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Sample Preparation for Flow Cytometry Benefits From Some Lateral Thinking

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• **Key terms**
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OVER the past 50 years, Flow Cytometry (FC) has established itself as one the most powerful analytical technologies for making controlled high throughput, multi-parameter measurements on large numbers of cells (1). As such FC has been pivotal in dissecting the phenotypes of key cellular populations from within complex, often highly heterogeneous samples both in the context of normal and disease states. The multi-parameter, high-throughput nature of FC very much relies on the fact that cells are first stained with fluorochrome labeled monoclonal antibodies specific for one of a panoply of key cellular antigens before being analyzed in liquid suspension by a flow cytometer system. The sample preparation process requires a significant degree of optimization, is labor-intensive and can be affected by a host of potentially confounding variables. As such it is certainly fair to say that the "journey" from specimen to measurement is fraught with danger as fear of artifact generation lurks at every twist and turn. For those who analyze cells liberated from solid tissue by FC the challenge of sample preparation can be daunting. Even before the staining process begins the biopsy has to be digested and disaggregated to generate a suspension of viable cells while trying to avoid stripping off key surface receptors or changing the cellular composition in a way that no longer reflects the original tissue-resident populations (2). However there are also significant challenges to overcome when working with liquid biopsies such as blood. Blood is one of the most important analytical mediums because it is relatively non-invasive and cost effective to collect. Moreover the phenotypic status of various immune cells in the peripheral blood circulation may provide a set of highly informative biomarkers for diagnosing and monitoring various diseases. It is therefore imperative that the true in vivo cellular state is preserved as much as possible during the sample processing steps for FC analysis.

The question any cytometrist must ask themselves when preparing samples for FC analysis is "what changes could I be eliciting in the cell population by treating the sample in this particular way"? There can be no debate whatsoever that the fewer manipulations carried out on a sample prior to analysis by FC the more likely it is to reflect the native cellular state. In this issue of *Cytometry A*, Civin and coworkers (page 1073) present a powerful microfluidics-based approach to aid in the analysis of blood samples that is based on a technology called Deterministic Lateral Displacement (DLD). The DLD-based microfluidics chip they describe is able to remove almost 99.99% of red blood cells (RBCs) and unbound monoclonal antibody (mAb) in under 20 min while still recovering ~88% of the target lymphocyte population from within the sample. The authors have described the use of DLD for this purpose previously (3) but now in this issue of *Cytometry A* (page 1073; DOI: 10.1002/cyto.a.23019) they report the performance of a reformatted, high precision plastic version of the microfluidic chip that will invariably lead to improved accessibility and more widespread adoption across different cytometry-reliant disciplines.

Processing a blood sample for FC can take one of several directions but is always dictated by the fact that RBCs outnumber lymphocytes by a factor of 1000:1 in peripheral blood. Typically, fluorochrome-conjugated monoclonal antibodies against various key phenotypic surface receptors are added for a period of incubation before a 1–2 mL volume of RBC lysing agent is added to the sample. The sample is then

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washed by centrifugation to remove unbound mAb, un-lysed RBCs, and to concentrate the sample for rapid acquisition by resuspending the cell pellet in a smaller volume. It has been shown previously that various RBC lysing agents can have deleterious effects on lymphocytes (4) and as Civin and coworkers correctly state in their manuscript, can even lead to the preferential loss of specific cells types from within the sample rather than a wholesale depletion. In addition, the process of washing cells by centrifugation also poses a significant threat to cell retention and viability whether it be after RBC lysis or when using the Ficoll approach to separate lymphocytes from whole, un-lysed blood (5). More recently so called "lyse no wash" protocols have been adopted whereby, as the name suggests, samples are not washed out of lysis buffer and are instead acquired immediately after the lysis incubation period. However this creates its own set of issues. First, there may still be excess mAb present in the sample thus creating ideal conditions for increased non-target binding that could decrease signal resolution by increasing background staining levels. Second, the increase in sample volume as a result of adding lysing agent can be extremely problematic for rapid sample acquisition by FC, particularly in situations of rare cell analysis whereby in order to satisfy Poisson statistics for confidence in the measurement, a minimum number of rare cell target events must be collected to obtain the desired precision (6). This problem of increased sample volume post lysis is well demonstrated by the CD34 stem cell detection and enumeration assay, designed to provide an absolute count of CD34+ cells in human blood (~0.1% of all lymphocytes). In this situation, blood samples are stained with a solution containing fluorochrome-labeled CD34 and CD45 mAbs. Counting beads are also present within the sample to derive an absolute cellular count using the so-called "single platform" FC-based approach (7). In order to avoid loss of either the cells or counting beads the "lyse no wash" protocol is used resulting in a 2 mL sample volume for acquisition by FC after lysis. However, in order to satisfy the statistical requirements for rare event analysis at cell target frequency of ~0.1% one must acquire a minimum of 80–120 *bona fide* CD34+ cells (following ISHAGE gating guidelines). The fact that there are time limits set in the protocol for the validity of key reagents such as the counting beads (acquire within 1 h of removing from foil pouch) coupled with a general concern for cell viability during the assay means that the increased acquisition time required for each sample can severely limit the throughput. In situations where the target population is rarer then there may even be a risk that the duplicated samples are not both fully acquired within the set time limits (personal observation). As such, overly dilute samples are not ideal for rare cell analysis, but volumetric concentration by centrifugation may result in cell loss.

In order to try and mitigate the risk of cell loss by lysis and/or centrifugation it is also possible to utilize a "no lyse no wash" technique. However such a protocol requires a strategy to distinguish lymphocytes from RBCs. One method is to take advantage of the fact that mature RBCs contain no nucleus by adding a cell permeant fluorescent DNA dye such as DRAQ5 or DyeCycle Violet to the sample. In this case nucleated lymphocytes will be stained whereas enucleated RBCs will not. Furthermore, RBCs do not express CD45 whereas lymphocytes do so one can also use a CD45 mAb coupled to a well resolved fluorochrome as a distinguishing parameter. However it should be noted that using either approach the RBCs will still be present in the sample and as such can cause issues with mAb staining. Moreover, RBCs will also dominate the detector electronics of the FC system and limit the number of target lymphocyte events able to be saved in a single data file. These two issues can be overcome by setting a threshold on the CD45 and/or nucleic acid fluorescence signal so that any negative RBCs are ignored by the system and will not be recorded as part of the FCS file. In practice, the optimal situation for processing blood for FC would seem to be the DLD approach presented by Civin and coworkers (page 1073) as it can remove both RBCs and excess mAb without the need for lysing agents or centrifugation while eluting the target cells in an appropriate volume for rapid FC-based analysis.

There is no question that microfluidics, microfabrication, and "lab-on-chip" based technologies have the potential to redefine the way we do cytometry. Certainly, it is currently having an impact right across the cytometric landscape (8–13). Microfabrication techniques present a method to produce fully enclosed cell sorting systems that overcome issues with both biohazard containment and therapeutic sterility that exist with classical electrostatic droplet sorters. They may even be gentler to the cells they sort in terms of preserving viability, not eliciting unwanted activation or altering transcriptomic profiles during the separation process (8). Imaging flow cytometry systems based on microfluidics (14) could easily rival existing imaging flow technologies for the analysis of rare cell populations such as telophasic cells that absolutely require imagery for accurate identification (15). Moreover high throughput analysis can be achieved by microfluidic-based cytometer systems using massively parallel processing techniques with multiple microfluidics chambers working simultaneously for a common purpose (16). DLD is a great example of one such "cytometry-enabling" microfluidic technology as it can physically separate particles or cells based on size (3). Briefly, microfabrication is used to produce an array of micro-posts that have a specific size and distribution pattern. The diameter and position of these posts, coupled with the tilt angle of the channels creates a critical diameter whereby cells or particles with diameters greater than this critical value are gently bumped and deflected in to a specified microfluidic channel. In the work presented by Civin and coworkers, the first "zone" contained posts with a 24 μm diameter that were each spaced 18 μm apart. This created a critical diameter of ~8 μm above which lymphocytes were bumped in to a centralized channel while the trajectory of smaller cells and particles (RBCs) remained unchanged and were carried out as waste. To increase the throughput, the authors created a second array within the chip that was tilted in the mirrored direction so that all lymphocytes were deflected in to a common central collection

channel. The performance data presented by the authors is convincing with an RBC depletion of nearly 99.9% and an almost complete removal of unbound mAb from the pre-stained samples. Moreover, they also show that the chip is able to recover close to 90% of the expected lymphocyte number and, importantly, did not bias or alter the cellular subtype composition based on phenotypic analysis before and after purification. It was also highly impressive to see that after depletion the lymphocytes could be identified by Forward and Side Scatter measurements alone with no requirement for either CD45 mAb staining or vital DNA dyes. Collectively, these results all but eliminate the various concerns mentioned previously that are associated with other blood sample processing techniques for FC analysis.

The work presented by Civin and coworkers does however raise some intriguing questions and possibilities, both for the present and future. For example, while the overall cellular composition of the sample was unaltered based on the few phenotypic surface markers analyzed, could contact with the microfluidic posts lead to lymphocyte activation? This could certainly be an interesting parameter to assess. If DLD purification not only preserved the accurate cellular composition of a blood sample but also preserved the true activation status as well then one could envisage it being used in conjunction with downstream assays looking at cell signaling and genomic/transcriptomic profiling. In terms of the hardware described by Civin and coworkers, one would also expect that in the future there would be a more "user friendly" chip interface for sample introduction and collection as well as a more streamlined process for preparing the chip to receive the blood sample. This would possible benefit from a dedicated hardware solution that could even integrate the mAb staining step either on or off chip. Furthermore if this was combined with on-chip fluorescence detection and cell phenotyping then one would have a highly portable and potentially powerful solution for blood analysis. There is no question that the cytometry landscape in changing and microfluidics, microfabrication, and lab-on-chip technology will be at the forefront of this revolution, as such the authors should be commended for their "lateral" thinking with respect to how we can improve blood sample processing for FC analysis.

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