IDENTIFYING MALARIA SPECIES SIMPLY:
THE EYES DON'T HAVE IT!

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I am posting this to the Purdue Cytometry Mailing List because, although it does not contain enough hard data to be readily accepted by a peer reviewed journal, it proposes an affordable, sustainable cytometric solution to a major diagnostic problem, that of identifying and characterizing malaria parasites. Malaria is an ancient disease, but it was one of the first few for which a cellular cause was identified, and that occurred less than a century and a half ago. An effective remedy had been discovered by happenstance a few centuries before. Over the past decade, the death toll from the disease has gone down dramatically, due in part to the Nobel Prize-winning work of To Youyou on artemisinins, currently the most effective therapy.

It is sobering that modern cytometry is now increasingly used to detect emerging resistance to these drugs, but also disturbing that the turn-of-the-century technology used annually to prepare and examine 50 million blood smears for malaria is from the turn of the 20th century. And it was only in 2010 that the WHO recommended that a parasitologic diagnosis, made either using this antique technology or the newer (1980s), but still problematic antigen-based Rapid Diagnostic Test (RDT) be done to support treatment in every case.

On the bright side, what I propose below to replace them could be used for drug development and sensitivity determination and for detection of malaria eradication as well as for diagnosis and monitoring treatment of disease at the point of care. It only requires whole cell measurements but, like PCR or LAMP, gathers its critical information from parasite genomes.

The required measurements can be done in almost any existing flow cytometer and in many imaging systems, including standard laboratory microscopes fitted with inexpensive digital cameras. They are:

1. Determining species of each parasite by measurement of total numbers of A-T and/or G-C base pairs present in its DNA using fluorescent dyes, and, optionally,

2. Determining stage of development of each parasite by measurement of its RNA content using fluorescent dyes and/or its hemozoin (Hz) content using intrinsic absorption in the far red (650-700 nm) spectral region.

For background, the reader is referred to the overview of malaria cytometry that my colleagues and I published in Current Protocols in Cytometry in 2013(1). In the current post, I shall include references to more recent significant work with direct bearing on what I consider to be the optimal current technical approach Six species of the genus *Plasmodium* are now known to cause malaria in humans. The genome of *P. falciparum*

(*Pf*) was assembled in 2002, and those of *P. vivax* (*Pv*) and *P. knowlesi* (*Pk*) in 2008. More recent data for the other species in the following table suggest that it will be possible to determine the species of any human malaria parasite on a solid surface or in liquid medium by a single measurement of the fluorescence of an A-T selective DNA dye. The measurement technology was developed beginning in the 1970s and 80s and applied to the identification and sorting of human chromosomes to generate the first libraries for the Human Genome Project. The method has also been shown to apply to bacteria and used to detect differences as small as a few percent between organisms with genomes less than 10 Mb in size.

Although the Genome Project work was done on very expensive, high-power high-speed sorters, and large sorters are now in commercial use in cattle breeding to separate X and Y chromosome-bearing bull sperm differing in total DNA content by about 3%, a standard fluorescence microscope was used in the 1980s to show differences in base composition between different strains of trypanosomes. Ironically, the authors of a 1987 paper found differences of about 16% in the fluorescence of Hoechst dye-stained ring forms of different malaria species examined in an arc-source BD FACS Analyzer, but concluded these were artifactual and did not investigate further.

Plasmodium Species	Genome Size (Mb)	AT Fraction	AT (Mb)	*Predicted AT Dye Signal (%*Pf*)
P. falciparum (Pf)	23.3	0.81	18.9	100.0
P. vivax (Pv)	29.1	0.60	17.5	92.5
P. knowlesi (Pk)	24.4	0.61	14.9	78.9
P. ovale curtisi (Poc)	33.5	0.71	23.8	126.0
P. ovale wallikeri (Pow)	33.5	0,71	23.8	126.0
P. malariae (Pm)	33.6	0.76	25.6	135.3
P. malariae-like (Pml)	23.7	0.70	16.6	87.9

*from cytometry with Hoechst 33342 or similarly A-T selective dye

Table 1. Size and AT content of some Plasmodium genomes.
H. Shapiro 2019 Data from Rutledge et al. Nature. 2017; 542:101.

Malaria had been known for millennia in Africa, Asia and Europe, but its cause was unknown, and there was no effective treatment. The disease was transmitted to the Americas around 1500 by European conquerors and colonists and their African slaves. Cinchona bark, a native remedy for chills, became known to missionaries in Peru, and was brought back by them to Rome, which was ravaged by malaria. The "Jesuit bark" became the first effective therapy for the disease, and soon found worldwide demand. The cause remained a mystery for another few centuries, but it was noted in

postmortem dissections of malaria victims that a brownish-gray pigment, now known as hemozoin, accumulated in tissues.

By the mid-1800s, cells and microorganisms had been discovered and the role of the latter in several infectious diseases had been established. A bacterium was suspected of causing malaria when, in 1880, the French army doctor Alphonse Laveran inspected fresh blood from a malaria patient under a microscope and saw larger, motile, pleomorphic hemozoin-containing bodies which he became convinced were parasites that caused the disease. This view was not widely accepted until two significant advances in microscopy were made. The oil immersion lens, developed by Zeiss, became widely available around 1884, and new synthetic dyes greatly increased the visibility of cellular structures.

The dyes themselves might not have come into existence without malaria. The active ingredient of cinchona bark, quinine, was isolated in 1820 but still had to be extracted from the bark to be useful in the treatment and prevention of the disease, and remained expensive. An abortive attempt in the 1850s to synthesize it yielded mauve, a superior textile dye, and spawned an early "high-tech" industry in Great Britain and Europe.In the 1870s, Paul Ehrlich, a medical student in Germany, developed the notion that different parts of cells would stain with different dyes depending on their chemical affinities. This made it possible to distinguish several different types of white cells in blood, and, eventually, to detect the presence and visualize the development of malaria parasites in red cells. In the 1880s, Malachowski, Romanowsky, and others raced to improve staining mixtures containing eosin Y and methylene blue in order to optimize detection of parasites; the trick was to add an azure dye to maximize contrasting color differences between parasite nuclei and cytoplasm (3). All three dyes are relatively nonspecific.

In 1904, Gustav Giemsa described the mixture that has since become the "gold standard" for hematologic microscopy in general. By then, of course, other blood diseases besides malaria were known, but it was the only one for which there was a treatment, once staining provided a diagnosis. Before it became available, Laveran had faced many skeptics, as he noted in his 1907 Nobel Lecture; Ronald Ross's 1902 Lecture also stressed the importance of staining.
Since 1891, there had actually been two treatments for malaria. Ehrlich, recognizing that methylene blue stained malaria parasites, thought it might also kill them, and in that year reported successful chemotherapy of two malaria patients with the dye. It is still occasionally used therapeutically in malaria when resistance to other drugs is encountered. This, and Ehrlich's later work on trypanosomes and syphilis, transformed the chemical industry into a drug industry. The two sources of medicinal methylene blue mentioned in his 1891 report were Merck, of Darmstadt, and another company, which later took the name of its location, Hoechst.

Ehrlich himself was largely uninvolved in the development of blood cell stains after 1882, when he joined Robert Koch, who had lately discovered the causative organism of tuberculosis, in his efforts against that disease. Most TB diagnosis still relies on the methods of the 1880s; I will have more to say about that later and elsewhere. We get back to malaria by traveling roads not taken.

The English words "cytometry" and "cytometer" were introduced into the language in the late 1880s to describe the counting of blood cells and the device in which the cells were counted. Hemoglobin's functions and dysfunctions had begun to be known, but the amounts present in a cell or solution could only be estimated by comparison with color charts, a procedure readily applicable only to fairly intensely colored materials. New vocabulary emerged over the decades; for example, the "AC" in ISAC was once "Analytical Cytology," which was the title of a book influential in our field from the 1950s on.

The problem with Giemsa's stain in the context of modern malaria diagnosis is that it is non-stoichiometric and variable in color to the extent that it is only useful to pick up morphological rather than biochemical information about parasites and cells. Until about 1990, novices' and trainees' diagnoses were evaluated based on agreement or lack thereof with those made by more senior and more extensively trained clinicians and microscopists. More recently, the standard comparison has been with the results of molecular biologic and immunologic tests, which have shown the experts to be more fallible than was previously thought.

One of Ehrlich's alternatives to eosin-azure dye mixtures for staining blood cells was a triple stain containing methyl green, an impermeant doubly charged cationic dye, pyronin Y, a reddish-purple permeant cationic dye, and, optionally, one of several orange impermeant anionic dyes. Methyl green stained nuclei, while the cytoplasm of blood cells blended various amounts of pink and orange. Since the literature of that era contained no color photographs, one cannot be sure, but almost everyone I know who has compared drawings of methyl green-pyronin- and Giemsa-stained blood cells, with and without malaria parasites, found the former stain combination to give superior contrast. Cost, staining time, etc. are approximately the same for the two stains, but methyl green-pyronin does not require the tweaking needed by Giemsa.

The early 20th-century saw an increased interest in the cell nucleus and in the chemistry of its constituents. Albrecht Kossel received the 1910 Nobel Prize in Medicine for his studies in this area. In 1924, Robert Feulgen reported on a procedure for quantifying the amount of DNA, then called thymonucleic acid, but the readout required removing bases from the nucleic acid and replacing them with absorbing (or, later, optionally, fluorescent) dyes.

Beginning in the 1930s, Einar Hammersten and Torbjorn Caspersson, in Stockholm, used UV absorption microspectrophotometry to quantify unstained proteins and nucleic acids in cells, with the proteins absorbing at 280 nm and nucleic acids (both DNA and RNA) at 260 nm. The amount of DNA alone could be calculated after cells were treated with RNAse. An informative technique, but very complicated and requiring very expensive equipment.

The information was made much easier to collect by Jean Brachet, who, in the late 1930s and 40s, established that, in permeabilized cells, methyl green absorption

measured DNA content and pyronin Y absorption measured RNA content. By 1952, Robert Lewert, a young parasitologist in Chicago, had reported (4; a paper I encourage you all to read) that UV microspectrophometry and methyl green-pyronin absorption were equivalent in documenting increases in RNA and DNA in parasitized red blood cells during schizogony, which had been known for decades to involve cycles of division in parasite nuclei. Lewert's work apparently did not generate "aha moments" among malariologists; he spent the rest of a long career on schistosomiasis.

Between the 1950s and 80s, work by numerous people in numerous places, notably Nils Ringertz in Stockholm and Zbigniew Darzynkiewicz, who moved from there to New York, established that RNA and DNA could also be quantified using a single dye, acridine orange (AO), in permeabilized cells under carefully controlled conditions with green fluorescence from DNA-bound dye monomers and red fluorescence of RNA-bound dye aggregates both excited by blue or blue-green light.

Fluorescence flow cytometers with small and large argon ion lasers ideal for excitation of AO came on the market in the 1970s; they and larger sorters fitted with more powerful argon lasers were used to examine malaria parasites by the end of the next decade. In 1986, Hare et al sorted parasitized cells stained with AO using Darzynkiewicz's technique to show that all developmental stages could be identified based on DNA and RNA content without recourse to morphologic information. This methodology, however, would have been difficult to implement in the field.

Those fortunate enough to have UV sources on their instruments were able to work with some then-new dyes from Hoechst, not always as productively as they might have. The bis-bezimidoles 33258 and 33342 and some others were used for chromosomal analysis, for detection of BrdU incorporation into DNA, and, especially, for quantification of DNA in permeabilzed and (especially 33342) intact cells.

The dyes, by virtue of their strong preference for binding to AT pairs (a property shared with methyl green), could be added to DNA mixtures to facilitate ultracentrifugal separation of DNAs of high and low AT content. This property was used in the late 1980s to extract P. falciparum DNA from infected blood to prepare some of the first sequence probes for malaria. As mentioned above, however, the ability of Hoechst dyes to stain malaria species differently went unrecognized.

I have a sin of my own to confess here. In 1980, when I was searching for a DNA/RNA dye combination compatible with fluorescein immunofluorescence, I settled on pyronin Y as the RNA stain, knowing the 488 nm laser would excite its orange fluorescence, but did not consider methyl green because it had long been reported to have problems with crystal violet contamination. So I chose Hoechst 33342 instead, continuing to avoid methyl green even in the later 80s and 90s, when I was eagerly pursuing red-excited dyes. Right now, there are enough AT-sensitive dyes, old and new, so that anyone interested can play the game I have suggested in flow or image cytometers using just about any available illumination.

A 2014 paper by Prieto et al (5) from the Pasteur Institute in Montevideo, Uruguay, has thoroughly rehabilitated methyl green (there was also a poster at CYTO 2015). Shaking with chloroform leaves a stable, bright DNA stain excited at 633 nm and emitting at 677 nm. Its red absorption makes it likely that it could be used to stain fairly thick blood films. And it's cheap (less than a penny per milligram)! Admittedly, so far there are no direct data on organisms with different base composition, but, at that price, I'm willing to take a flyer.

UV lasers remain expensive, but, largely because of Blu-ray discs, violet diode lasers, which first emerged around 2000, became a commodity by 2002. They are more or less standard in entry level cytometers, usually described as emitting at 405 nm, but often as far up as 415 nm. Even at 405, they're marginal for exciting Hoechst 33258 or 33342 (both of which cost about 80 cents/mg) in human cells, which contain about 300x as much DNA as malaria parasites; those two dyes, excited at 405, would not be very useful for malaria. DAPI, which costs about 10 times as much, is only a few times better, but may form fluorescent complexes with RNA. The dye which seems most promising for use with violet lasers is nuclear yellow (Hoechst S769121), about the same price as DAPI, but with a slightly longer emission and many timesbetter excitation above 400 nm.

Perhaps most exciting, if you pardon the pun, is the new probe QCy-DT (6)\, from the lab of Prof. T. Govindaraju at the Jawaharlal Nehru Centre for Advanced Scientific Research in Bangalore <tgraju@jncasr.ac.in>. This permeant dye has already been shoWn to exhibit AT-specific fluorescence enhancement in malaria parasites. It has excitation peaks at 463 and 530 nm, emits maximally at 680 nm, and would be excited with better than 50% efficiency by any source operating between the violet and red. The potential applications go far beyond malaria, but those in malaria alone might keep a lot of older entry-level cytometers purposefully employed.

You might ask why, if whole-genome based malaria parasite identification is so easy to do, nobody's doing it? I've been playing games with this for around 15 years, during which there might have been six times when specimens of two or more species of human malaria were available to me and my colleagues. We have anecdotal evidence from flow that Hoechst dye fluorescence from *P. falciparum* is substantially brighter than that from *P. knowlesi* and from flow and laser scanning of slides that it is brighter, but not that much brighter, than that from *P. vivax*. Frankly, the methodology could be validated with slides, with FISH (7) used to provide the ground truth. I would not rule out washing Giemsa stain out of old smears (including any of Giemsa's) with methanol, but cover slips might be a problem. A lot of cytometer development can be done with stained beads and surrogate samples, but you'd like to see how the gadgets handle their intended normal diet before sending them overseas.

The "malaria box" I have been trying to put into the field uses three sources. A 365 nm, 5W LED excites Hoechst 33342, a 460 nm, 5W LED excites Pico green when base composition is being measured, or thiazole orange to measure RNA when species identification is not needed. All three illuminators use color glass filters for wavelength

selection, and aspheric lenses with a diffusing surface for collimation. UV and blue are delivered obliquely from above; in the third, substage illuminator, a low-power 680 nm LED provides light from 660-710 nm for a transmission measurement (8,9) that quantifies hemozoin (Hz) more simply and cheaply than the depolarized slide scatter measurements that have been traditional for this purpose (10). A low-power (0.3-3x) image of a slide or other specimen carrier is made on a l/3", 1.2 MP monochrome camera chip with bandwidth restricted to 530-710 nm by color glass and dichroic filters. Light sources are turned on and off in sequence.

The simplest version of the instrument need not have any movable parts, and no version requires focus adjustment or stage motion during the analysis of a single sample, which can be done in minutes. This instrument was designed to analyze a methanol fixed slide containing 3 uL more of blood, detect and count any malaria parasites present, and identify each by species and stage of development. It would, of course, be controlled by a networkable computer (the inexpensive and popular Raspberry Pi is what we have been using in the design stages; it can run all of the popular open source biomedical image analysis software also available for PCs and Macs) and therefore, without any additional modification, be programmed to run any additional assays requiring UV- and/or blue-excited fluorescence and/or far red transmission measurements.

Among those relevant to malaria are red and white cell and reticulocyte counts, hematocrit and hemoglobin determinations, and fluorescence-based G6PD tests, e.g., to manage P.vivax patients. One could also do simple immunophenotyping, such as CD4 counting, and simple multiplexed ligand binding assays, acid-fast staining for TB, and antimicrobial susceptibility tests for various bacteria, fungi and protozoa. 3-D printing will be used to produce a robust, fieldworthy mounting structure for the optics.

Automated diagnostic instruments for blood cell counting had begun to make their appearance in clinical laboratories by the late 1950s, and apparatus that could perform the white cell identification tasks normally done by human observers looking at Giemsa-stained slides began to be developed around that time. These were refrigerator sized, complex instruments usually incorporating early minicomputers, with selling prices close to US $100,000.

It was not expected that there would be a large market for such devices in the resource poor countries most burdened by malaria. The smaller, less expensive, flow cytometers that began to appear in the 1980s, in large part in response to the HIV epidemic, seemed to provide a jumping off point for the development of diagnostic instruments for HIV, malaria, and TB, which together killed millions of people per year in those countries. Within the past few years, however, a few companies have pursued diagnostic instruments for malaria, primarily benchtop systems (11-13).

Over the past decades, flow cytometry manufacturers have produced successively smaller and less expensive machines for doing CD4 counts and other immunologic tests related to AIDS diagnosis and management. In 2015, the WHO recommended that

routine CD4 counting be replaced by viral load measurements for AIDS management. Each of the countries that has done this is likely to have a few hundred flow and/or image cytometric instruments, costing a few tens of thousands of dollars for the flow systems and a few thousand each for the imagers, lying idle.

I have been asked whether these instruments could be repurposed for diagnosis of malaria, TB and other diseases. They probably could, for a price, but they have been designed specifically for one purpose, and it might cost thousands more dollars per machine to modify them. One would have to consider at the outset whether a newer, more sustainable multipurpose system could be made from scratch. This is the direction in which WHO now seems to be pointing with last year's introduction of a List of Eessential In Vitro Diagnostics. We in ISAC need to join the discussion about which boxes can bring the most bang for the buck to what are currently points of no care; Vancouver might be a good place to start. And we could teach a lot of people how to build something useful, rather than train them to “diagnose” malaria by eye.

Love,

-Howard

P.S.- Just weeks ago, my email contained an ad for Sensirion's laser scattering aerosol particle counter module - for <$50!
-HMS

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