Absolute Cell Counting

Applications
In the research and especially the clinical setting, there are situations that require not just the percentage positive of a particular cell population but the actual absolute number positive cells. There are three main areas in the clinical setting that require the precise and accurate determination of absolute cell counts.

CD4+ Lymphocyte Enumeration
The absolute CD4+ T lymphocyte count has long been recognized as a useful laboratory tool for the staging of HIV infected patients. It can also be used to assess the likelihood of opportunistic infections, the timing of the administration of preventative treatment and, more recently, for monitoring the effect of new antiviral therapies. Absolute CD4+ T lymphocyte counts of less than 200 cells/μL are generally accepted as being the cut-off point for the laboratory diagnosis of HIV infection. It should be noted that diseases other than HIV can result in a reduced CD4+ T lymphocyte count, and that a diagnosis of HIV infection cannot be made on the CD4+ T lymphocyte count alone.

CD34+ Hematopoietic Progenitor Cell Enumeration
Mobilization, harvesting and transplantation of CD34+ progenitor cells are now well-recognized techniques. By monitoring absolute CD34+ cell levels after growth factor-induced mobilization, it is possible to ensure the maximum number of cells can be collected with the minimum number of time-consuming and expensive harvesting procedures.

Residual White Blood Cell Enumeration
The presence of white blood cells (WBCs) in blood products has been shown to lead to febrile reactions, alloimmunization, as well as the transmission of infectious agents (e.g. EBV, CMV and CJD). Many countries have now adopted a policy of filtering all red cell, platelet and fresh plasma products for transfusion to remove the WBCs. Quality control of the procedure involves screening a number of randomly selected filtered units and performing a residual WBC (rWBC) count. In Europe, the upper limit is 1×10^6 WBCs per unit, which equates to a count of around 3.3 WBCs/μL for an average 300 mL unit.

In all the above applications, it is essential to accurately identify the cell population of interest, and this population may be present at very low levels. The development of strategies using multiple gating regions, employing both light scatter and fluorescence parameters, have aided in this process. For example, the use of CD45 (pan leucocyte marker) versus side scatter results in a more reproducible and accurate lymphocyte gate.\(^1\)
Likewise, the use of the ISHAGE gating protocol for CD34 determinations has resulted in more reproducible results.²

Methods
There are two flow cytometric methods that can be employed to derive absolute counts.

Dual-Platform Approach
The dual-platform approach employs the use of three separate measurements obtained from two different instrumentation platforms. A flow cytometer is used to determine the cells of interest as a percentage of a “reference” population (e.g. CD3+/CD4+ cells as a percentage of the total lymphocyte population). Then a hematology analyzer provides an absolute white blood cell count (WBC) and the lymphocyte percentage. The absolute CD4+ T Lymphocyte count is, thus, the product of the absolute WBC, the lymphocyte percentage and the CD4+ T lymphocyte percentage.

With the dual-platform approach, it is important to ensure that the cell population of interest, as well as its reference population, can be accurately identified. Different gating strategies have been employed to aid the identification of lymphocytes for CD4+ T lymphocyte enumeration. Initially, light scatter gating using FSC vs. SSC was used. The main drawback of this method is the increased likelihood of non-lymphocyte contamination in the gate, including cell debris, platelets, monocytes and basophils. This method is now not recommended for CD4+ T lymphocyte determinations³ and has been superseded by the use of light scatter and an immunological marker, for example SSC vs. CD45. Cell debris and platelets are CD45 negative, monocytes have a slightly higher side scatter signal than lymphocytes, and basophils have a dimmer expression of CD45 than normal lymphocytes and, thus, can easily be excluded (Figure 1).
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Figure 1. The importance of the correct identification of the reference population in dual-platform determinations. A and B show the potential contamination in a Forward vs Side Scatter gate drawn around the lymphocyte population. This gate includes cell debris and platelets, (yellow), monocytes (cyan) and basophils (green). Note how the basophils (green) sit in the middle of the lymphocyte population on Forward Side Scatter. In C and D, CD45 vs Side Scatter is used to gate the debris, and monocytes and basophils are excluded.

It has been well documented that the dual-platform method has a higher inter-laboratory coefficient of variation (CV) due to the way different hematology analyzers derive the absolute WBC and lymphocyte count, and not to the CD4+ T lymphocyte percentage from the flow cytometer. The variance is seen to increase dramatically with WBC counts less than 0.1×10^9/L. That means this approach cannot be used for rWBC determinations, where very low counts in the order of 0.001×10^9/L may be encountered.

**Single-Platform Approach**

In the single-platform approach, the absolute cell count is derived from the flow cytometer itself, without the need for a hematology analyzer. A very precise, known volume of sample is mixed with relevant antibodies. A gating strategy is employed to accurately identify the cell population of interest and exclude any contaminating cells; identification of a "reference" population is not required. The cell population of interest can then be related back to the original blood volume by a variety of different methods.
Microbeads. The use of microbeads is by far the most common approach to single-platform absolute counting. Microbeads are small fluorescent latex particles with a diameter of 5-10 µm. There are two basic methodologies for using microbeads. The first uses a tube containing a lyophilized pellet of an exact number of microbeads, to which a known volume of sample is accurately added. In the second method, equal volumes of sample and microbeads are accurately added to a tube. In this approach, the microbeads are in a liquid suspension medium of known concentration, which is supplied by the manufacturer. By counting the number of cell events and the number of bead events and knowing the initial concentration of beads, it is possible to determine the absolute cell count using the following formula:

$$\text{Absolute Cell Count} = \frac{\text{Number of cell events}}{\text{Number of bead events}} \times \frac{\text{Concentration of beads}}{(\text{cells/µL})}$$

Using time as an acquisition parameter can give a lot of internal quality control information about the preparation and acquisition of that sample. A histogram plot of time will show if the count rate of the sample has been stable over the acquisition period (Figure 2). Recent studies have demonstrated that the number of beads acquired in a fixed time period is remarkably constant for most flow cytometers. If all the sample preparation pipetting has been performed accurately, then for each tube acquired, the number of beads counted in a fixed time should be constant. It is then possible, for each batch of beads used, to derive a Mean ± 2 Standard Deviation (SD) range of beads counted in a fixed time period. Bead counts outside of this range could be indicative of potential pipetting errors, and the sample should be restained.

Volumetric. With the volumetric method, an exact and reproducible volume of stained sample is passed through the flow cell, and the number of positive cell events can be directly related to the known volume of sample acquired. The exact volume of sample can be measured using either precision syringes or by utilizing two sensing electrodes that monitor the movement of the sample and, hence, sample volume. With volumetric systems, all pipetting steps must be made with great precision. The final dilution of the sample must also be calculated and taken into account.

Microfluorimetry. With this method, blood stained with the relevant antibody is placed into a capillary tube of precise known dimensions. A laser scans the capillary, and the number of positive events is counted. This can be related to the precisely known volume of the capillary.

Flow Rate Calibration method. This is a recently developed method aimed at reducing costs in resource poor countries. The method utilizes the fact that with most bench top flow cytometers, the flow rate of sample through the flow cell is remarkably constant. Hence, the volume of sample acquired in a fixed time period will be constant. An accurately prepared dilution of counting microbeads in lysing reagent can be used to calculate the volume of sample acquired in a defined fixed time period. Accurately prepared stained samples without counting beads
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can then be acquired over the same time period. The number of events in the cell population of interest can be expressed per volume acquired, and if the dilution factor for the blood sample is known (i.e. ratio of blood volume to total volume), then the cell count per microlitre of blood can be calculated. A correction factor for the increased viscosity between the tube containing beads plus lysing reagent and the tube containing sample is required. This factor varies for different lysing reagents used.

The use of single-platform absolute counting methods are becoming more popular, especially the use of microbeads. With accurate pipetting techniques, the results can be precise and reliable.

Figure 2. The plots illustrate the value of using time for data acquisition. The first plot shows a normal steady flow rate, indicated by the level appearance of the histogram. The second plot shows that towards the end of the data acquisition period, the sample flow rate began to decrease due to a partial blockage in the flow cell.

Technical Considerations

Since the use of microbeads is the most common approach to single-platform absolute counting, the technical considerations discussion will focus on that methodology. Accurate and precise pipetting is perhaps the most important factor in obtaining reliable results with this methodology. It is recommended that a "wet tip" reverse pipetting technique be employed for the dispensing of sample and liquid phase beads. A description of that technique follows:
Press plunger to the second stop and place tip in the fluid and aspirate the sample. Press the plunger to the first stop dispensing the sample. The pipette tip should contain excess fluid. Repeat this step at least two times.

The pipette tip is now ready to use. Press plunger to the second stop and place tip in the fluid and aspirate the sample. Carefully remove the pipette tip from the sample and wipe the pipette tip gently with tissue to remove any excess fluid, being careful not to touch the tip orifice and inadvertently removing sample from inside the pipette tip.

Try to keep the pipette as vertical as possible. Check for the presence of air bubbles in the tip. If air bubbles are seen, then dispense the fluid back into the sample and repeat the sampling procedure.

Prior to dispensing, place the pipette tip on the tube wall near the top of the sample. Slightly angling the tube and pipette will aid this step. Dispense to the first plunger stop. Residual fluid should be seen in the pipette tip after the fluid has been dispensed. When removing the tip do not touch the walls of the test tube.

The same pipette should be used to dispense the sample and beads. Adopting the same technique for dispensing both sample and beads will ensure more consistent results. Dispensing pipettes should also be well-maintained and regularly checked for accuracy and precision (CVs < 2%).

Following are some additional tips for the use of microbeads that will help to ensure reliable, precise results.

Ensure the stock microbead vial is at room temperature and has been well mixed before sampling. Harsh vortexing of the stock microbead vial will induce foam formation, which attracts the microbeads, and can also introduce micro-air bubbles into the fluid, which will reduce the expected volume of microbeads pipetted, resulting in increased absolute cell counts.

Care must be taken not to lose any of the suspending fluid before re-suspension, otherwise the stated microbead concentration becomes invalid.

It is not advisable to use FSC as the thresholding parameter. Most microbeads have a low FSC signal and can easily be excluded when thresholding on FSC. A fluorescence parameter may be used, for example the one used for CD45. Another option is the use of a "not" gate, placed in the lower left hand corner of a FSC vs. SSC dot plot. This ensures that all the microbeads are acquired, but debris is excluded (Figure 3).

To ensure that the microbead-to-cell ratio is maintained, a lyse no-wash procedure should be used.

A minimum number of 1,000 microbead events should be acquired.

To ensure that the ratio of microbeads to cells is constant during long acquisition periods, it is best to pause acquisition and gently mix the tube every 7-8 minutes.
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- Care must be taken when gating the microbead population to exclude non-microbead events. Some protocols require that all the microbeads should be counted and others only the singlet microbead population. Please refer to the manufacturer’s product insert.

- Microbeads should be added to the sample just prior to acquisition to prevent bead loss due to settling, sticking to the tube, or sticking to cells. Some microbead preparations contain detergents to prevent bead aggregation. These can damage cells over prolonged incubation.

- Vortexing of the microbead/sample mixture should be performed with care to decrease foam and micro-air bubble formation.

Figure 3. A) Illustrates that care must be taken when using Forward Scatter as the threshold parameter, as the counting beads (green) can be easily eliminated. B) Illustrates that by using a threshold on CD45 the majority of debris (blue) can be eliminated, but the counting beads (green) are left intact. In some samples, the level of debris can still be seen interfering with the lymphocyte/CD34 region. C) Illustrates that using a CD45 threshold and acquiring through a “not gate” removes the majority of the debris that can interfere with the lymphocyte/CD34 populations.
For more detailed information on the practical use of microbeads please see the two articles by Brando and Mandy.⁷⁻⁸

References