FlowCam® Technical Brief

FlowCam VS Series Auto Focus Methodology and Benefits

Summary: The FlowCam VS-Series has an auto focus option which focuses the optical system algorithmically without operator intervention, assuring repeatable focus position and therefore measurements.

Definitions:

Focal Plane: The focal plane is the distance from a camera's sensor at which objects are in sharpest focus (object space, Figure 1).

Image Plane: The image plane is the location where the optics focuses the object, and where the sensor is positioned (image space, Figure 1).

Depth of Field (DOF): In any imaging system, there will be a distance in front of and behind the focal plane in which objects remain in *acceptable focus* (Figure 1). The key word here is "acceptable": this means that there is no *absolute* DOF definition, it is only defined by some qualitative (acceptable to the eye) or quantitative (acceptable modulation level on an MTF curve) description of "acceptable."

DOF is proportional to illumination wavelength and numerical aperture (NA) in a microscope (simplified equation):

$$D_{Field} = \lambda / NA^2$$

(where λ= illumination wavelength and NA = n[sinα], where n is the index of refraction [usually 1.0 for air], α is the half-angle of the objective's opening angle, which decreases significantly with magnification).The direct result of this is that the DOF *decreases* exponentially with magnification as indicated in the following table:

Magnification	Numerical Aperture	Depth of Field (µm)
4x	0.10	15.5
10x	0.25	8.5
20x	0.40	5.8
40x	0.65	1.0
60x	0.85	0.40
100X	0.95	0.19

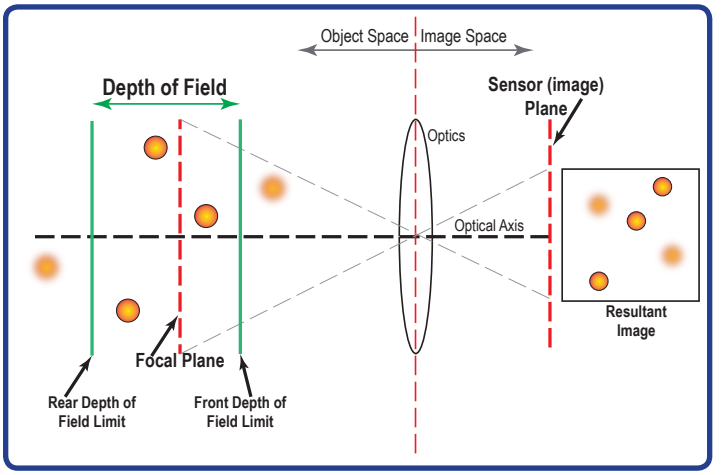

Figure 1: Side-view diagram showing focal plane, image plane and depth of focus for a simple optical system. To the right of the sensor plane is a depiction of the resultant image.

The values shown in the table are for a single set of conditions: a single wavelength and unknown definition of "acceptable" focus (as defined by MTF or circle of confusion). (Source: http://www.olympusmicro.com/primer/anatomy/objectives.html). Because the acceptable condition is unknown, these numbers should only be used for reference, not as absolutes.

Flow cell geometry and fluidics considerations: Because of the limited depth of focus found in microscope systems, dynamic imaging particle analysis (DIPA) systems such as FlowCam must *physically restrict* the depth parallel to the optical axis in which the particles are flowing in order to keep them in as sharp focus as possible. This restriction is called the flow cell *depth*, which is matched to the objective lens being used.

One might simplistically think that the particles flowing through the flow cell would distribute themselves evenly through the flow cell depth. This turns out to be incorrect due to the *laminar flow* conditions set in place by the flow cell geometry. Fluid Imaging has gone to great lengths to mathematically model and experimentally verify the actual behavior of the particles within the flow cell. What we found is that the particles actually end up separating into

two different "sheets" positionally within the flow cell as shown in Figure 2. This behavior of the particles in the flow cell has important ramifications for optimally focusing the instrument.

Manual Focus: Because of the actual organization of the particles within the depth of the flow cell as described above and shown in Figure 2, when focusing of the FlowCam is done manually (by eye), there is a tendency to focus on one of the two "sheets" of particle focus. The result of this is that manual focusing may not be repeatable from the standpoint of which "sheet" the operator has focused on. The focal plane chosen can have a direct effect on both concentration and sizing calculations for the particles, thereby can be a cause for reduced repeatability.

Auto Focus Mechanism: One of the many benefits of the FlowCam VS-Series systems is the ability to easily change to different magnification objectives and flow cell sizes in the same instrument. However, each time one of these changes is made, the system must be refocused. To overcome the potential repeatability issues found by manually focusing, an auto focus hardware option has been introduced that provides for repeatable focusing of the instrument. It removes any subjectivity introduced from the operator by manually focusing.

How it Works: To use the auto focus hardware, a software assistant is activated which directs the operator through the procedure:

1. Operator does coarse focus by eye using focus beads, and locks the flow cell carriage down.
2. Focus beads are pipetted into the sample introduction port, and the software process is initiated.
3. The flow cell carriage is moved to position the focal plane through the entire depth of the flow cell while constantly acquiring bead images and recording the edge gradient values. Edge gradient is a quantitative way of measuring edge sharpness, therefore degree of focus.
4. The final carriage position for best focus is determined by a proprietary algorithm in FlowCam's VisualSpreadsheet® software based upon the position where the highest edge gradient was found for the most number of particles.

Measurement of Auto Focus Repeatability: In order to quantitatively show how repeatable this procedure is, the following experiment was conducted for each different FlowCam magnification:

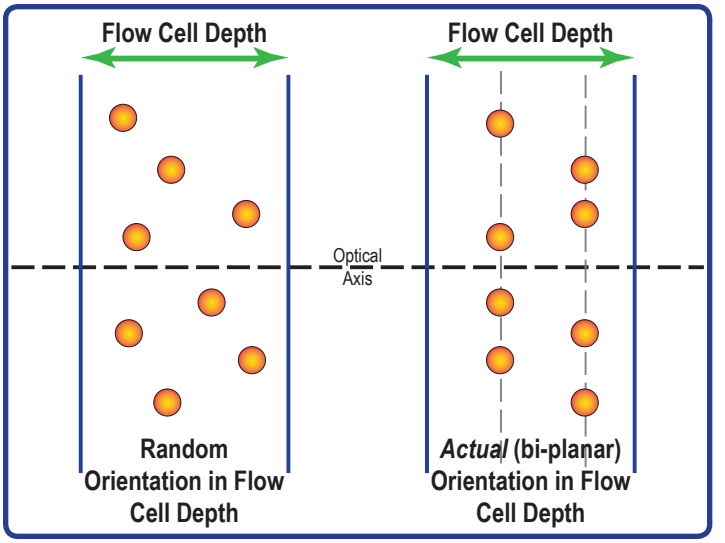

Figure 2: Side-view of flow cell depth dimension along the optical axis, showing random particle distribution through the depth on left, and actual bi-planar distribution on right.

1. Operator does coarse focus by eye using focus beads, and locks the flow cell carriage down.
2. Five repetitions of the auto focus function are performed, with exact physical location of the final carriage position recorded, and standard deviation around the mean calculated.
3. Steps 1 and 2 are repeated two more times, and average standard deviation for the total 15 runs is calculated:

Magnification	5-run Std. Dev (1) (µm)	5-run Std. Dev (2) (µm)	5-run Std. Dev (3) (µm)	Average
4x	2.9734	1.7269	3.1882	**2.6295µm**
10x	2.4626	2.1877	2.4174	**2.3559µm**
20x	1.3443	1.7641	2.7099	**1.9394µm**

The results show a high degree of precision and accuracy.

Conclusion: The FlowCam VS-Series auto focus option yields exceptional positional focus repeatability, far superior to what could be achieved manually by eye. Because the exact positioning of the focal plane inside of the flow cell may affect both particle size and concentration calculations, it is critical that this position be very repeatable.

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