum well capacity; these and pixels adjacent to them should be excluded from analysis as instances of blooming, which should not occur unless a pixel captures signals from two or more cells or from a noncellular source of interference.

All pixels with intensities in the range between the threshold level and the top of the scale can then be identified as constituents of constellations. Any constellation comprising more than four pixels, or four pixels not arranged in a quad, must represent two or more cells; such constellations, like those containing pixels with intensities at the top end of the scale, may be identified as "coincidences" but are best eliminated from further analysis. In cellular astronomy, as in flow cytometry, a high fraction of coincidences should prompt the experimenter to dilute and/or filter the sample and/or to take appropriate measures to reduce cell aggregation.

Note that the procedure just described does not depend on the background intensity level being at or near zero, as long as there is reasonably good contrast between the intensity of the cellular "trigger" signal and the background. So, for example, white cells can be detected based on the fluorescence of a DNA-specific dye even when they are surrounded by red cells, as long as these do not interfere with the excitation of the dye or the detection of its emission.

Doublet Discrimination in Cellular Astronomy and Flow Cytometry

Nuclear staining has been used previously in flow and image cytometries to facilitate analysis of blood samples without the need for red cell lysis. DNA staining is particularly useful for doublet discrimination in work with unstimulated peripheral blood cells because, under normal circumstances, more than 98% of the cells have a G_0/G_1 DNA content. When flow cytometry of DNA content is done on growing cell populations, comparison of peak and integral signals and/or mathematical modeling may be used to identify doublets. However, many immunophenotyping applications of flow cytometry are routinely performed without the inclusion of any doublet discrimination procedure.

Doublet discrimination in flow cytometry based on comparison of peak and integral signals is not generally applicable when the cells or particles in the sample are substantially smaller than the illuminating beam height; because optical design considerations generally prevent use of beam heights lower than $5\text{ }\mu\text{m}$, an alternative strategy is needed to discriminate single bacteria from aggregates (22). Nucleic acid staining may be sufficient for this purpose in flow cytometry and cellular astronomy, and the discrimination task may be unnecessary when the object of analysis is the detection of "colony forming

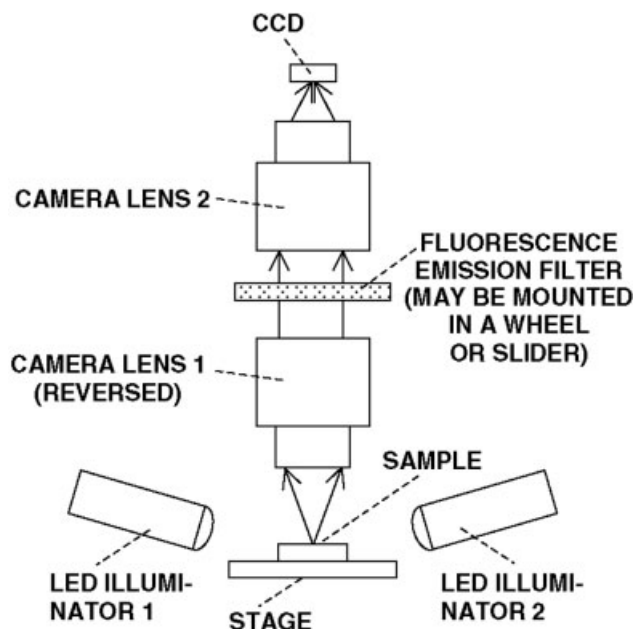


Fig. 2. Optical layout of a low-resolution "cellular astronomy" imaging cytometer.

units" of microorganisms, which may be single cells or aggregates.

ANATOMY OF A "CELLULAR ASTRONOMY" IMAGING CYTOMETER: CONFIGURATION AND COST

At present, the most practical CCD detector for the low-cost instrument illustrated in schematic form in Figure 2 may be Kodak's microlensed KAF-0402ME, with 768×512 pixels, each $9\text{ }\mu\text{m}^2$, and a peak quantum efficiency of 77%. This chip, with a capacity of 100,000 electrons/well, allows a measurement dynamic range of just under four decades for each pixel and is available without major defects for around \$250. Camera electronics and mechanics and a Peltier cooler and controller cost no more than a few hundred dollars; complete cooled camera systems incorporating the KAF-0402ME can now be bought for \$1,495 (Model ST-7XMEI, Santa Barbara Instrument Group, Santa Barbara, CA, USA).

We want the pixel size in the specimen to be about $20\text{ }\mu\text{m}^2$, i.e., larger than a typical cell, and matching the "pixel size" or observation area typically used in a flow cytometer. The image on the chip must thus be approximately half the actual size of the cell. This is more the province of close-up or macrophotography than of microscopy, and the time-honored technique of using two lenses, one reversed, as was done in the FAD (19), is effective here for several reasons. The fluorescence emission filter is placed between the lenses, where the collected light is essentially collimated. The 55 lines (or pairs) per millimeter of optical resolution required of the lenses is not difficult to obtain with good-quality photographic optics, which cost at most a few hundred dollars and

which, even at a relatively high f -number, achieve better numerical aperture than low-power microscope lenses ($f/4 = 0.13$ numerical aperture). Depth of field ($>100\ \mu\text{m}$) is sufficient to obviate focus adjustment, and the field of view is large enough to make stage motion unnecessary.

The long working distance of the optics allows the specimen to be excited by one or more LED illuminator modules, which incorporate collecting, collimating, and focusing optics and a filter to restrict excitation bandwidth. As many as four excitation modules, each emitting a different wavelength, can be mounted around the stage, with only one active at any time. By using Luxeon V high-intensity LEDs (LumiLEDs, San Jose, CA, USA) and inexpensive molded plastic optics (Fraen Corporation, Reading, MA, USA), it is possible to deliver over $10\ \text{mW}/\text{cm}^2$ at 450 to 490 nm to a $1.5 \times 1\text{-cm}$ viewing area with no more than 20% variation in intensity. Each LED excitation module should cost no more than \$150, including a regulated power supply suitable for operation from line voltage or a battery; the excitation filter would account for at least half of the cost. High-power (3- and 5-W) Luxeon LEDs are available with peak emission at wavelengths ranging from about 460 nm to about 530 nm; 1-W LEDs with peak emission at around 590, 610, and 630 nm are also available, and a high-intensity ultraviolet emitter is due to be added to the product line some time in 2004.

The use of external excitation rather than epi-illumination eliminates the need for dichroics. For the simplest tasks, one might need only a single, fixed emission filter; for multicolor measurements, it would be most practical to mount multiple filters in a wheel driven by an inexpensive stepper motor. The mechanical and electronic components needed would probably cost no more than \$100, and filters should be no more than \$100 each.

If only a single excitation wavelength and a single emission wavelength are needed, it is relatively easy to implement a "cellular astronomy" instrument on an epi-illuminated fluorescence microscope with an LED substituted for an arc or halogen lamp as the light source and standard fluorescence excitation blocks incorporating excitation and emission filters and a dichroic. Imaging is done with a $4\times$, 0.1 numerical aperture lens; a video camera mounting adapter that reduces the image to approximately one-third size (Thales Optem, Fairport, NY, USA) yields a magnification of $1.33\times$ on the CCD chip, making the field of view smaller than that of the externally illuminated system using camera lenses but still providing a feel for the technology. If multiple illumination wavelengths are needed in a microscope-based system, it becomes necessary to provide motion control for LEDs, excitation filters, and dichroics and for emission filters, which makes the mechanics of the apparatus somewhat more complicated than they are in a system using external LED illuminators. Further, because the illumination in a microscope-based system comes through the imaging lens, fluorescence induced in this lens by the illumination is more likely to interfere with measurements than is the case in an externally illuminated system.

An inexpensive laptop computer, which has on the order of 1,000 times the processing capacity and storage of a 1960s mainframe, is more than adequate for instrument control, data collection, processing, and communication; a production instrument could have an inexpensive single board computer built in. The component costs appear compatible with a selling price of a few thousand dollars for the simplest instruments (one light source, one emission wavelength range) and well under \$10,000 even for more complex systems with three or four excitation sources and five or more emission wavelengths. The basic instrument could readily be adapted to scan microarrays on slides or, with the addition of low-precision stage motion control, samples in multiwell plates.

LOW-RESOLUTION IMAGING VERSUS FLOW CYTOMETRY

At this point, readers may suspect they are being offered a free lunch; this is not the case. To detect optical signals, it is necessary to collect photons from the specimen, and, in most fluorescence measurements, the measurement quality is determined by the number of photons collected, which is in turn dependent on the number of excitation photons reaching the specimen during the observation period. In a typical benchtop flow cytometer using a 20-mW laser source, between 1 and 2 mW of excitation power actually impinges on a cell during the 10 μs or so it takes to pass through the illuminating beam (5, pp. 131-132); this power level is typically sufficient to produce between 10 and 100,000 photoelectrons at the detector photomultiplier photocathode, depending on the amount of fluorescent material associated with the cell. However, the actual analysis rate is usually no more than 4,000 cells/s, meaning that cells are in the beam for only 40 ms of every second; during the remaining 960 ms, the laser power is wasted because it illuminates only sheath and core fluid.

Although the LEDs used in the imaging system may emit over 400 mW of light, it is only possible to direct a few tens of microwatts from an LED through the area of a $10\text{-}\mu\text{m}$ cell; this, incidentally, makes LEDs relatively poor light sources for flow cytometers. However, the observation time in the imaging cytometer is longer, and all the cells remain in the field of view the entire time, so, even allowing for the relatively low light collection efficiency of the optics, with all other things being equal, in an observation time no longer than a few seconds, the imaging system can accumulate as many photoelectrons per well (10-100,000) as are generated at the photocathode of the flow cytometer photomultiplier. This allows the imaging system to achieve roughly the same measurement precision and, provided background is comparably low, the same sensitivity as the flow cytometer. The imaging instrument makes multiparameter measurements by changing emission and/or excitation filters as necessary and collecting multiple images, but, because the illumination intensity is relatively low, bleaching is not as big a problem as it may be in flow cytometry.

A flow cytometer and a low-resolution imaging system eventually must characterize cells one at a time. In the flow cytometer, information about cells is derived “on the fly” as they move through the instrument; in the imaging system, software processes images including all the cells to sequentially identify and extract information about individual cells. A simple imaging system is unlikely to match the sample throughput of a high-speed cell sorter but, given its cost, is likely to represent an acceptable alternative to a benchtop flow cytometer or scanning laser cytometer for many kinds of measurements. The imaging system is particularly well suited for examining samples in which a very small number of target cells or particles is sought in a relatively large volume of material, as is frequently the case in clinical, environmental, food, and water microbiologies. Microorganisms are conveniently collected by filtering samples through low-fluorescence black polycarbonate membrane or aluminum oxide filters and examined after they are stained with fluorescent dyes.

No laws of physics are being broken by the “cellular astronomy” imaging system; it might best be conceptualized as the electro-optical or cytometric equivalent of the personal computer. The IBM personal computer introduced in 1981, which fit on a desktop, drew a few hundred watts of house current, cost about \$4,000, and had computing power and speed and storage capacity equal to or slightly greater than those of 1960s IBM mainframes, which occupied entire rooms, required kilowatts of electrical power, and cost millions of dollars. Current desktop and laptop computers have a thousand times as much computing and storage capacity and are smaller, more energy efficient, and cheaper. Without the affordable personal computer and its descendants, we would not have word processing, spreadsheets, e-mail, or the Internet; we can expect that affordable imaging cytometers likewise will find applications that would never exist if today’s “mainframe” flow and image systems were the only ones available.

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