Cell sorting capabilities as diverse as your applications

Morphology – Here I Come Again

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"WHAT is this good for," was the comment of our clinical cooperation partner, when we showed him the first imaging flow cytometry results using a microfluidic system and a customized quantitative phase microscope for high-throughput blood cell analysis. What went wrong? We have shown the hematology-oncology expert phase images of various leukocyte types. Our eyes were trained on these images showing round-shaped cells of different sizes with rather low-contrast but we could interpret the image quality and even differentiate leukocyte types. The clinician, however, looked at blood cell images he was never trained on and expected contrast similar to stained blood smear. Our first disappointment vanished when we showed him dot plots from image analysis, which pleased him as he started to become familiar due to his own expertise in flow cytometry. Showing him additional results on the discrimination potential of leukemia samples finally gave us a mark that we are looking at something with clinical relevance (1, 2). This meeting was a really good experience for the team working on phase imaging flow cytometry for next-generation hematology analyzer and showed us again that expectations can differ dramatically between an engineer and a clinical user. The reader might ask himself, why is label-free cell imaging of interest in hematology offering only low-contrast images.

When we look into the field of flow cytometry, hematology analyzer are somewhat the automated high-throughput beasts for blood cell analysis requiring only nonspecific staining to discriminate leukocyte or more challenging analytes, such as reticulocytes. The high-throughput for a complete blood count (CBC) and a leukocyte differential (Diff) as well as the deep hematological information make these tools unique in the world of in vitro diagnostics (IVD). With low costs per test and high statistical power for blood cell biomarkers, a CBC/Diff is today one of the most requested clinical tests in the world and literally every patient receives a CBC/Diff. This achievement was the result of a few elegant technical solutions in the past starting with the Coulter counter principle in the early days and later light scatter analysis, which allows discriminating monocytes, lymphocytes, and different granulocytes by means of size and morphology without requiring manual microscopy of blood smears. Contrary to fluorescence flow cytometry (FCM) no specific and costly antibody labeling is required, which is key for integration, standardization, and robustness of the flow cytometry workflow. Even today, the unique automation level in flow cytometry and the wealth of biomarker information from a single IVD instrument are outstanding in a very mature central laboratory market. The defined CBC/Diff is also a much easier task for IVD certification compared to the numerous FCM assays. However, the hidden champion is—as it is very often in the IVD space—the magic chemistry of the reagents for hematology analyzer. Only with the right chemistry on board analyzer can achieve standardized sample preparation in seconds, such as the sphering of blood cells, erythrocyte lysis, the granulocyte differentiation, the extremely accurate high-throughput counting, and even the hemoglobin concentration measurement on single erythrocyte level (3, 4). These developments make hematology analyzer after decades of usage in the clinical routine even today an amazing piece of engineering and still an active field of research (5, 6).

The caveat of the success story is the sample preparation limiting the morphological information for instance due to sphering, leukocyte artifacts from staining. With increasing number of reagents on board, the fluidics become complex as well as the maintenance effort to operate the analyzer. Such

systems work effectively in central laboratory environments but are not suitable for point-of-care testing (POCT). In addition, the image information is missing and only indirect cell morphology information is obtained. Today, new blood cell biomarker classes, such as highly dilute circulating tumor cells (CTCs, 7) or blood cell aggregates (8), covering a large dynamic concentration range became of clinical interest. However, due to the indirect cell analysis, sample preparation limitations, and fluidic conditions, many new cellular biomarkers cannot be covered by conventional hematology analyzer depending on fixed sample volumes requiring medium to high target cell concentrations and rapid processing cycles to achieve economical high-throughput. Moreover, in the clinical routine, only a few of the provided biomarker parameters of a CBC/Diff are used for routine analysis which is in reality too complex for routine diagnosis. Such market conditions are generally unfavorable to develop new technologies. Due to the plethora of data provided by these systems, multivariate data analysis and artificial intelligence algorithms are applied to reduce the effort for data interpretation and to fully exploit the analytical power of analyzer for differential diagnosis. This data mining can lead to unexpected results as we have shown for malaria testing (9).

Most research on new blood cell biomarkers, such as CTCs or cell aggregates, is performed today with FCM requiring multidimensional immunophenotyping for preclinical research, which is not matching the requirements for economical cell analysis for the clinical routine. Imaging flow cytometer, such as the famous Amnis system, has only found niche applications in the life sciences and preclinical research (10). One could assume that the current flow cytometry trends are representative for a "retreat" from morphology information due to the dominating high-dimensional immunophenotyping methods. But none of these methods is compatible with the brutal economics of the IVD market and in particular for hematology analysis. This is also true for automated blood smear analysis, which still is considered the gold standard in hematology analysis. Even sophisticated automated microscopy systems with integrated sample preparation, such as the Bloodhound, can barely compete against the efficiency of hematology analyzer (11). Most recently, the integration of advanced microfluidic solutions with viscoelastic focusing and image analysis allows the Hemoscreen from Pixcell Medical to achieve clinically comparable CBC/Diff quality for POCT (12). The main advantage of using imager is the parallelized imaging flow cytometry of blood cells in a highly defined focal height, which allows researcher to decouple from serial cell analysis and compensates for usually low flow rates to achieve sufficient statistical power. However, the sample preparation and reagents remain essentially the same as in today's analyzer of the central laboratories.

Quantitative phase imaging (QPI) is a label-free imaging opportunity to replace sample preparation in hematology analysis (13). Instead of measuring refractive index changes within and around the suspended blood cells by scatter analysis, phase images are acquired, which allow to visualize the individual cell with integrated phase information on an imager. Knowing the phase conditions quantitatively allows to reconstruct whole cells or respective slices depending on the optical measurement conditions (Fig. 1A). In this way, the morphological information of cells is preserved. With appropriate imaging conditions hematology analysis can now be performed in principle without any sample preparation reducing the fluidic complexity of automated analyzer but gaining access to nonstable biomarkers, which are not accessible with today's workflow, such as blood cell aggregates (Fig. 1B). In other words, in silico image analysis replaces chemical sample preparation, which distorts the cellular morphology (14). One example is the opportunity to resolve label-free the *Plasmodium falciparum* life cycle in erythrocytes
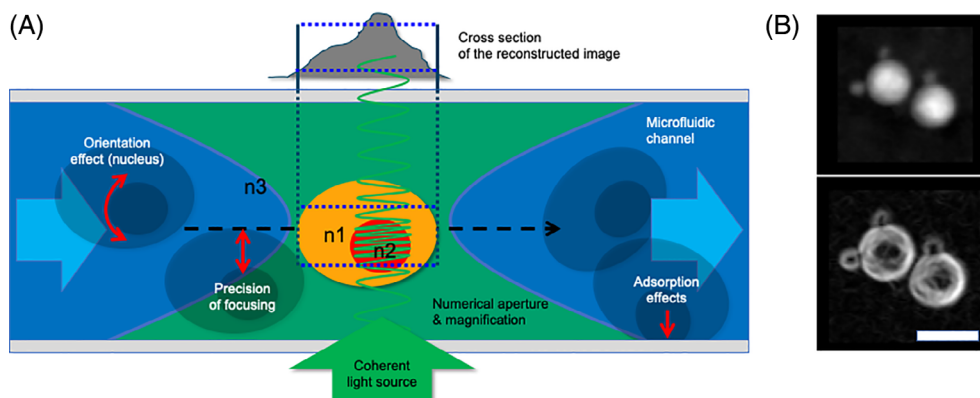

Fig 1. Hematology analysis using a DHM and a rectangular microfluidic channel. (**A**) The cross-sectional view shows challenges for high-throughput imaging, such as robustness of the image analysis independent of the random cell nucleus orientation with respect to the depth-of-field, precision focusing of all blood cell sizes or matrix effects. For parallelized analysis of a submonolayer of blood cells constant phase resolution should be achieved over the entire field of view (not shown). (**B**) Exemplary platelet-leukocyte interaction and first-derivative of the reconstructed image. The phase contrast quantifies the delay of the optical path length that is caused by areas with different refractive index (n) in cells in comparison with the surrounding medium. The resolution allows to even count the number of platelets interacting with a leukocyte as biomarker for inflammation (scale bar: 10 µm).

including the clinically relevant ring-stage (15). With high statistical power and access to the life cycle of parasites, one could imagine that future hematology analyzer can even support to monitor the efficacy of treatments and identify resistances beyond the limits of costly molecular analysis. However, we have to admit that QPI will be insufficient to replace a regular CBC/Diff. We need to find and integrate additional optical solutions for reticulocytes and measuring effectively the hemoglobin concentration in an erythrocyte to match the state-of-the-art biomarker panel to claim a CBC/Diff by physical methods only. The less elegant alternatives would be a mix of partial sample preparation and phase imaging solution or an analyzer for dedicated new hematological biomarkers. One critical piece of the puzzle for a next-generation hematology analyzer is microfluidics. Only with appropriate solutions to enrich rare cellular analytes at low shearing conditions and to deplete erythrocytes, we can cover the dynamic concentration range of new biomarkers and minimize image analysis effort for redundant information. Storing Gigabytes of images data for offline analysis is only an option for research. The last piece of the puzzle is, therefore, real-time image analysis and the respective image analysis algorithms. Due to this dependence, future imaging-based hematology analyzer will be a partial digital healthcare product.

Digital holographic microscopy (DHM) is an interferometry-based variant of QPI that typically uses the classic holographic principle, with the difference that the hologram recording is performed by a digital image sensor using a coherent light source (16). Although a rather old methodology, DHM has taken off only recently due to the computational power for image reconstruction and robust "off-axis" imaging tools replacing classical interferometer setups. With tomographic information, one can resolve amazing details of cellular compartments (17-19), but for clinical high-throughput operations a single image acquisition of blood cells is the only option to match today's hematology analyzer. With DHM, we have an attractive platform technology for the field of hematology analysis and parallelized analysis of >3,000 cell/s at 100 frames per second (1). With full access to undistorted morphological information and low shear stress, we can visualize and quantify the morphology of erythrocytes and platelets in plasma and we can resolve the differences in granularity and nuclei of leukocytes replacing forward and side scatter analysis. Second, we can visualize fragile megakaryocytes and observe cell–cell interactions in blood without depending on harsh sample preparation conditions, which interfere with these logistically nonstable biomarkers. Third, being potentially independent from sample preparation, we are not bound to a defined blood sample volume and the respective statistical power for a given cell concentration. Clinical users could run hematology analyzer by simply defining the statistical power they want to achieve rather than looking at a Diff from a few microliters of blood only. Last, with the appropriate cell enrichment tools, we can potentially look at any concentration range covering even very dilute biomarkers, such as CTCs, from even several milliliters of blood. The group of Natan Shaked at Tel Aviv University (Cytometry A. 2020 Sep 10. doi: 10.1002/cyto. a.24227. Online ahead of print) reports in this issue a proof-of-concept of rare CTCs detected with DHM. Future clinical studies will show if the cellular phase contrast allows sufficient sensitivity and specificity for CTCs. In this way, a new generation of imaging-based hematology analyzer could cover even liquid biopsy marker.

Much more work is ahead of us to create smart and integratable workflows to match the accuracy of conventional hematology analyzer and to add new, derisked hematology biomarkers for the clinical routine. To achieve this goal, we need interdisciplinary work between engineers and clinicians requiring a deep understanding of biomarkers, clinical workflows, and technology to avoid surprises for translation. With new biomarkers of clinical relevance, DHM and additional optical solutions could outperform today's analyzer and disrupt both the clinical and POC market in a similar way as was the introduction of the platelet count in hematology analyzers in the past.

AUTHOR CONTRIBUTIONS

Christian Klenk: Formal analysis.

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