Obtaining Accurate Concentration Data with FlowCam

SUMMARY

This technical note will explain how counts and concentrations are determined when using a Flow Imaging Microscopy (FIM) system like FlowCam when equipped with a field of view (FOV) style flow cell. This applies to FlowCam 8000/8400, FlowCam Cyano, and FlowCam LO models. It is not relevant to FlowCam 5000 or FlowCam Nano as these models do not have FOV flow cells, and it also does not apply to FlowCam Macro which uses an external peristaltic pump instead of a high-precision syringe pump. This note will also provide tips for optimizing system settings and sampling techniques to ensure reported concentrations are as accurate as possible.

DEFINITIONS

Particle Count: The number of particles imaged during a sample run. Particle capture is subject to any user-defined capture and acquisition filter settings.

Particle Concentration: A normalized number of particles per 1 mL of sample, calculated by dividing count by fluid volume imaged. Reported as particles/mL in VisualSpreadsheet® software.

Efficiency: The percentage of fluid volume imaged relative to fluid volume processed during a sample run. Efficiency is dependent upon magnification, flow cell dimensions, flow rate, and AutoImage rate. A 100% efficiency would indicate that the measured counts are equal to the actual counts of a given sample aliquot.

COUNTING PARTICLES WITH FIM

In an FIM system such as FlowCam, particles are imaged in a two-dimensional plane as they are pulled through the flow cell and past the camera's field of view (FOV) using a high-precision syringe pump (Figure 1). The instrument's camera captures images of its FOV at a set frequency in frames per second—a metric referred to as AutoImage rate in VisualSpreadsheet. These camera frames are then processed to obtain images of individual particles. During an analysis, particles will spend some period of time inside the camera's FOV while the camera is capturing images at set time intervals. To ensure accurate particle counting, we want to capture no more or less than a single image of each particle; the particle should enter the FOV, be imaged, and exit the FOV prior to the next image being captured. Achieving this behavior depends on the flow rate needed for a specific flow cell and the AutoImage rate—settings specified by the user in VisualSpreadsheet.

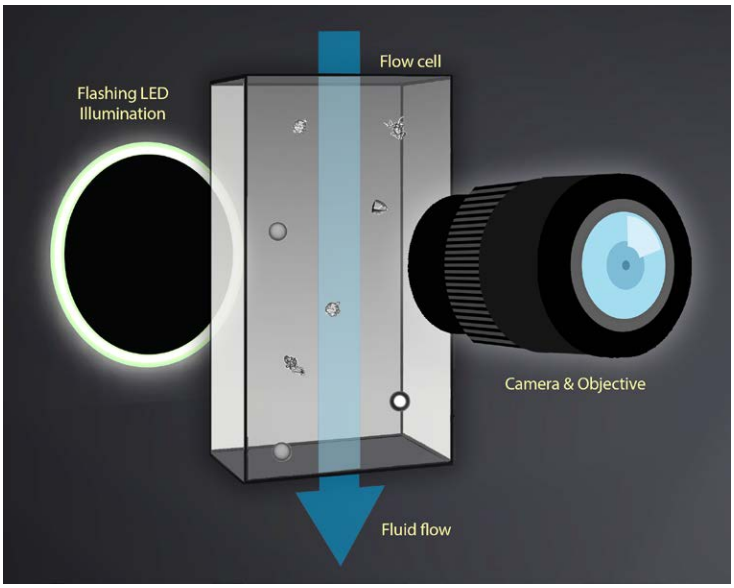

Figure 1. Flow path and optical components of FlowCam system

For every objective and flow cell combination, it is important to optimize flow rate and AutoImage rate to obtain the most accurate counts and concentrations possible. If the AutoImage rate is set too high relative to the flow rate, some particles may not exit the camera's FOV after an initial image is captured and before the next image is taken. This results in individual particles being imaged multiple times, resulting in oversampling. If the AutoImage rate is set too low, some fraction of the sample volume and any particles contained within will enter and exit the camera's FOV without being imaged. This results in FlowCam undercounting particles in the sample, as shown in Figure 2.

In VisualSpreadsheet, efficiency is an estimate of the fraction of the total sample volume that is imaged at a specified flow rate and AutoImage rate. Efficiency can be used as a metric to determine an appropriate AutoImage rate for a given flow rate.

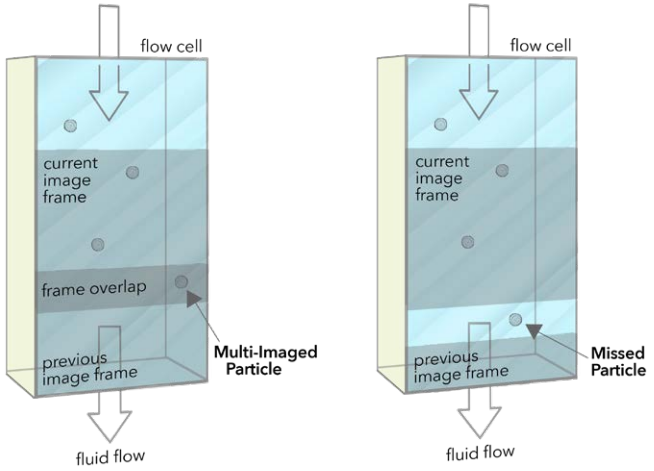

Figure 2. The effect of mismatched flow rate and AutoImage rate. On the left the system is oversampling, and single particles may be counted multiple times. On the right the system is undersampling, resulting in missed particles. Both scenarios produce inaccurate counts.

An efficiency equal to 100% commonly results in oversampling due to parabolic flow. Parabolic flow is exhibited by Newtonian fluids like water and organic solvents when friction introduced by the walls of a flow channel creates drag, slowing the fluid at the outer edges of the channel. In the context of flow imaging, this slowing of fluid near the flow cell boundaries can cause oversampling when efficiencies are set too high.

To prevent this, the recommended practice for FlowCam is to set the frame rate as high as possible for a given volume without triggering the efficiency warning in the Fluidics tab in context settings (Figure 3). The efficiency warning occurs at ~70% efficiency and indicates that multiple imaging of single particles and overcounting are likely to occur at the current settings.

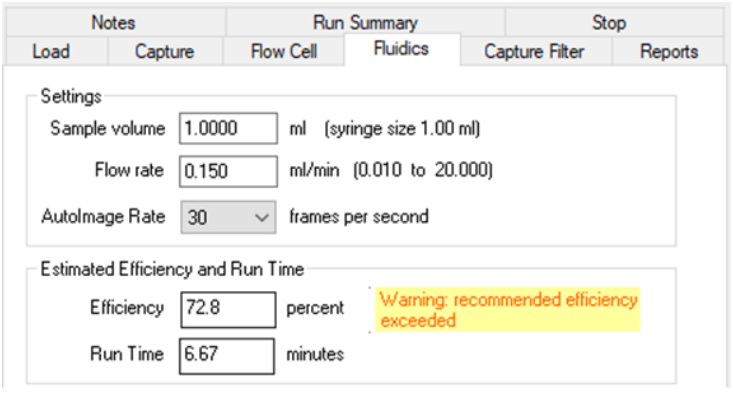

Figure 3. The efficiency warning indicates the AutoImage rate is too high relative to the flow rate. To prevent oversampling, increase the flow rate (if appropriate) or decrease the AutoImage rate until the warning no longer appears.

Note that the efficiency displayed in Figure 3 is an estimated efficiency calculated from the flow rate and AutoImage rate. The actual efficiency is calculated at the conclusion of a run using the following calculation.

$$\text{Efficiency } \% = \frac{\text{fluid volume imaged (mL)}}{\text{sample volume processed (mL)}} \times 100\%$$

Where fluid volume imaged is the total volume of sample imaged by the camera and sample volume processed is the volume pulled by the syringe minus any volume used for background calibration.

Because it is not practical to achieve efficiencies of 100% without oversampling, FlowCam is not typically used to measure absolute particle *counts*. However, it is possible to obtain and compare relative counts among samples analyzed using the sample capture and fluidics settings.

FlowCam can provide accurate *concentration* (particles/mL) measurements because this calculation relates count to the exact fluid volume imaged during a sample run:

$$\text{particles/mL} = \frac{\text{count}}{\text{fluid volume imaged (mL)}}$$

While the fluidics settings and instrument performance are important considerations in obtaining accurate concentrations, there are several software and sample-specific factors that can affect reported particle concentration.

CONSIDERATIONS FOR ACCURATE CONCENTRATION CALCULATIONS

Capture Settings

Capture settings as defined in the Context dialog can greatly impact reported concentrations, and it is important to optimize these settings so that individual particles are treated as such. Suboptimal capture settings can result in multiple particles being captured as an individual particle (Figure 4), resulting in artificially low concentrations. Conversely, they can also result in single particles being erroneously segmented into multiple particles, resulting in falsely inflated concentrations (Figure 5).

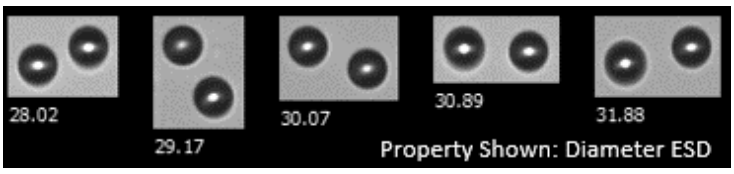

Figure 4. Particle images with incorrect capture settings. Two particles are being captured and counted as a single particle, resulting in artificially low concentrations. In this case, DNN is too high and should be lowered to 0 or 1 µm.

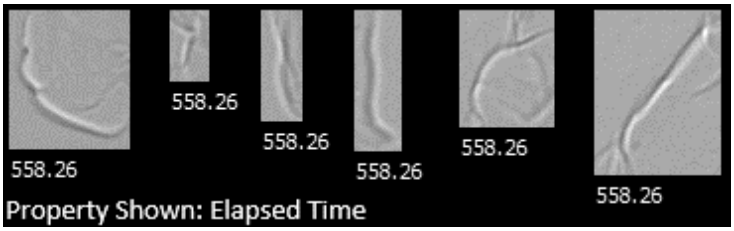

Figure 5. A single transparent particle has been segmented into multiple, partial particle images due to incorrect capture settings. Note that the elapsed time values below the images are identical. In this case DNN is too low and should be raised.

The most critical capture setting in this regard is distance to nearest neighbor (DNN). This parameter defines the distance (in µm) required between two particles for them to be imaged separately. For compact, discrete particles like the beads shown in Figure 4, DNN should be very low (0 or 1 µm) meaning that even beads that are very close together will be treated as separate particles.

For more transparent particles, like protein aggregates or phytoplankton, using a low DNN often segments a single particle into multiple partial particles, resulting in inflated concentration values (Figure 5). For these types of particles, setting DNN between 4-8 µm (depending on concentration and degree of transparency) typically resolves the issue.

Other capture settings like Segmentation Threshold and Close Holes can also affect counts and concentrations. Segmentation Threshold defines the intensity of a particle relative to the initial background calibration and determines what VisualSpreadsheet will consider a "particle" based on how much lighter or darker it is than the background. Close Holes implements a standard morphological operation used to fill small pixel gaps in an image caused when threshold settings are too high. If suboptimal parameters are used for either of these settings particles may be cropped or missed entirely, affecting concentrations.

For this reason, we recommend using File Processing mode to optimize the capture settings when you are first setting up your FlowCam system or running a novel sample type. For more information about using File Processing mode to determine optimal capture settings, please refer to the VisualSpreadsheet user guide found on your FlowCam's desktop.

Sample Concentration

For highly-dilute samples, over or undercounting even one particle may be enough to significantly impact the calculated concentration. In this situation, it is better to err toward overcounting and then delete replicate images during post-processing than to risk missing a particle. If sufficient volume is available, then higher sample volumes can be analyzed to capture a more significantly robust number of particles.

Highly concentrated samples can also introduce error into reported concentrations. If a sample is so concentrated that multiple particles are being captured together as a single particle image (Figure 6), reported concentrations will be skewed low.

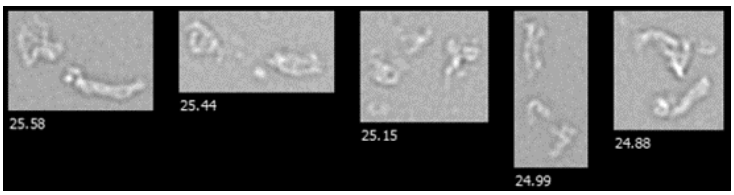

Figure 6. Protein aggregate images from an over-concentrated sample. Note that every image contains two or more distinct particles. This will result in an artificially low reported concentration.

Maximum sample concentration is dependent on several factors including objective used, flow cell depth, particle size, and capture settings required to obtain accurate particle morphology.

The primary method of decreasing sample concentration is dilution, provided it is not expected to alter particle behavior or morphology. Ideally, dilution is done with the sample's native solvent or a compatible buffer like phosphate-buffered saline or ultrapure water. Because optimal sample concentration is dependent on many factors, it is not possible to provide dilution requirements based on specific concentrations. Instead, this is something that is usually learned through training and experience.

Generally, if a relatively low percentage of particle images contain coinciding particles (like the ones shown in Figure 6) then a lower dilution factor (on the order of 1:2, 1:5 or 1:10) will be sufficient. If many images contain coinciding particles, then greater dilution factors (1:100, 1:1000, 1:10000) may be required to obtain accurate concentration data.

If dilution is required, VisualSpreadsheet allows for entry of a dilution factor that will be used to adjust the reported concentration accordingly. This feature can be found in the Fluidics tab of the Context dialog (Figure 7).

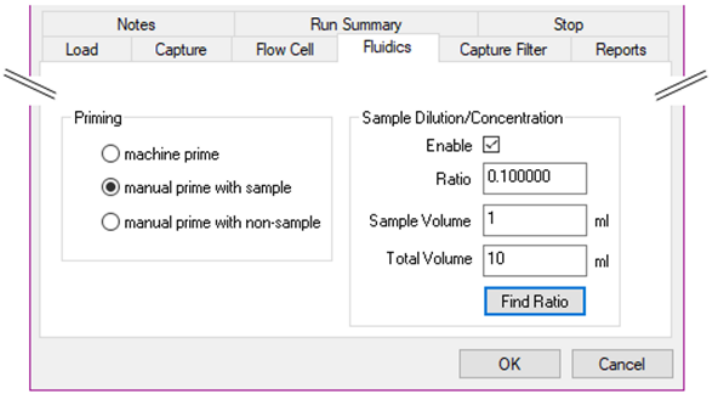

Figure 7. Using the dilution factor feature of VisualSpreadsheet to automatically adjust reported particles/mL values.

Sample Handling

To obtain accurate concentrations, it is important for particles to be distributed as evenly as possible prior to FlowCam analysis. For most samples we recommend hand-inversion of the sample container or mixing via pipet prior to aliquoting sample for a run. For protein therapeutics or other samples sensitive to mechanical stress, a very gentle side-to-side rocking of the sample vial is recommended.

It is necessary to consider how the density of particles contained within a sample might affect reported concentrations. Higher density particles will tend to settle more quickly which can impact concentration data. When handling this type of sample, it is important to instantly remove a sample aliquot from the container after mixing and to start a data run immediately after sample introduction.

Below are some additional techniques for samples that settle quickly:

1. Run multiple aliquots of smaller sample volumes so that there is less time for particles to settle.

2. Increase the flow rate to accelerate sample runs.

3. Try using a solvent with a slightly higher density so that particles settle less quickly.

CONCLUSION

FlowCam 8000 series instruments provide count and concentration data for a wide variety of fluid samples. Relative counts can be compared reliably among different samples, while concentrations provide particles/mL values based on the actual fluid volume imaged by the instrument. By understanding and considering the various factors that can affect reported concentrations, FlowCam users can ensure they are obtaining accurate concentration data.