Cytometry in Malaria—A Practical Replacement for Microscopy?

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ABSTRACT

Malaria, caused by protozoan *Plasmodium* parasites, kills ~800,000 people each year. Exact figures are uncertain because presumptive diagnoses are often made without identifying parasites in patients' blood either by microscopy, using Giemsa's century-old stain, or by simpler tests that are ultimately dependent on microscopy for quality control. Microscopy itself relies on trained observers' ability to detect subtle morphological features of parasitized red blood cells, only a few of which may be present on a slide. Quantitative and objective flow cytometric measurements of cellular constituents such as DNA, RNA, and the malaria pigment hemozoin are now useful in research in malaria biology and pharmacology, and can provide more reliable identification of parasite species and developmental stages and better detection of low-density parasitemia than could microscopy. The same measurements can now be implemented in much smaller, simpler, cheaper imaging cytometers, potentially providing a more accurate and precise diagnostic modality. *Curr. Protoc. Cytom.* 65:11.20.1-11.20.23. © 2013 by John Wiley & Sons, Inc.

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INTRODUCTION

Malaria has to date had far more influence on cytometry than cytometry has had on malaria. Although cytometry—in the sense of detecting, counting, measuring, and characterizing cells—was initiated by van Leeuwenhoek, Hooke, and other early microscopists in the late 1600s, the cellular culprits involved in malaria and other diseases were not identified for another 200 years. The clinical features of malaria had been described millennia earlier, however. Molecular biology recently confirmed (Hawass et al., 2010) that Tutankhamun harbored the same protozoan malaria parasite, *Plasmodium falciparum*, that has killed large numbers of other young Africans annually since his time. Even today, the precise numbers of malaria infections and deaths worldwide remain uncertain because diagnoses are often made without confirming the presence of parasites in patients' blood (World Health Organization, 2011; Murray et al., 2012).

The current less-than-24-karat "gold standard" method for malaria parasite detection uses high-magnification microscopy to examine blood smears stained with a dye mixture developed by Gustav Giemsa in 1904. Quantification of parasite density, i.e., the number of parasites per unit volume of blood, provides some measure of the severity of malaria infection, and sequential density determinations are recommended to assess treatment progress in severe cases. Density measurements also provide critical objective data to malaria researchers studying the effects of drugs and biologicals on parasite growth and development in cultures and in animals, and to clinicians and epidemiologists analyzing data from trials of vaccines and other preventive and therapeutic interventions.

Quality assurance programs for diagnostic malaria microscopy now aim at a lower limit of detection of 100 to 200 parasites/μl (World Health Organization, 2008, 2010). Although

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experienced microscopists can detect fewer than 50 parasites/μl, they typically examine no more than 0.1 μl of blood, making quantification imprecise, even at the higher densities (O'Meara et al., 2006, 2007). For some purposes, e.g., the determination of whether or not malaria has been eliminated from a geographic region or population, investigators would need to examine blood from asymptomatic individuals. They would also need to be able to detect densities no higher than 5 to 10/μl. The time requirements alone make it impractical to consider microscopy for this application; molecular methods, although effective, have not yet been made robust and affordable enough for routine use in the field.

Until about 1950, cytometry was microscopy and microscopy was cytometry, providing the only means of detecting individual cells—malaria parasites included—in blood or any other material. By 1960, the first simple flow cytometers—electro-optical and electronic instruments capable of counting red blood cells (erythrocytes, RBCs) and white blood cells (leukocytes, WBCs)—had found their way into both clinical and research laboratories in places in which they were affordable and in which trained personnel and the infrastructure needed to keep the apparatus functioning were available. The identification of different types of WBCs and other nucleated cells and of malaria parasites in blood, however, still required microscopy of stained smears, in both rich and poor countries.

Since the 1970s, increasingly sophisticated apparatus and techniques for flow and image cytometry—using reagents far more specific than any available in Giemsa's time—have become well established in other contexts, and have been used for the detection and characterization of many cell types found at low densities in blood and other specimens. Malaria microscopy remains unchanged; even in well-equipped clinical and research laboratories in affluent countries, malaria diagnosis, epidemiology, and the development of new diagnostics, drugs, and vaccines still critically rely on a technique that is essentially neither informed nor transformed by any scientific or technological advances made during the past 108 years—including electric lighting!

Although cytometric apparatus has been commercially available for over 50 years, and more than 140,000 papers involving its use have been published to date, fewer than 300 have described flow or image cytometry of malaria parasites, and only a handful of investigators and laboratories worldwide combine expertise in both malaria and cytometry. During the past decade, however, modern cytometry has come into increasing use in malaria research for investigations of the physiology of parasites and their responses to antimalarials. It is also now feasible to take relatively compact cytometers into the field for epidemiologic work, e.g., the detection of low-level parasitemia using either cell-based (Campo et al., 2011) or molecular methods, in many places in which malaria causes significant morbidity and mortality.

A great deal is now known about which cytologic characteristics, or parameters, of malaria parasites can be best used to detect them, identify different species and developmental stages, and determine the effects of pharmacologic and immunologic agents on growth and development. Different groups of investigators have developed increasingly sophisticated protocols for multiparameter cytometric analysis of the parasites (Hare, 1986; Hare and Bahler, 1986; Pattanapanyasat et al., 1993; Jouin et al., 1995, 2004; Wongchotigul et al., 2004; Grimberg et al., 2008, 2009; Izumiyama et al., 2009; Karl et al., 2009; Ch'ng et al., 2010; Apte et al., 2011; Campo et al., 2011; Gerena et al., 2011; Malleret et al., 2011; Boissière et al., 2012; Jogdand et al., 2012; Jun et al., 2012; Kaushansky et al., 2012; Philipp et al., 2012; Clark et al., 2013; Sinnis et al., 2013). There have been no systematic multicenter collaborative studies to establish the relative merits of different protocols, however, nor have serious attempts been made to determine which of a variety of various reagents, or probes, and reagent combinations would be optimal for use with a given instrument.

It is important to note that, although conventional cytometric technology has been too costly and complex to be considered as a replacement for malaria microscopy until recently, now some newer field-capable apparatus—primarily imaging instruments using light-emitting diodes (LEDs) for illumination and digital camera chips for detection—can implement the same proven measurements that are being done in flow cytometers. The new systems are sufficiently small, simple, robust, energy efficient, and inexpensive to be feasible for use in routine malaria diagnosis, even in resource-limited settings.

Similar instruments are already in use in such environments for CD4+ T cell counting in HIV-infected patients. The actual

implementation of CD4 counting on a worldwide scale required decades of effort on the part of hundreds of scientists and clinicians in dozens of countries (Mandy et al., 2002). Initially, it was necessary to know what cellular characteristics to measure, what reagents to use, and how to analyze the data. Equally important, it was necessary to implement training and quality assurance programs. The same processes will be required to make malaria cytometry more widely usable and reliable as a research tool and eventually enable it to replace microscopy, yielding substantial improvements in sensitivity, accuracy, and precision of parasite density determination, whether for diagnosis, epidemiology, or research.

As a first step in this direction, we convened a brief Workshop on Malaria Cytometry at the CYTO 2012 Meeting of the International Society for the Advancement of Cytometry (ISAC), held in Leipzig, Germany, in June 2012. At the workshop we agreed to cooperate on further work and to document the current state of malaria cytometry and its prospects for further development and application. The present work, in which we have tried to provide background information comprehensible from the points of view of both malariologists and cytometrists, represents our consensus.

Practical Flow Cytometry (Shapiro, 2003), now available online in free downloadable PDF format, provides details of cytometry hardware, software, and the associated reagent technology. Specific cytometry applications in malaria are the subject of an extensive recent review by Grimberg (Grimberg, 2011). Malaria diagnosis and some relevant biology are discussed at length in Garcia's text (Garcia, 2007).

BEGINNINGS: MIASMA TO GIEMSA

Malaria had a profound influence on the early development of modern medical science. A number of authors have provided enjoyable narratives about this time (de Kruif, 1926; Desowitz, 1991; Garfield, 2001). More scholarly accounts (Clark, 1983; Bosch and Rosich, 2008; Gaynes, 2011) supply additional detail.

Giemsa, Paul Ehrlich, and the others who optimized mixtures of synthetic dyes to improve the visualization of malaria parasites in blood from the 1880s on had the disease to thank for their palette of stains. The Americas, free of malaria until the conquistadores and their African slaves brought parasites there, yielded an often-curative tree bark that made its way across the Atlantic via native healers and European priests. The "Jesuit powder" and extracts containing its active ingredient, quinine, remained the only effective agents for the treatment and prophylaxis of malaria (or any other serious disease) for centuries thereafter. Quinine, with or without gin added to increase its palatability, sustained the British Empire and others, but producing enough of the drug was not a simple matter of growing money on trees.

By the mid-1850s, William Perkin, a teenaged chemistry student in London, had attempted to synthesize quinine from aniline and had failed. His reaction product was the intensely colored purple dye, mauve. The dye was the first of a long line of commercially successful synthetic textile dyes, and the impetus for an international chemical industry initially driven by the demands of fashion. Within a few decades, some synthetic dyes would be used to stain cells, including those harboring malaria parasites, and, shortly thereafter, to treat malaria and other infectious diseases. Many of the dye-manufacturing companies would become more widely known as producers of drugs, some of which even now double as biological stains.

By the 1850s, improvements in microscopes had facilitated the development and acceptance of the cell theory. In Germany, Rudolf Virchow played a central role in moving the study of disease to the cellular level. Virchow's assistant, Karl Weigert, Ehrlich's older cousin, was among the first to use synthetic dyes to improve the visualization of cells and tissues by microscopy, inspiring his younger relative to explore differential staining of cells and their components by dyes with differing chemical properties and contrasting colors. By 1878, Ehrlich had used combinations of acidic dyes such as eosin and basic dyes such as methylene blue to identify and classify different types of WBCs in peripheral blood.

The identification of disease-specific cellular pathogens also began in the 1870s. Robert Koch led the way, applying dyes to stain bacteria, and taking advantage of what would now be called beta-tester status to obtain substage condensers, oil immersion objectives, and other newly developed products from the Zeiss works before they became commercially available. He also introduced photography to document results more objectively than could be done with drawings. With the help of Ehrlich's methylene blue staining technique, Koch discovered the causes of anthrax and tuberculosis. By 1882, Ehrlich and Koch had developed stains for the "acid-fast"

Mycobacteria, different species of which caused tuberculosis and leprosy. They inspired Christian Gram's subsequent work on bacterial staining.

Stains were not involved in Alphonse Laveran's 1880 discovery of malaria parasites in blood, however. Malaria was itself named for the "bad air" thought by many to be its primary cause; by 1880, several microbiologists claimed to have identified a causative bacterium, but had not made their case. Laveran, a French military doctor working in Algeria, examined fresh unstained blood, attempting to discover the origins of a dark pigment then known for over a century to be present in the tissues and blood of malaria sufferers. His observation of motile, pigment-containing particles substantially larger than bacteria led him to believe that a highly pleomorphic parasite was responsible. Laveran's findings were viewed skeptically for several years, until more widespread use of oil immersion objectives and staining enabled others to confirm the existence of malaria parasites and identify different stages of their development (Laveran, 1907).

It also became possible to detect subtle differences in parasite morphology that correlated with long-known differences in the clinical course of malaria, suggesting that more than one species of parasite might be involved in the human disease. Working with a bird malaria, the British military doctor Ronald Ross elucidated the complex development of the parasite and its transmission through the bites of specific mosquito species. Ross and Laveran received Nobel Prizes in Medicine in 1902 and 1907, respectively. Ross's bitter rival, Giovanni Battista Grassi, who, with others in Italy, established the causative role of *P. falciparum* in the most severe form of the human disease and its transmission by mosquitoes of the genus *Anopheles*, was not similarly rewarded; the feud, over a century old, still provides fuel for disputes among malariologists.

Having found that malaria parasites take up methylene blue, Ehrlich speculated that the dye might exert selective toxicity against them and in 1891 reported antimalarial action in several cases. He had already established close ties to the dye industry in his search for better stains to facilitate diagnosis; his subsequent successful demonstration of what he named chemotherapy motivated companies to modify molecules to treat diseases. The new drug industry provided Ehrlich with over six hundred compounds to test for activity against syphilis; this first "high-throughput screen" yielded two effective treatments.

Methylene blue itself is still occasionally used as an antimalarial, but does not, either by itself or combined with eosin, produce optimal staining of malaria parasites in blood smears. Between 1891 and 1904, several improvements in staining were made, initially by Malachowski and Romanowsky, and ultimately by Giemsa. All involved the addition of azure dyes—themselves oxidation products of methylene blue—to the eosin-methylene blue mixture. Dye interactions in the azure-augmented stains color parasite cytoplasm an intense blue and nuclei a contrasting red, facilitating identification of the earliest stages of malaria parasites in RBCs. Giemsa's dye combination, still almost universally used for malaria diagnosis by microscopy, is among the easiest to prepare and among the most consistent in its staining properties.

This stain has also remained the standard for morphologic hematology. Over a century later, we might wonder how the malaria tail managed to wag the hematology dog. Many people now have many reasons to look at cells in blood, in both the clinic and the laboratory, and relatively few of us have any occasion to look for or at malaria parasites. That was not the case at the turn of the twentieth century, however. To be sure, the anemias and the leukemias were known by then, as were elevations in the numbers of WBCs associated with infection. In addition, the utility of the first hemacytometers, which counted blood cells in graduated chambers of defined volume, was appreciated. However, although microscopy could provide diagnostic and prognostic information about a range of blood disorders, at that time no treatments were known for most of them. Malaria, considerably more widespread than it is today, could be treated with quinine if diagnosed by microscopy, with subsequent clearance of parasites from the blood confirmed by further microscopy. It thus made sense to optimize staining procedures for blood to facilitate malaria diagnosis.

THE PARASITES: THEN AND NOW

In 1904 the staining patterns observable in blood cells and malaria parasites revealed chemical differences among different cell types and among different intracellular structures, but the biological, chemical, and biochemical details were almost completely unknown. Few suspected that the component

molecules of cells were as large as we now know proteins and nucleic acids to be. In addition, most of their lower-molecular-weight building blocks had not been discovered. The details of cellular and nuclear division had only been worked out in the previous two decades; it was clear that cell nuclei contained an acidic component that would bind basic dyes such as methylene blue and azure B, and that both nucleus and cytoplasm contained varying amounts of proteins, which bound eosin, and acidic materials, which bound the basic dyes. The parasites themselves have changed little since 1904; the acquisition of genes for resistance to antimalarials, perhaps the most significant difference, had no discernible effect on morphology.

By Giemsa's time, it was known that malaria parasites go through only part of their life cycle in the blood. Sporozoites, each containing a single nucleus, are the infective stage resulting from the parasite reproductive cycle in the mosquito vector. Sporozoites are introduced into a human or animal host's blood by a mosquito's bite. The sporozoites invade liver cells, becoming trophozoites, which accumulate cytoplasm, and later schizonts, which undergo multiple rounds of asexual nuclear reproduction (schizogony), and segment into multiple merozoites, each with one nucleus. Rupture of the infected liver cell releases merozoites into the blood, where one or more can infect a RBC.

Within RBCs, merozoites become haploid "ring stage" trophozoites, and then mature into schizonts, all the while feeding on the RBCs' hemoglobin and converting it into hemozoin, the "malaria pigment." Like those in the liver, trophozoites in RBCs initially accumulate cytoplasm. Most develop into schizonts, which segment into uninucleate merozoites, each of which can initiate additional rounds of schizogony in a previously uninfected RBC. A minority of trophozoites develop into male (micro-) and female (macro-) gametocytes, which are ingested by mosquitoes to continue the life cycle, eventually yielding sporozoites.

The overall theme of parasite development described above has recognizable variations; among other things, the timing is different for different species. The development of parasites in both the liver and the blood is synchronized, and the release of parasites and cellular debris produces the characteristic "ague"—a symptom complex including chills, sweating, and fever. Although malaria is frequently diagnosed based solely on the presenting clinical features and their time course, it is recommended that parasites be demonstrated in blood for definitive diagnosis and that density be monitored by microscopy to establish the efficacy of treatment in severe cases.

Human Malaria Parasite Species: Now at Six

Three distinct clinical pictures of malaria had been known for centuries before the parasite was discovered. By 1904, correlations between these and parasite morphology in patients' blood had allowed investigators to distinguish three species of the genus now called *Plasmodium*.

P. falciparum (Pf)

Most malaria deaths result from the "malignant tertian (i.e., occurring roughly every third day) malaria" caused by Pf, in which symptoms may show 36- to 48-hr periodicity or may occur continuously. The parasite infects RBCs of all ages. Pf also differs from other parasite species in that RBCs infected with trophozoites and later intraerythrocytic stages express surface antigens that bind to endothelium; these infected cells are thereby sequestered, i.e., removed from circulating blood, and may obstruct small blood vessels in various organs, notably the brain and placenta. Cerebral malaria is involved in most deaths caused by Pf.

Plasmodium vivax (Pv)

Pv is responsible for "benign tertian malaria," a relatively mild disease in which symptoms occur at intervals of roughly 48 hr. Pv can produce hypnozoites, a stage that can remain dormant in the liver for long periods of time (months to decades). Pv infects young RBCs (reticulocytes), and was thought to require the presence of the Duffy blood group antigen on the host cell for infection. It is now known, however, that there are Pv strains that can infect Duffy-negative individuals.

P. malariae (Pm)

Pm causes "benign quartan malaria," which is relatively mild but has a cycle of 72 hr, or every fourth day. Pm infects older RBCs.

Since 1904, three other species of *Plasmodium* have been demonstrated to cause malaria in humans.

P. ovale (Po)

Po, identified in the 1920s as the cause of a mild tertian malaria, has recently been demonstrated to comprise two distinct species,

P. ovale curtisi and *P. ovale wallikeri* (Fuehrer et al., 2012). They are not distinguishable by morphology and both resemble Pv. Like Pv, the Po species infect younger RBCs and may produce long-lived hypnozoites.

P. knowlesi (Pk)

Pk (Cox-Singh, 2012) was identified in the 1920s as a cause of malaria in monkeys; occasional human cases have been reported from the 1960s on. In the past decade, substantial numbers of human Pk infections, some severe, have been recognized to occur in Southeast Asia; many were initially misdiagnosed as due to *P. malariae*. The range into which the parasite is likely to expand is limited because it is only transmissible by a small number of geographically restricted mosquito species. Pk has a 24-hr cycle and can infect RBCs of all ages.

Pk and Pf can be maintained in long-term culture in monkey and human RBCs; efforts are now under way to develop long-term culture methods for Pv, which has the broadest geographic distribution and probably infects the largest number of people. The great majority of the 600,000 to 1,000,000 malaria deaths estimated to occur annually however, are due to Pf. Pm and the Po species, which altogether account for no more than 10% of malaria infections, have not been grown in culture.

BIOLOGY, MICROSCOPY, AND CYTOMETRY: PARAMETERS, PROBES, AND PROTOCOLS

A substantial fraction of malaria microscopy and almost all malaria cytometry involve the detection and characterization of small numbers of parasites or parasitized blood cells against a background of much larger numbers of normal blood cells. A necessary first step in developing an appropriate methodology is to identify the physical and chemical characteristics, or parameters, that are most useful in distinguishing parasites and parasitized cells from other constituents of blood and in discriminating among parasite species. In microscopy or cytometry of cultured parasites or blood cells from experimental animals or human subjects, it is generally not necessary to identify the parasite species involved; in diagnosis, this is critical.

None of the dyes used in the Giemsa stain and its relatives is specific for any chemical constituent of a malaria parasite or, indeed, of any other cell type. For that reason, it is necessary in malaria microscopy, as it is in identifying different types of WBCs in Giemsa-stained smears, to discern the sizes, shapes, colors, and textures of various constituents of cells and to combine that information to identify a parasite or cell. In malaria, information relevant to species identification may come not only from the size, shape, and texture of the nucleus or nuclei and cytoplasm, and the presence or absence of various cytoplasmic structures, but also from the numbers of different developmental stages encountered, the modal number of nuclei per schizont, and the presence or absence and form of the malaria pigment, hemozoin. Discerning the morphologic details of both blood cells and parasites either visually or with interactive or automated image analysis requires that a well-prepared and -stained smear be examined at high magnification (1000×) using an oil immersion lens.

Species Identification: Cell-, Sequence-, and Antibody-Based Diagnostic Approaches

The visual identification of parasite species relies on morphologic clues that are generally more subtle than the ones microscopists need to distinguish among developmental stages. There is no guarantee, however, that every example of a given stage of a given parasite species will match those "textbook pictures" of that stage and species that are typically found in instructional materials for microscopists. As a respected text (Garcia, 2007) puts it: "Note: Without the appliqué forms, Schüffner's dots, multiple rings per cell, and other developing stages, differentiation among the species can be very difficult. It is obvious that the early rings of all four species can mimic one another very easily." Simultaneous infection with more than one parasite species is not uncommon; such mixed infections are difficult to diagnose with microscopy.

Sequence information collected to date has yielded molecular reagents that are usable for the identification and discrimination of all six known human malaria parasite species. Such reagents are frequently employed in multiplexed bead assays done by flow cytometry and, more recently, by image cytometry. Molecular assays using DNA amplification can detect very low parasite densities and are viewed as more reliable than microscopy for species identification. At present, however, such assays are too complex and costly for routine use.

Specific parasite proteins provide the targets for antibody-based rapid diagnostic tests (RDTs), which were introduced in the 1980s.

The tests detect parasite antigens with "dipsticks" or other simple visual indicators. Although acceptable for the diagnosis and, to some extent, identification of parasite species, RDTs are ineffective at densities below 100 to 200/μl, and do not quantify accurately. They are less effective at detecting Pv than Pf, and unreliable for the detection of Pm and the Po species (Murray et al., 2008; McMorrow et al., 2011). Because they deteriorate when improperly stored, RDTs are reliable only when quality control can be provided by microscopy.

Pf can be identified by RDTs detecting histidine-rich protein 2 (HRP2), which is expressed in and on blood stages. This protein, however, can remain detectable in blood for several weeks after an infection has been adequately treated. It has also been established that some strains of Pf, notably several found in South America, do not express HRP2 at all; this further limits the utility of the protein as a marker.

Nonspecific antibodies to the malaria parasite aldolase have been used in RDTs and combined with HRP2 antibodies in tests that discriminate Pf infections from mixed infections and from infections with other parasite species. There are also RDTs that use monoclonal antibodies against parasite lactate dehydrogenase (pLDH); levels of this protein correlate sufficiently well with the presence and level of viable parasites to be used for antimalarial drug screening and the assessment of antimalarial antibody activity as well as for diagnosis. The range of available anti-pLDH antibodies now includes some that are monospecific and others that are reactive with all or a subset of parasite species.

If one accepts the accuracy of species identification now available using molecular reagents, cumulative anecdotal evidence suggests that even the best and most experienced malaria microscopists fall short of infallibility (Barber et al., 2013). We therefore think it unlikely that computer processing of high-resolution images of Giemsa-stained material, advocated in some quarters, will provide an effective replacement for malaria microscopy. Instead, we favor an approach based on simpler but far more reliable quantification of relevant parameters of parasites and parasitized cells that takes advantage of what has been learned since Giemsa's time.

The Cellular and Molecular Ecology of Blood

By the mid-1800s investigators understood that counting blood cells could provide clinically useful information. RBCs are the most abundant (~5,000,000/μl whole blood); their very numbers require that a sample be diluted a hundredfold or more. This permits a microscopist to count individual cells in a known volume using a hemacytometer and thereafter calculate the RBC concentration in whole blood from the number counted and the known dilution factor.

Circulating RBCs do not normally contain DNA. Like other blood cells, RBCs are formed in the bone marrow; unlike WBCs, they normally extrude their nuclei before entering circulation. At this point, some of the ribosomes used earlier in RBC development for the synthesis of hemoglobin and other proteins remain in the cytoplasm. Ehrlich himself established that basic dyes would precipitate a stained network, or reticulum, in these young RBCs, giving them the name of reticulocytes. The dye-binding material, which disappears during the cells' first day or two in circulation, is now known to be ribosomal RNA.

Blood platelets (thrombocytes) are actually cell fragments that break off from large multinucleated megakaryocytes in the bone marrow, and contain neither nuclei nor detectable DNA. They may, however, contain small amounts of ribosomal RNA during their first days in circulation. Platelets, normally present at concentrations of 100,000 to 400,000/μl, are much smaller (volume a few tens of fl) than either RBCs (typical volume ~90 fl) or WBCs (typical volume at least ~200 fl) and are easily distinguishable from RBCs and WBCs by their size.

The typical WBC concentration in normal blood is 5000 to 10,000/μl, meaning that only one or two WBCs accompany each 1000 RBCs. Although their hemoglobin content makes RBCs simple to discriminate from WBCs by microscopy or cytometry, most modern automated cell counters, which simply measure cell size, do not make the distinction and instead include WBCs in RBC counts, with negligible effects on accuracy. WBC counts with either hemacytometers or automated counters are typically done on whole blood diluted ~1:10 with a solution containing chemicals that lyse the RBCs. WBCs contain nuclei, each of which carries two copies of the genome, or approximately 6000 Mbp of DNA in humans.

Since it is not necessary to distinguish subcellular details when using hemacytometers to visually count RBCs and WBCs, total magnifications of 100× or less are typically used. Visual discrimination and counting of

different types of WBCs—the differential leukocyte count or "diff"—requires substantially higher magnification, as noted above.

Giemsa's and the related dye mixtures derived from Ehrlich's work produce characteristic staining patterns in WBCs, defining five major types. The three types, called granulocytes, contain cytoplasmic granules and have lobulated nuclei when mature. The granules of eosinophils stain most intensely with acid dyes, and those of basophils stain most intensely with basic dyes. Those of neutrophils, the most common granulocytes, stain with both acid and basic dyes. Mononuclear cells include lymphocytes, which typically are smaller and rounder than granulocytes and have relatively round nuclei and scanty cytoplasm; and monocytes, which generally are larger than granulocytes, less round, and have larger and less round nuclei.

Although color information allows different types of granulocytes to be identified unequivocally, assessment of the maturity of granulocytes by their degree of nuclear lobularity and discrimination between lymphocytes and monocytes require careful examination of morphologic details. This is typically done at 1000× on a slide made by smearing 1 to 2 μl of blood in a thin film occupying an area of 2 to 4 cm^2. The quality with which morphology is preserved varies in different areas of a smear; in those areas best suited for cell identification, most WBCs fit within a circle ~20 μm in diameter, and most RBCs fit within a circle <10 μm in diameter.

The relatively small size of malaria parasites—particularly ring stages, whose detection is critical in diagnosis—also demands high-magnification microscopy of high-quality smears, which allows an observer to best appreciate the morphologic characteristics necessary to detect parasites and distinguish species. Ring stages, which are actually biconcave discs, are typically about 1/3 the diameter of the RBCs in which they are found. The optical resolution practically attainable with oil immersion lenses is on the order of 0.25 μm; if this resolution were to be maintained in a digitized image of a ring-form parasite, the image would contain no more than 200 pixels, many of which would represent the clear central space and therefore provide relatively little significant morphologic information. The option of making digital images became available only in the late 1950s; until then, identification of blood cells, parasites, and any other formed elements that might be found in blood required microscopy.

Significant Parameters in Malaria Cytometry

Nucleic Acids: DNA and RNA content

Although the involvement of nuclear replication in the developmental cycle of malaria parasites was recognized by 1904, the quantification of RNA and DNA would have been uninformative at that time; the nature of these macromolecules and their critical roles in cell growth were unsuspected then and indeed not fully appreciated until the 1960s. The staining characteristics that have made it possible for microscopists from Ehrlich on to distinguish RBCs containing malaria parasites from unparasitized RBCs depend on the binding of basic dyes to nucleic acids, but neither methylene blue nor azure B, the basic dyes essential in Giemsa's stain, has particularly specific staining properties. Both of these dyes stain DNA, without a strong preference for A-T or G-C base pairs, and also RNA, making precise quantification of either nucleic acid in Giemsa-stained material impossible.

By 1952, both UV microspectrophotometry and staining with the DNA- and RNA-specific methyl green-pyronin Y dye combination had been used to document increases in both DNA and RNA content during schizogony in RBCs (Lewert, 1952). By 1986, a flow cytometric method for DNA and RNA quantification using acridine orange was used to confirm this, and to demonstrate by cell sorting that all parasite developmental stages in RBCs could be identified based on DNA and RNA content alone, without recourse to any morphologic information (Hare, 1986; Hare and Bahler, 1986). This was again shown in 2008 (Grimberg et al., 2008), using a combination of Hoechst 33342, a compound that stains DNA stoichiometrically in living cells, and thiazole orange, originally developed for flow cytometric reticulocyte counting and established to be RNA-specific in the presence of the Hoechst dye (Jouin et al., 1995, 2004; Grimberg et al., 2008). A schematic of DNA and RNA content levels observed in a culture of *P. falciparum* (Pf) in human RBCs appears in Figure 11.20.1.

Uninfected mature RBCs, as noted above, contain little or no DNA or RNA, and their Hoechst 33342 and thiazole orange fluorescence signals are essentially indistinguishable from those of unstained RBCs. Cells with one or two rings contain little RNA (they are estimated to have only ~10,000 ribosomes) and one or two copies of the genome (23.3 or 46.6 Mbp DNA in the case of Pf). Uninfected

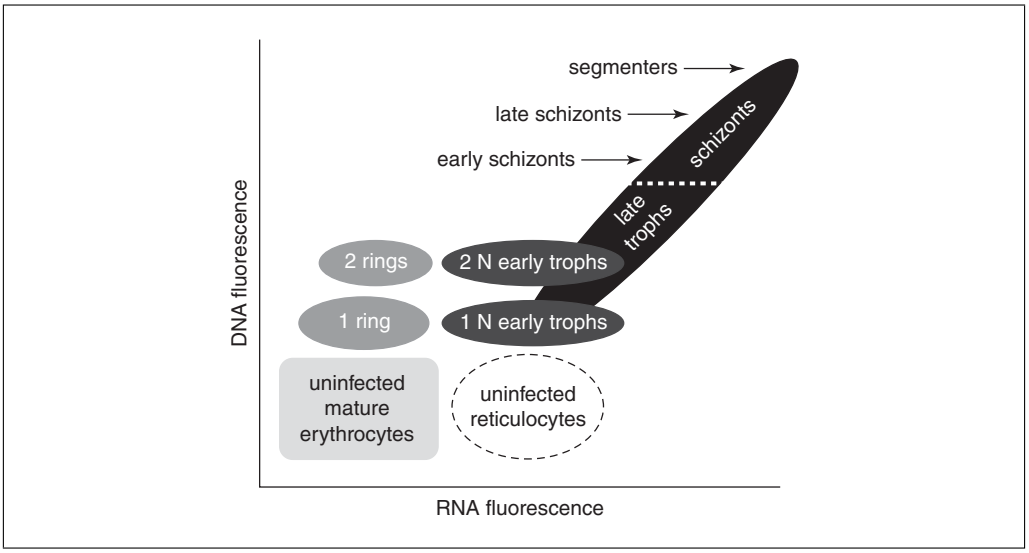

Figure 11.20.1 Typical appearance of a plot of DNA content (UV-excited blue Hoechst 33342 fluorescence) and RNA content (blue-excited green thiazole orange fluorescence) of a population of RBCs infected with Pf. Both DNA and RNA content are displayed on logarithmic scales. Gametocytes are not shown. From Grimberg et al. (2008).

reticulocytes are estimated to contain 50,000 to 100,000 ribosomes, and thus show considerably more thiazole orange fluorescence than is detected in either uninfected mature RBCs or RBCs containing ring forms. RBCs containing early trophozoites contain one or two copies of the genome, and therefore have DNA content equal to those of the RBCs containing corresponding ring stages; their RNA content corresponds to that of uninfected reticulocytes. Both DNA and RNA content increase as parasites pass from the trophozoite to the schizont stage. The overall pattern of increasing DNA and RNA content with parasite development, originally observed by microscopy in Pv decades ago, and shown in Pf above, is also apparent in flow cytometry of cultured Pk. The correlation between morphologic features of these species and those of Pm and the two Po species strongly suggests that the latter three species, which thus far do not appear to have been subjected to multiparameter cytometric analysis, would manifest similar patterns of DNA and RNA content during development.

In various contexts, DNA and RNA content can be used to detect malaria parasites and/or to determine the effects of drugs and immune manipulation on their growth and development. Any of these tasks can be done far more effectively by cytometry than by microscopy, primarily because cytometry is far more accurate and precise for quantification, but also because of the limitations of some of the probes used for detection. DNA could originally be quantified only by Feulgen staining (which removed the bases and thus lost sequence information) or by its UV absorption near 260 nm (which required treatment of samples with RNAse, since RNA absorbs at the same wavelength). Instrumentation was necessary; visual quantification is imprecise and 260 nm is invisible to humans. The methyl green/pyronin dye combination (introduced, ironically enough, by Ehrlich, although its advantages were not appreciated until well after his death) stains DNA green and RNA reddish purple with great specificity, facilitating relative comparisons of DNA content by microscopy, as is done when schizont nuclei are counted.

In current practice, however, quantification of DNA and RNA is most frequently done using fluorescent dyes. Those first demonstrated to be effective were, for the most part, compounds developed early in the 20th century by the dye companies that by then had evolved into drug companies. The Hoechst 33342 dye now widely preferred for DNA staining in unfixed cells, and referred to in Figure 11.20.1, is one such compound. Many newer dyes, however, were designed specifically for analytical purposes, usually by systematic modification of the structures of compounds previously found useful. Thiazole orange, the RNA dye of Figure 11.20.1, coincidentally exemplifies this class of dyes; it was made to allow blood reticulocyte counting to be done on the large base of flow cytometers equipped with 488-nm lasers. Pyronin Y itself can be used as a fluorescent RNA stain when combined with DNA stains

such as Hoechst 33342 (which, incidentally, has DNA-binding properties nearly identical to those of methyl green) (Shapiro, 1981).

A single 23 to 27–Mbp malaria parasite genome can easily bind several million molecules of a DNA dye; larger genomes, such as those in human cells, may bind hundreds of millions. A single ribosome can bind hundreds of molecules of an RNA dye, suggesting that RNA signals from a ring-form-parasitized cell would come from at least one million molecules. Thus, DNA and RNA detection can easily be accomplished by simple fluorescence microscopes and imagers as well as by flow cytometers.

Hemozoin

The association of the "malaria pigment" hemozoin (Hz) with the disease (Hänscheid et al., 2007) antedates the first observation of the parasites by more than a century, and played a direct role in Laveran's initial discovery. It is now known that Hz formation serves the parasite by detoxifying heme, which is produced as it feeds on the hemoglobin in RBCs. The amount of Hz present in parasitized cells therefore increases as the parasite passes through succeeding developmental stages; little or none is typically detectable in ring forms, while late-stage schizonts and gametocytes contain large quantities.

Hz can be detected and at least partially quantified without the use of reagents. It is paramagnetic, making it possible to use magnets to enrich and separate parasite stages containing the pigment (Karl et al., 2008, 2009; Kim et al., 2010). Hz is also optically birefringent, i.e., capable of changing the plane of polarization of incident light. When observed in unstained or stained blood by transmitted light microscopy using unpolarized light, it appears as brownish-yellow particles. Inserting crossed polarizing filters in the illumination and observation paths of the microscope darkens the field of view except where Hz is present; its birefringence produces bright spots (Lawrence and Olson, 1986). Suitable polarizing filters are widely available and relatively inexpensive; lenses from polarizing sunglasses have been used successfully (Maude et al., 2009). Hz particles also appear as relatively bright spots when observed using dark-field microscopy without polarization. The most pronounced contrast with the background is obtained when polarized light is used for dark-field illumination and samples are observed through a filter with a polarization plane perpendicular to that of the incident light.

This combination is also employed for Hz detection in flow cytometers, in which a side scatter (SSC) signal is measured in a plane of polarization orthogonal to that of the illuminating laser beam (conventional SSC signals are measured in the same plane as the beam). Until recently, the only flow cytometers equipped to do this were Abbott's (*http://www.abbott.com/*) Cell-Dyn hematology analyzers. The depolarized SSC signal in these analyzers was used to detect eosinophil WBCs, which have birefringent granules, although they do not contain Hz. A now-lapsed patent on depolarized SSC measurement, exclusively licensed to Abbott while in effect, prevented other flow cytometer manufacturers from implementing the feature in their machines.

From the 1990s on, several groups of investigators found that in addition to detecting eosinophils, the Cell-Dyn instruments would register depolarized SSC signals from granulocytic and monocytic WBCs that contained Hz by virtue of having phagocytized parasitized RBCs (Mendelow et al., 1999; Grobusch et al., 2003; Hänscheid et al., 2011). Such cells had previously been visualized on slides; their relative rarity makes it difficult to estimate their abundance precisely enough to clarify their clinical significance. Flow cytometry, in principle, made it possible to quantify Hz-containing WBCs more precisely by analyzing much larger blood samples; however, the hematology analyzers were not readily modifiable to do this. Depolarized SSC measurements can now be implemented in other flow cytometers without legal impediment; many commercially available instruments can be modified to do them (Frita et al., 2011). In some cases, e.g., the Accuri (BD Biosciences) C6 instrument and some from Partec, a user can make the modification in minutes using inexpensive optical components.

The use of Hz detection alone in either blood or tissue for malaria diagnosis, potentially manageable without the use of a reagent, has attracted numerous investigators, and substantial funding has been made available for the approach. Some cautions are in order, however. It is already known that detectable Hz can be found in human WBCs for many weeks after an active malarial infection has resolved, and that the material can persist in animal tissues for months (Frita et al., 2012). Furthermore, Hz may be produced by parasites other than malaria, e.g., schistosomes and some filaria. While they are much less common causes of infection than are malaria parasites, they

occur with significant frequency in many places heavily burdened by malaria.

Lastly, in *P. falciparum* (Pf) infection, RBCs harboring the late trophozoite and schizont stages—which contain most of the Hz—also express surface antigens that adhere to endothelial cells and lead the parasitized cells to be removed from circulation. As a rule, >95% of circulating parasitized RBCs in Pf-infected patients are ring forms containing little or no detectable Hz, although some will be encountered in circulating gametocytes. A flow cytometric study measuring depolarized SSC and DNA in blood from Pf-infected patients found no parasitized RBC with detectable Hz signals (Rebelo et al., 2012). Since the use of magnetic fields to concentrate malaria parasites—in or outside RBCs—from blood depends entirely on whether the parasites contain Hz, magnetic separation would be unlikely to increase the sensitivity of a solely Hz-based Pf diagnostic.

Cytometric detection and quantification of Hz can nonetheless be valuable in malaria diagnosis when combined with the measurement of other parameters, notably DNA and RNA content. Moreover, although the literature now contains descriptions of several highly complex and expensive measurement methods, Hz detection using polarized light can, as noted above, be implemented simply and cheaply in either image or flow cytometers. As one might expect, given the established utility of Hz observation in microscopy, cytometric quantification of Hz improves the ability to discriminate among various developmental stages and is likely to aid in distinguishing species. It is already known that a number of antimalarials interfere with Hz production in susceptible parasite strains, and recent evidence suggests that monitoring this by cytometry may produce a relatively simple and effective means of detecting the emergence of drug resistance (Rebelo et al., 2013).

Mitochondrial Membrane Potential ($\Delta\Psi_m$) and Other Physiologic Characteristics; Viability

Malaria parasites contain mitochondria; the single mitochondrion that enters an RBC in a merozoite is reproduced during schizogony. Each of the resulting new generation of merozoites is haploid and contains a single mitochondrion. Energized mitochondria in all cell types maintain a difference in electrical potential of ~120 mV between their interior and the cytosol, with the interior negative; this mitochondrial membrane potential ($\Delta\Psi_m$) decreases to zero or near zero in the presence of metabolic inhibitors such as the proton ionophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). The mitochondria in blood stages of plasmodia, like those in trypanosomes and unlike those in almost all animal cells, are not primarily involved in energy metabolism but instead use their membrane potential-derivable energy in biosynthesis, primarily of pyrimidines.

Note that $\Delta\Psi_m$ can be measured cytometrically using fluorescent dyes that carry a single positive charge and are sufficiently lipid-soluble to be able to pass through cytoplasmic and mitochondrial membranes. The less lipophilic representatives of this class of dyes, e.g., tetramethylrhodamine ethyl ester (TMRE), partition across membranes in accordance with the Nernst equation; at 37°C, the ratio of dye concentrations inside and outside the membrane increases by a factor of 10 for every 60 mV of membrane potential. More lipophilic dyes, e.g., rhodamine 123 and cyanines, exhibit higher ratios of interior to exterior concentrations than would be predicted by the Nernst equation.

Mitochondrial de-energization and the accompanying decrease in $\Delta\Psi_m$ occur early in apoptosis in many cell types, and have also been observed in parasites exposed to a number of antimalarial drugs, including chloroquine and atovaquone. Both dihexyloxacarbocyanine ($DiOC_6(3)$) and hexamethylindodicarbocyanine ($DiIC_1(5)$) can be used as $\Delta\Psi_m$ indicators (Shapiro et al., 1979; Grimberg et al., 2009; Grimberg, 2011). In cells exposed to concentrations of 1 to 10-nM dye, most fluorescence comes from mitochondria. The dye now in widest use, however, is 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1). When applied at micromolar concentrations, this normally green fluorescent dye becomes sufficiently concentrated in energized mitochondria to form red fluorescent aggregates. Measurements of red and green fluorescence and the ratio of both provides a more precise indicator of $\Delta\Psi_m$ than can be obtained by measuring a single emission wavelength. JC-1 has been used to examine $\Delta\Psi_m$ in malaria parasites (Ch'ng et al., 2010).

Most eukaryotic cells have many mitochondria; microscopy or image cytometry would be necessary to detect a condition in which some were energized and some not. The mitochondria in RBCs typically lose function as the cells mature; a parasitized RBC will therefore

typically contain only one energizable mitochondrion for each nucleus, allowing a measurement of DNA content to be used to normalize fluorescence measurements of $\Delta\Psi_m$. The presence of an energized mitochondrion in a parasitized cell provides evidence that the parasite is metabolically viable, but does not speak to its reproductive viability.

Furthermore, the loss of $\Delta\Psi_m$ itself does not establish that an intracellular parasite is apoptotic. The characteristics commonly used in conjunction with $\Delta\Psi_m$ as apoptosis indicators in cultured animal cell lines include membrane phosphatidylserine (PS) exposure, detected with fluorescent annexin V, and permeability to various nucleic acid dyes. The cytoplasmic membranes of parasitized RBCs, although known to contain exposed PS and to therefore bind annexin V, retain sufficient structural integrity to prevent annexin V from reaching the parasite cytoplasmic membrane. Similarly, it is the RBC membrane that determines whether a permeability indicator dye such as propidium iodide can reach the outside of the parasite.

Other parameters measurable by cytometry, e.g., intracellular and intraorganelle pH, content of ions such as Ca^{++}, and levels of redox-related metabolites such as reactive oxygen species (ROS), glutathione, and total –SH groups, are of interest with respect to the physiology of parasites. The multicompartmental nature of parasitized RBCs, composed as they are of RBCs containing membrane-bounded parasites within which there are a variety of membrane-bounded organelles, may make it necessary to use relatively high-resolution imaging apparatus to distinguish signals from molecules of the same indicator in different compartments.

Physiologic parameters such as $\Delta\Psi_m$ are meaningful only in cells that are at least potentially viable. There are some probes that can be applied to cells in that condition that will bind covalently and withstand subsequent fixation, thereby providing at least some indication of the cells' initial physiologic status (Jogdand et al., 2012). This approach would almost certainly be necessary if a parameter such as $\Delta\Psi_m$ were to be considered for diagnostic applications.

Activities of some intracellular enzymes may be detectable however, not only in nonviable cells but also in those that have been permeabilized and/or fixed, using chromogenic or fluorogenic substrates. The fluorogenic substrates most widely used in cytometry are simple esters of fluorescein and its carboxylated derivatives carboxyfluorescein and calcein; the esters are nonfluorescent and sufficiently lipophilic to diffuse readily through intact cell membranes. Once inside, they are hydrolyzed by nonspecific esterases present in almost all cells, forming fluorescent products containing negatively charged carboxyl groups, which do not readily cross intact membranes. Although esterases in cells with damaged membranes may hydrolyze the esters, the resulting products do not accumulate in sufficient quantity to render these cells fluorescent. Thus, intracellular fluorescence after exposure to fluorescein esters, although often described as an indicator of "viability," actually only establishes the integrity of the cytoplasmic membrane. Other chromogenic and fluorogenic substrates, e.g., tetrazolium dyes, have been used to determine metabolic activity; still others detect the activity of hydrolytic enzymes unique to unique cell types such as monocytes and more general classes such as stem cells.

Protein Structure: Labeled Antibodies and Fluorescent Proteins

By the late 1960s, when analytical flow cytometers and cell sorters were first applied to the problem of differential WBC counting, it was clear that the simple acid-basic dye mixtures in Giemsa's and related stains could not provide the information necessary to distinguish classes of lymphocytes that had been shown to play different roles in the function of the immune system. The fluorescent antibody technique, first developed in the 1940s, provided an obvious means of identifying small numbers of protein molecules in and/or on cells using the high-powered lasers that had recently become available. From the late 1970s on, initial successes in this area led to the exploitation of monoclonal antibodies as specific reagents. When these were used in the early 1980s to define the cellular basis of HIV infection and AIDS and provide a reliable indicator of disease progression, flow cytometry became an essential clinical method, at least for those who could afford and maintain the apparatus.

Today's most complex flow cytometers can simultaneously detect a few thousand molecules of each of ten or more well-defined antigens on single cells. Their simpler predecessors facilitated the discovery of effective antiviral therapies for HIV. From 2000 on, the global effort to extend treatment to HIV

patients in resource-poor countries has motivated the development of a range of progressively simpler apparatus for counting CD4+ T lymphocytes. As noted above, such apparatus for these regions now includes small field-portable fluorescence imaging cytometers that are orders of magnitude smaller, simpler, and cheaper than the far more elaborate flow systems that are now used in malaria research. The imaging instruments can detect signals from fewer than 5,000 fluorescently labeled antibody molecules above background; newer antibody labels should provide even higher sensitivity.

Monoclonal antibodies that detect species-specific parasite protein epitopes are now used in RDTs. Although levels of proteins such as aldolase, HRP2, and pLDH may vary considerably from case to case, antibodies to the latter have been detected by fluorescence microscopy, and biochemical analysis suggests that at least a few tens of thousands of molecules are expressed even in ring-form-parasitized cells. They should therefore be detectable by simple imaging systems as well as by flow cytometers, potentially making it possible for thousands of instruments now in place in the field to be adapted for parasite density determination, diagnostic and otherwise.

The practical development of fluorescent proteins as research tools was, like that of monoclonal antibodies, facilitated by the availability of cytometric apparatus, including high-resolution imaging and confocal microscopes as well as flow cytometers. Although the proteins have proven useful for research applications in malaria (Tilley et al., 2007; Vorobjev et al., 2012), they are substantially less likely than antibodies to be practical for diagnosis.

Nucleic Acid Sequence and Base Composition

Complete genome sequences are now available for three of the six human malaria parasites. The *P. falciparum* genome contains 23.3 Mbp of DNA, with 80.6% in the form of A-T pairs; few other organisms approach this A+T percentage. The *P. vivax* genome contains 26.8 Mbp of DNA, 57.7% in the form of A-T pairs. The *P. knowlesi* genome contains 23.5 Mbp of DNA, 62.5% as A-T pairs. The genomes of *P. malariae* and both *P. ovale* species are estimated to be between 25 and 27 Mbp in size; A+T percentages for these species are not precisely known. Haploid nuclei contain a single copy of the parasite genome.

Although DNA sequence information is widely regarded as capable of providing definitive malaria diagnoses, molecular methods are not yet sufficiently simple and inexpensive enough to replace microscopy. The earliest molecular reagents demonstrated to provide highly sensitive malaria species identification were probes for ribosomal RNA (rRNA) sequences specific to *P. falciparum*. Although autoradiography was initially used for their detection, it is now relatively commonplace to employ fluorescent in situ hybridization (FISH) and flow or image cytometry for the purpose in other contexts. The FISH probes are typically combined with DNA stains, allowing nonspecifically clumped probes to be identified as such by their lack of association with small amounts of DNA comparable in size to the genomes of target organisms. The principal drawback to the use of either FISH probes or fluorescent antibodies lies in the relative lack of abundance of binding sites for them in individual parasites, which typically contain no more than a few tens of thousands of copies of either rRNA sequences or species-specific proteins. Even ring-form parasites can, by contrast, bind millions of molecules of DNA dyes and at least hundreds of thousands of molecules of RNA dyes, providing much stronger fluorescence signals, making detection easier and quantification more precise. Also, nucleic acid dyes are, in general, substantially cheaper and more stable than antibodies and nucleic acid sequence probes, and staining procedures are considerably faster and simpler.

Old and newer cytometric data now suggest that base composition–selective DNA dyes could be used to achieve a genome-based species-specific diagnosis in both flow and image cytometers. Molecular biologists found in the early 1960s that the melting temperature at which double-helical DNA could be broken into single strands depended on the percentages of A-T and G-C pairs, which, respectively, are held together by two and three hydrogen bonds. This allowed the base composition of the DNA of a variety of organisms to be determined relatively simply from melting curves. The nucleic acid binding characteristics of a number of different dyes and drugs could then be determined by analysis of their spectra when associated with different natural and synthetic polynucleotides.

The Hoechst dyes 33258 and 33342 and some related compounds, all originally developed and evaluated as antimalarials, were found to fluoresce strongly only when bound to multiple A-T pairs in DNA. Other drugs such as chromomycin A3 and mithramycin were found to bind with resultant fluorescence only to G-C pairs in DNA. The addition of Hoechst 33258 to mixtures of DNA with substantially different percentages of A-T pairs facilitated ultracentrifugal separation, and was employed in late 1987 to purify *P. falciparum* DNA subsequently used to create sequence-specific probes for the organism (Dame and McCutchan, 1987).

Hoechst 33258 had also been used earlier in 1987 to stain human RBCs infected with *P. falciparum* and *P. vivax* for flow cytometry (Janse et al., 1987), with the former yielding fluorescence signals >21% higher than the latter, precisely as would be predicted from the A-T percentages and genome sizes, neither of which were known at the time. The predicted signal intensities from RBCs infected with *P. knowlesi* would be almost 27% lower than those from Pf-infected RBCs. Although intensity differences of less than 30% are not reliably detectable by eye, much smaller differences are routinely measured cytometrically. Breeders now inseminate cattle using bull sperm sorted into highly enriched X-chromosome-bearing and Y-chromosome-bearing fractions based on a difference of less than 4% in Hoechst dye fluorescence.

Even smaller base composition differences are detectable using combinations of an A-T selective dye with one that has a G-C preference. Two-parameter sorting of human metaphase chromosomes stained with Hoechst 33258 and chromomycin A3 was used to prepare the first chromosome-specific libraries used in the Human Genome Project. This dye combination was also used to identify different bacterial species by base composition and was shown to provide reliable quantification of A+T percentage. Such analyses do not demand use of high-powered, high-priced cell sorters; base composition differences between trypanosomal strains, which would be expected to yield roughly the same fluorescence intensities as would malaria parasites, can be detected on smears using relatively modest fluorescence imaging cytometry apparatus (Mühlpfordt et al., 1985; Mühlpfordt and Berger, 1989, 1990).

The best information now available suggests that base composition measurements done in such apparatus can reliably discriminate *P. falciparum* from *P. vivax* and P. *knowlesi* and probably distinguish between the latter two species, achieving a specificity equivalent to that obtained with sequence-specific probes but using only relatively inexpensive, simple dyes. The imaging instruments could easily be used to measure the genome sizes and base compositions of the two *P. ovale* species and *P. malariae* by analyzing blood smears from patients with diagnoses confirmed by molecular methods. The utility of DNA base composition measurements for identifying these species could therefore be determined within a few months.

REPLACING MALARIA MICROSCOPY WITH CYTOMETRY: WHAT TO MEASURE AND HOW

Microscopy and Cytometry: Multiparameter Analysis in Stage Identification and Diagnosis

Cell identifications made on the basis of morphologic characteristics visualized by microscopy are of necessity a level removed from those that are made using flow or image cytometry to quantify specific cellular constituents. However, both observers and cytometrists, whether identifying cells by stage and species or determining their functional status, typically consider more than one cellular characteristic. An observer will identify a malaria parasite as a ring stage because of its size and shape, relatively scant cytoplasm, the presence of a single nucleus, and the absence of Hz. Cytometric identification of trophozoites and schizonts can be made by quantification of DNA and RNA content. The former is very highly correlated with the number of nuclei and the latter reflects the amount of cytoplasm present; Hz might or might not be measured, but size and shape information are unnecessary.

The visual identification of gametocytes, particularly the crescent forms that gave the species name to *P. falciparum*, is almost entirely based on morphology, although the presence of Hz also provides a clue. Cytometric quantification of DNA and RNA would reveal a DNA content about twice that of a single genome (Janse et al., 1988), and also would demonstrate the presence of a much larger amount of RNA in female (macro-) than in male (micro-) gametocytes. The difference in RNA content accounts for the more

pronounced blue color of the cytoplasm in Giemsa-stained macrogametocytes. Cytometry would also detect relatively large depolarized SSC signals from gametocytes because of their substantial Hz content; they are the only parasite forms with near-diploid DNA that contain significant amounts of the pigment. Morphologic information would, as in the case of ring forms, be unnecessary for cytometric identification of gametocytes.

All of the parameters mentioned above as relevant to the detection and identification of malaria parasites, i.e., DNA content, base composition, and sequence; and the contents of RNA, hemozoin, and various specific proteins, can be measured accurately and precisely by light transmission, fluorescence, and/or scattering. Whether the measurements are made on liquid samples in a flowing stream or a counting chamber, or on dry samples in smears on slides, the basic analytical problem remains one of detecting and quantifying small amounts of light originating from small regions of space. In 1904, the detection task could only be done by a human observer using a powerful microscope. A century later, although humans remain severely limited in their capacity to quantify light intensity, the more accurate and precise instrumental methods for doing so do not depend on either high magnification or high resolution.

Problems with Microscopy 1: The Sampling Statistics Numbers Game

Even before Giemsa developed what became the definitive stain for blood smears, it had become clear that malaria diagnosis presented problems different from those encountered in blood cell counting, because the levels of parasites present in blood were frequently far lower than was the case for even relatively uncommon types of WBCs and RBCs.

If it is assumed that any malaria parasites seen on a patient's blood smear come from the patient, detection of even a single parasite would indicate the presence of parasitemia. Reliable quantification of parasite density, however, requires counting a number of parasites in a defined volume of blood, and precision depends on the number actually counted. The relevant statistical distribution is Poisson's: the standard deviation (SD) of a count of n objects is $n^{1/2}$ (i.e.,),

$$\sqrt{n}$$

even in the absence of any other sources of variability. The percent coefficient of variation (CV), i.e., 100 times the SD divided by the count, defines the best attainable precision of the count. In order to reduce the minimal attainable CV on a density measurement to 10 percent, it is necessary to count 100 parasites; at a density of 100/μl, this would require the examination of 1 μl of blood.

In a modern microscope, the area of a high-power field (HPF) seen with 10× eyepieces and a 100× oil immersion lens is ~0.025 mm^2; in older instruments, a single HPF encompassed ~0.02 mm^2. Thus, a 10 × 20–mm area of a thin smear or film containing 1 μl of blood would contain 10,000 HPF. At a density of 100/μl, the target lower level of detection for microscopy, only 100, or 0.002%, of the ~5,000,000 RBCs in 1 μl of blood contain parasites; it would be necessary to examine 0.01 μl of blood, or 100 HPF, to encounter a single parasite.

Ronald Ross himself, realizing that the detection of parasites at low densities in thin blood smears would require hours of observation, suggested in 1903 (Ross, 1903) that in addition to the thin smear, a thicker one be made that contained 10 to 20 μl of blood. He observed that washing an unfixed thick smear with distilled water would remove the hemoglobin from most of the RBCs, enabling parasites to be seen. It remains common practice to prepare both thin and thick smears, examining 200 HPF in both and 500 HPF in the thick smear if no parasites are seen in 200 HPF. By 1910, Ross had refined his methodology (Ross and Thomson, 1910), allowing the actual volume of blood in a thick smear to be estimated relatively precisely by dispensing it from a calibrated capillary tube, typically distributing 1 μl over a 5 × 5–mm area, comprising 1250 HPF. Allowing 3 sec/HPF, the entire volume of blood could be searched in an hour, although, as Ross noted, "the identification of the plasmodia [in the thick smear] requires considerable practice."

In recent years, it has been established that the distilled water wash step may remove 60% to 90% of parasites from an unfixed thick smear; gametocytes, typically accounting for only a small percentage of parasites in any case, appear to be lost preferentially in washing. Since even the best thick smears typically do not present parasites in a fashion in which morphological differences between species are as easily distinguished as is possible with thin

smears; however, a good case can be made for the use of fixed thin smears alone (Ohrt et al., 2008).

Problems with Microscopy 2: The Resolution Requirement

The relative nonspecificity of the Giemsa stain demands that the human observer process high-magnification, high-resolution images to extract information about the species and stage of any parasites encountered on the slide. We have already noted that equivalent or superior information, independent of morphology, can be obtained using flow cytometers, without the need for high magnification or resolution. Assuming for the moment, however, that computer image analysis of Giemsa-stained slides could be made as accurate as flow cytometry, the collection of data itself would require relatively complex and expensive hardware. It would be necessary for an automated stage to move in increments of no more than a few micrometers in two directions to bring hundreds of high-power fields into the range of the objective lens, and for an automated focusing mechanism to move in submicrometer increments to acquire a properly focused image of each field. A high-power microscope made from a mobile phone, using a spring clip in which to hold a slide and human rather than binary digits for motion control, is unlikely to provide any significant improvement in malaria diagnosis.

Problems with Microscopy 3: Fluorescence Cannot Be Used Optimally

Almost all staining procedures for flow cytometry employ fluorescent dyes and/or labels; it is the quantification of multiple cellular constituents by whole-cell fluorescence measurements in different spectral regions that provides the information needed to identify different cell types and characterize their physiologic states.

The light collection lenses used in flow cytometers are similar in their optical characteristics to the "high dry" and oil immersion lenses used in microscopes. The lens characteristic critical in flow cytometry, however, is numerical aperture, or light-gathering power. Although there are flow cytometers available at premium prices that can provide medium-resolution images of single cells, a more typical instrument collects light equally accurately and precisely from the thin "slice" of a cell on which it is focused and from the adjacent out-of-focus areas. These instruments do not form an image in which cellular details can be recognized.

Fluorescence measurements of stained cells using either flow cytometers or imaging systems typically yield signals high enough above a background level to make it substantially easier to detect small numbers of dye molecules in or on cells than would be the case with absorption or extinction measurements. Absorption and extinction by particles near or below the optical resolution limit are essentially undetectable; fluorescence and light scattering by particles substantially smaller than the resolution limit, down to the level of individual virions, microvesicles, and even single dye molecules, can be detected in suitably configured apparatus.

Fluorescence microscopes were not available in 1904; until around 1940, they required expensive and relatively power-hungry light sources such as carbon arcs and mercury and xenon arc lamps, and were therefore inaccessible in many places with a high incidence of malaria. From the 1940s on, improvements in incandescent lamp technology made microscopes more affordable and somewhat more energy efficient; since the "Giemsa Centennial" in 2004, high-power LEDs, efficient enough to be run for hours on batteries, have become preferred illumination sources for fluorescence (and other) microscopy as well as for automotive, home, and industrial lighting. Although an LED-illuminated fluorescence microscope must still be run in a dark environment, the addition of a video camera allows a human observer to work in a lighted area.

From the 1920s on, fluorescent dyes were evaluated as stains for blood cells, malaria parasites, and bacteria. Their most notable recent application to public health problems in resource-poor countries has been for the diagnosis of tuberculosis based on the acid-fast nature of the mycobacterial cell wall. It had been known for almost 70 years that using the fluorescent dye auramine O rather than the carbol fuchsin Ziehl-Neelsen absorption method to stain sputum slides typically allowed smaller numbers of organisms to be detected more rapidly using lower (high-dry instead of oil immersion) magnification. A number of manufacturers offer microscope adapters and inexpensive fluorescence microscopes that are equipped with blue LEDs for excitation and yellow filters for observation. This apparatus

is suitable for detecting malaria parasites in acridine orange-stained blood as well as for TB microscopy with auramine O. At least one microscope, the CyScope (Partec), can be equipped with a UV LED for excitation and a blue emission filter, allowing the detection of parasites using the relatively DNA-specific stain DAPI.

Since a human nucleus contains several hundred times as much DNA as a ring-form parasite and almost ten times as much as a schizont with 20 to 30 nuclei, it is not a challenge for a human observer to distinguish parasites from WBC on the basis of the fluorescence intensity of a DNA stain. It would be substantially harder to discriminate between DNAs of different base compositions using a pair of DNA stains. As long as the human observer is involved, it is also necessary to work with a magnified image. Although a single field observed at 40× encompasses 6.25 times the area of a 100× field, allowing observation time to be shortened by roughly the same factor, the human, even dark-adapted and sitting in a dark room, would be unlikely to be able to detect fluorescence from a stained ring-form parasite when looking at a "life-size" (1× magnification) slide.

A further disadvantage of fluorescence microscopy vis-à-vis fluorescence cytometry lies in the tendency of fluorescent dyes to bleach and fade, especially when exposed to the high-intensity excitation used in microscopy and cytometry. Giemsa and other absorption stains leave a much larger amount of dye in stained cells than do typical fluorescence staining procedures; this in itself often makes it difficult to quantify stain intensity by absorption because Beer's law is violated. The large amounts of absorption dyes in cells are, however, easily observable using much less intense illumination than would typically be optimal for fluorescence excitation, so significant fading does not occur even after prolonged illumination. Although there are some chemical treatments that retard the bleaching and fading of fluorescent stains, one typically notices a progressive decrease in the intensity of cells after even a minute or two of scanning a slide. This effectively prevents visual quantification of fluorescence with any precision. In a flow cytometer, the sample flow rate is controlled; cells are not exposed to an illuminating beam until they pass through it for measurement, and all cells spend nearly the same time in the beam, allowing fluorescence intensities from different cells to be compared.

Fluorescence Image Cytometry: Throughput Going Up, Costs Going Down

Quantitative fluorescence microscopy at the whole-cell level can be done by restricting both the illumination and the microscope field of view to an area not too much larger than the area of the cells to be measured, and sequentially positioning individual cells using phase microscopy or an equivalent dye-independent, contrast-enhancing technique and illumination at a wavelength at which the relevant fluorescent dyes do not absorb significantly. Fluorescence measurements can then be made when an excitation source is turned on, or its light is allowed to reach the cell, for only a relatively brief, defined period of time.

This slow process, typically consuming at least several seconds for the measurement of a single cell, was unavoidable when photodiodes or photomultiplier tubes (PMTs) were the only detectors available and extended sources (e.g., arc lamps) were the only available excitation sources. It is possible to speed up the analysis process considerably by examining a larger field of view containing more than one cell, provided cells are well enough separated in space for signals from them to be distinguished. A diode or PMT detector can be used if a laser, focused to a small spot and scanned across the area of the field, is used for illumination; this is analogous to a flow cytometer except that the beam is brought to the cells instead of the cells being brought to the beam. Although such laser-scanning cytometry reduces analysis time to a small fraction of a second per cell, the apparatus incorporates both the costly and complex illumination and light detection components used in a flow cytometer and the precision stage motion and focus control hardware needed in an automated microscope.

If a lamp or LED is used to illuminate a field containing several cells, it becomes more logical to use one or more digital cameras as fluorescence detectors. In this case, it is necessary to use a fluorescence standard such as a dyed slide to measure variations in excitation intensity over the field, providing a normalization factor for raw fluorescence intensity measurements.

This allows reasonably sensitive and precise measurements to be done in standard fluorescence microscopes; it has, moreover, been established that, as is also the case in flow cytometry, the precision of such measurements may be maintained when cells are out of focus.

Using a multimegapixel digital camera chip as a detector overcomes the limitations of the human visual system, demonstrably allowing precise and sensitive quantitative whole-cell measurements of large numbers of cells dispersed on a slide or in a counting chamber to be made at 1× magnification, with all cells in the sample being illuminated simultaneously. Such wide-field imaging cytometry was first demonstrated in the early 1990s when the available digital cameras cost over $10,000 and the laser and arc lamp sources suitable for excitation were only slightly less expensive, making the apparatus very much simpler than a flow cytometer but not significantly less costly. An equivalent instrument could now be made using LEDs—available at wavelengths ranging from UV to IR and costing only tens of dollars—for illumination and similarly priced camera and microprocessor chips for detection and the very rudimentary image analysis required. Wide-field imaging cytometry requires exposure times no longer than a few seconds; data from thousands of cells in a field can be analyzed in minutes.

Based on the well-established relationships between component costs and finished product costs for similar biomedical apparatus, an image cytometer of the type just described could be sold for no more than a few thousand U.S. dollars. The device could examine 1 μl of blood smeared over a 1 cm^2 area of a slide without requiring either sample movement or focus adjustment, making detection of one or more parasites 95% likely even at a density of 5/μl. Obviously, the device could be put to use for a wide range of other measurements now made by flow cytometry.

Problems with Flow Cytometry

Reviews of experimental approaches to improving malaria detection, density determination, and diagnosis frequently describe flow cytometry as not only complex and expensive, which it is, but also as imprecise and not particularly sensitive for detecting low parasite densities. We regard the latter characterization as unfair. The small sample of publications dealing with this application, with the earliest dating to the 1970s, have used a wide range of preparative procedures and instruments, with the choice of dyes restricted more often than not by the capabilities of the apparatus available. Much of the earlier work was done using flow cytometers that had only one light source: a 488-nm argon ion laser. The flow cytometers also used nucleic acid dyes that were not particularly specific for DNA or RNA. This made it difficult to distinguish cells bearing early-stage parasites from reticulocytes. As noted above and illustrated in Figure 11.20.1, the use of DNA- and RNA-specific dyes facilitates this distinction. The addition of measurement parameters, e.g., Hz content, would be expected to increase the specificity and sensitivity of detection, and the small amount of data accumulated by the few laboratories now capable of making such measurements points in this direction. Long ago, flow cytometry was shown to be capable of detecting tumor cells seeded into blood at densities of 1/10,000,000 WBC (Gross et al., 1995); equivalent performance in malaria would detect 1 parasite/μl.

Although flow cytometers designed for research and relatively sophisticated clinical immunophenotyping procedures are reasonably well suited to measuring cells from human blood and the immune system, and flow cytometers designed for clinical hematology labs are optimized for the examination of blood cells, no production apparatus of either type comes equipped to measure DNA, RNA, and hemozoin content, let alone DNA base composition and the sequence and content of proteins such as pLDH. Almost all instruments could measure DNA and a specific protein; many could also be adapted to measure RNA and hemozoin. Base composition measurement is problematic because the required base-sensitive DNA dyes require excitation with UV or violet light. Neither wavelength is available on most flow cytometers; adding UV illumination also typically adds at least $10,000 to the cost of an instrument.

The effect of anemia, particularly iron deficiency anemia, on the course of malaria has been of great interest for some time; a recent paper describes flow cytometric measurement of the labile iron pool in parasitized RBCs (Clark et al., 2013), and it would be of great interest to examine the growth of parasites in RBCs with different hemoglobin contents. However, although one type of existing flow cytometric hematology analyzer (Advia; Siemens Healthcare) can measure hemoglobin, the procedure uses relatively complex optics that cannot readily be added to any existing research flow cytometer, and the hematology analyzers cannot readily be adapted for fluorescence measurements of other relevant parameters such as DNA and RNA. Although an instrument capable of the combined measurements could be built for substantially less than the cost of a

moderately sophisticated commercial research flow cytometer, it has been difficult to convince anybody to pay for it.

At a more mundane level, we note that flow cytometers, even those adapted to use less fluid in their specimen transport mechanisms, consume fairly large volumes of clean, particle-free water or saline, and that access to clean water is limited in many of the malaria-prone areas in which affordable cytometric apparatus for malaria diagnosis and research is most needed. Wide-field image cytometry, which is now capable of measuring the relevant parameters, requires minimal quantities of water and reagents, and the apparatus is less complex and costly, and more robust and portable, than flow cytometry, and is likely to remain so for the foreseeable future.

THE CYTOMETRIC FUTURE IN MALARIA DIAGNOSIS, RESEARCH, AND EPIDEMIOLOGY

At this writing, we are unaware of the existence of any wide-field image cytometers optimized for multiparameter measurements of malaria parasites. We have defined the capabilities of the necessary apparatus during development projects targeting other diagnostic and research applications. Fine-tuning an instrument configuration for malaria work could be accomplished within months, given a supply of unstained patient blood smears containing parasite species identified by nucleic acid sequence and smears from cultured *P. falciparum*, *P. knowlesi*, and—should it become available—*P. vivax*. It would then become possible to put prototypes into the field.

We have been asked about the feasibility of using the installed base of flow cytometers for malaria diagnosis. There are, at present, several thousand such instruments now in use in the field for CD4 counting; most of these relatively simple systems measure fluorescence in two spectral regions and some add a forward or side scatter signal. The fluorescence measurements are sufficiently sensitive to detect a few thousand molecules of each of two antibody labels. Any more complex fluorescence flow cytometer used in either a clinical or research laboratory can almost certainly measure the same two spectral regions with at least equivalent sensitivity.

As noted above, determination of a parasite density of 100/μl with a theoretical minimum percent CV of 10% requires counting at least 100 parasites, which will be distributed among the roughly 5,000,000 RBCs in 1 μl of whole blood. The simpler flow cytometers, e.g., CD4 counters, may count no more than 5,000 "events" per sec, where an "event" is defined as occurring when a selected "trigger" signal rises above a preset threshold level. In almost all malaria cytometry, the trigger signal is a scatter signal, with the threshold set to include RBCs, but to exclude platelets and smaller particles. Analysis of 5,000,000 RBCs in this case will require 1,000 sec, or 16.7 min. Since the actual flow rate of diluted sample through the cytometer is likely to be no more than 1 μl/sec, the blood sample will have to be diluted 1:1,000 before it is introduced into the cytometer.

The BD Biosciences FACSCount CD4 counter measures only two fluorescence signals. The trigger signal comes from the label on an antibody against the CD3 antigen, the presence of which defines a T cell. The CD4+ T cell count is obtained by counting only those "events," i.e., T cells, for which there is corresponding signal in the other fluorescence channel, indicating expression of the CD4 antigen. The blood sample is not lysed; however, since the RBCs do not express CD3 antigen, the apparatus is "blind" to them. If the blood sample is diluted 1:10, and the CD4+ T cell count is 1000/μl, it will take only 10 sec to detect 1000 CD4+ T cells, and, even at a count of 50/μl, 100 cells would be counted in 20 sec.

In attempting to detect malaria parasitemia at low levels, it would be advantageous to use a relatively low dilution of blood in order to be able to analyze 1 μl in a minute or two; the trigger signal would have to be chosen to keep the event rate below 5000/sec. The use of a signal from a DNA-specific fluorescent dye would be optimal. RBCs and platelets do not contain DNA; WBCs and nucleated RBCs contain several hundred times as much DNA as do ring-form parasites and almost ten times as much as do the latest-stage schizonts. If blood were diluted 1:10, 0.1 μl of blood would be analyzed each second. With a normal WBC count, no more than 1000 trigger events would come from WBCs, whereas, at a parasite density of 100/μl, only 10 trigger events would be generated by parasitized RBCs. Although small amounts of free DNA are present in blood, their size is typically well under 1 Mbp, so a threshold setting of ~15 Mbp would prevent either free DNA or any bacteria present in blood from generating trigger events because of septicemia or contamination. Nucleated RBCs present at levels of tens of thousands/μl might require that blood be

further diluted; the events they generated, however, would not be confused with any generated by parasitized RBCs. DNA events associated with malaria parasites in the range of 20 Mbp to a few hundred Mbp could also arise if other parasites, e.g., trypanosomes, were present in blood; potential interference would, however, come from micronucleated reticulocytes, which are not normally found in blood.

Micronuclei, also called Howell-Jolly bodies, are chromosomal fragments that remain in RBCs as they enter circulation after incomplete extrusion of the nucleus. Micronucleated reticulocytes typically contain a few times as much DNA as does a single malaria parasite genome; the actual amount is highly variable, however, whereas ring-form-parasitized RBCs contain one, sometimes two, and occasionally three copies of a genome of well-defined size. Many CD4 counters use green (532 or 546–nm) lasers for excitation; these are typically effective at exciting a number of DNA-specific dyes, including DRAQ5 and Vybrant DyeCycle Ruby. Both of these fluoresce at ~670 nm, allowing the shorter fluorescence wavelength (~580 nm) to be used to detect a weaker signal, e.g., one from a fluorescent antibody.

Although the signal from a DNA dye would detect any parasites present in a blood sample, that signal alone would be unlikely to permit reliable discrimination among parasite species; it might also not completely distinguish parasitized RBC from micronucleated reticulocytes. The base composition–specific dyes thus far shown to be usable for discriminating *P. falciparum* DNA from *P. vivax* and *P. knowlesi* DNA require UV or violet excitation—capabilities that are not widely available in simpler flow cytometers. Although one could use sequence-specific rRNA probes for species identification, they are relatively scarce and expensive, and sample preparation would require nucleic acid hybridization. It therefore seems preferable to us to adapt monoclonal antibodies to parasite antigens, which are already well characterized as components of RDTs, for species identification by flow cytometry. Since the diagnosis of *P. falciparum* takes precedence, one might first run a sample stained with DNA dye and antibody to the pLDH of that species, or to HRP2. If specific antigens were not found, one could stain additional aliquots with antibodies to multiple species. If antigen expression is sufficiently uniform, it might also be possible to use different dilutions of panspecific and/or species-specific antibodies together in a multiplex assay that would allow detection and species identification to be done from a single tube.

The strategy just described could be used with even the simplest flow cytometers for diagnosis, for following parasite clearance on therapy, and for determining low parasite densities for epidemiologic purposes. A simple flow cytometer could also be used with a combination of DNA- and RNA-specific dyes (the yellow fluorescent pyronin Y probably represents a good choice for the latter) to determine resistance to antimalarials by examining the growth of patient-derived parasites in short-term culture. This represents the cytometric analog of the schizont maturation assay (World Health Organization, 2001), which was first developed for *P. falciparum*, but it was also subsequently modified to work with *P. vivax*, eliminating the drawbacks of the associated microscopy.

Of course, more complex flow cytometers can be used for more complex assays, for example, combining DNA, base composition, RNA, hemozoin, and membrane potential measurements. We have already noted that several of these measurements could also be done simultaneously in a much simpler and less expensive wide-field image cytometer, overcoming the economic and infrastructure problems associated with flow cytometry (Basco, 2007), but that no such image cytometers now exist. We should now also point out that we do not know of more than ten flow cytometers worldwide that are presently configured for the full range of measurements. Dozens of these image cytometers could be built for the cost of a single additional high-end flow cytometer.

Economic realities have recently forced substantial reductions in the funds available to deal with all aspects of malaria; we remain optimistic that the right kind of cytometry might actually make it easier for microscopists to work within current fiscal constraints, putting affordable and sustainable technology where the malaria is and empowering those most directly affected by the disease to deal with it more effectively.

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