FlowCam®

FlowCam Quantitative Metrics and Camera Settings

The effect of background intensity on particle image analysis

SUMMARY

In flow imaging microscopy, background intensity is an important factor to consider for image segmentation and overall particle characterization.

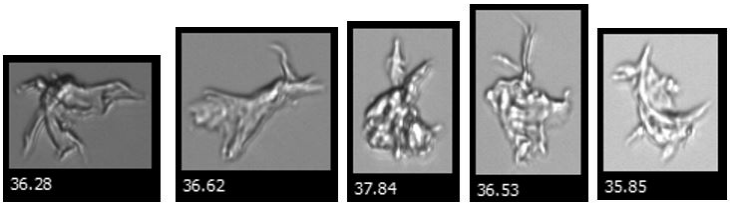
FlowCam instrument default camera settings are optimized for particle image extraction to minimize the need to adjust background intensity levels when imaging various types of particles. To demonstrate the robust imaging capabilities of FlowCam, the characteristics of various particle sample types were profiled over a series of background intensity values. The findings show comparable quantitative metrics across the range of background intensities tested.

The expected effect of edge gradient values increasing with increasing background intensity for opaque bead samples was observed. And while particle count values for more translucent protein-like particles varied with background intensity, the greatest particle count values were achieved at background intensity levels within the recommended range of 160-170.

THE BASIC PRINCIPLE OF IMAGE SEGMENTATION WITH FLOWCAM

Efficient image segmentation is achieved with VisualSpreadsheet digital imaging processing. VisualSpreadsheet captures digital images of the flow cell and uses pixel values in the raw image data to partition these images into individual particle images. Background intensity mean (BIM) is an important parameter in effectively discerning foreground particles from the background. A value between 0 and 255 indicates how bright or dark the background of the flow cell is, with values closer to zero representing darker shades and values near 255 representing lighter shades for the background. When background intensity levels are optimized, more highly resolved image segmentation is achieved, which yields more accurate sample calculations for more precise sorting, filtering, and classifying of individual particle images.

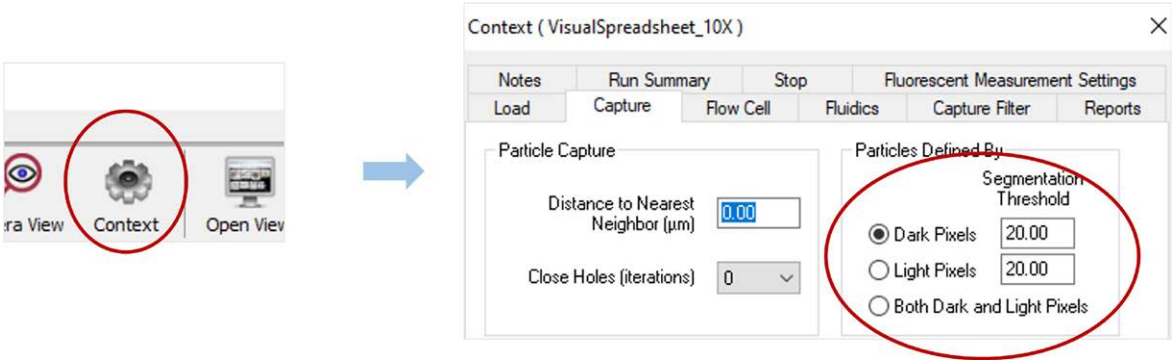
Background intensity can also impact several of the particle properties FlowCam measures even at fixed segmentation accuracy. For example, edge gradient, a property that measures the change in pixel values between the background and the outermost edge of each particle, will generally increase with BIM since a larger change in pixel values is possible with a brighter background. However, at fixed background intensity, edge gradient is often useful as a measure of image quality.



Example FlowCam images of particles (ETFE protein standards) captured at different background intensity settings

Background intensity is one of several parameters that can influence how accurately FlowCam and VisualSpreadsheet can segment particles. In VisualSpreadsheet, most of the settings that control segmentation such as thresholds, distance to nearest neighbor, and close holes (iterations) can be found in the context settings window (Figure 1A). These settings are often adjusted between experiments based on the properties of the samples and the particles they contain. Dark and light pixel thresholding and VisualSpreadsheet's neighborhood analysis are necessary to accurately analyze particles with different degrees of transparency[1]. For example, users will often utilize different threshold values for samples that primarily contain opaque particles (e.g., polystyrene latex calibration beads) than for samples that mostly contain transparent particles (e.g., plankton, biotherapeutics). Most users will generally use a single background intensity value regardless of their "context" settings. However, the BIM adjustment is found in a different location in VisualSpreadsheet—namely, in the camera view under setup → camera (Figure 1B). During measurements, the average background intensity is displayed in the lower left-hand corner of the live camera view (Figure 1C). To achieve optimal imaging, the recommended background intensity range for FlowCam instruments is between 160-170. Regardless of what BIM value is chosen for a protocol, it is highly recommended that users ensure their background intensity is kept at the chosen value before performing measurements to ensure consistent instrument performance between measurements.

A.



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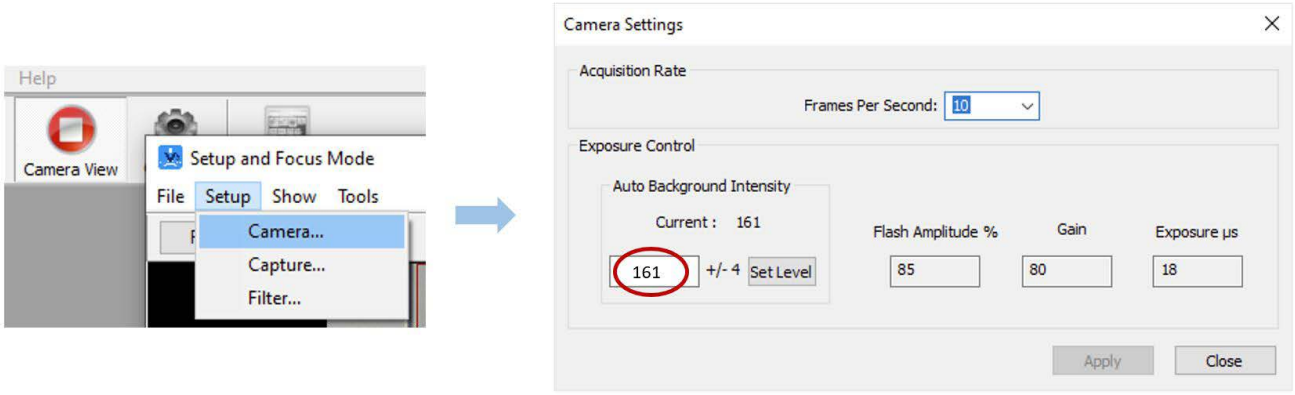

C.

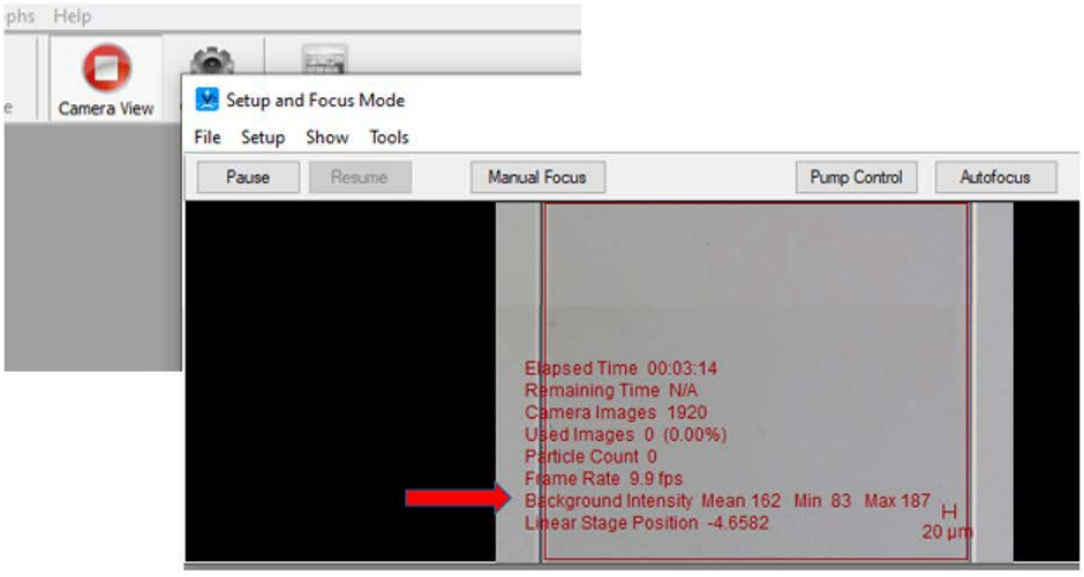

Figure 1. Options for setting image segmentation and capture parameters in VisualSpreadsheet 6

Segmentation thresholds can be adjusted in the "Capture" tab of the "Context" window accessed from the "Context" button in the VisualSpreadsheet main window, **A**. The background intensity exposure control factor can be adjusted in the "Camera Settings" window, which can be accessed by clicking the Camera View button in the VisualSpreadsheet main window and then selecting Setup -> Camera from the Setup and Focus Mode drop menu, **B**. The current background intensity mean value is displayed in the lower left-hand corner of the live camera view, **C**, (indicated by the red arrow).

EXPERIMENTAL DESIGN

Two FlowCam 8000 series instruments, one with a color camera and one with a black and white camera, were used to demonstrate how background intensity camera settings impact quantitative data metrics, particle segmentation, and image quality. Four different representative types of particles were imaged in triplicate at five background intensity levels between 100 and 200. Particle diameter, particle concentration (in #/mL), and edge gradient mean values were analyzed.

Materials

- NIST Traceable Polymer Microspheres (Diam: 14.97 µm+/-0.12 µm, Approx 1 x 107 #/mL) diluted to ~5,000 #/mL in ultra-pure water (Duke Standards, Cat No: 4K-15)
- Particle Size Standards (Certified Diam: 10.02 µm +/- 0.06 µm (NIST Traceable), Size Distribution: 0.09 µm Std Dev. 0.9% CV, Count: 3,000 Particles/mL (Count-CalTM, Cat No: CC-10)
- *Gloeocapsa* in Allen's medium (Carolina Biological Supply Company, Cat No: 15-1800) filtered through 100 µm PluriStrainer (PluriSelect, CA, Cat No: 43-57100-03)
- Protein Surrogate Standard (Ethylene tetra fluoro ethylene, ETFE) (Micro Measurement Laboratories, Inc., P/N: 061P) filtered through 100 µm PluriStrainer (PluriSelect, CA, Cat No: 43-57100-03)

FlowCam Setup

The following context settings were used for all particle types:

- 100 µm field of view (FOV) flow cells
- Magnification objective: 10X
- 1 mL pump syringe
- Autoimage mode
- Autoimage rate: 23 frames/sec
- Estimated efficiency: 70.1%
- Machine prime
- Flow rate: 0.15 mL/min
- Sample volume: 250 µL
- VisualSpreadsheet 6

The capture and filter context settings differed between particles as described in Table 1.

FlowCam Methods

Each material was imaged using each FlowCam instrument using the described setup details at each of the following grayscale background intensity levels: 120, 140, 160, 180, and 200. Note: background intensity levels were set following the steps shown in Figure 1B.

RESULTS

To illustrate background intensity's role on image segmentation in flow imaging microscopy, four different sample materials were imaged in triplicate runs using FlowCam instruments equipped with color and black and white cameras, respectively. The values calculated at each of the five grayscale background intensity levels (120, 140, 160, 180, and 200) included particle diameter, particle concentration (in #/mL), and edge gradient means.

Particle sizing across background intensities for both camera types was relatively consistent with low standard deviations over triplicate runs for all samples. The standard bead materials, 4K-15 (size standard), and CC-10 (count standard) showed comparable values across background intensity means (BIMs) (Figures 2A, 3A), with particle diameter values within 2-4% across BIMs (Table 1 and Table 2). The average diameter ABD mean values on the FlowCam color camera were within 5% for *Gloeocapsa* and 2% for ETFE across BIMs (Table 2). Similarly, average diameter ESD mean values on the FlowCam black and white camera were within 3% for *Gloeocapsa* and 7% for ETFE across BIMs (Table 3).

The impact of background intensity on particle concentration was sample-dependent (Figure 2B and Figure 3B). While the bead samples and *Gloeocapsa* showed particle concentration differences with background intensity levels between 2-8%, ETFE showed differences of 14% and 21% across BIMs on the FlowCam color camera and FlowCam black and white camera, respectively, with an appreciable maximum at 160 background level (Tables 1 and 2). It is important to note that ETFE was the only sample where particles smaller than 10 µm were captured and included in the analysis (Table 1). These results suggest that of the BIM values used in this study, the best detection of 2-10 µm particles was observed at a BIM of 160—a value within the recommended BIM range for the instrument.

Sample Material	Distance to Nearest Neighbor	Close Holes	Segmentation Threshold	Diameter minimum, maximum
Beads (e.g., 4k-15 and CC-10)	0	0	20 dark pixels	ESD 10 µm, 10,000 µm
Phytoplankton (e.g., Gloeocapsa)	8	1	17 dark pixels 17 light pixels	ABD 10 µm, 10,000 µm
Protein (e.g., protein surrogate)	4	3	15 dark pixels 15 light pixels	ESD 2 µm, 10,000 µm

Table 1. Particle type-specific context settings for image segmentation and capture

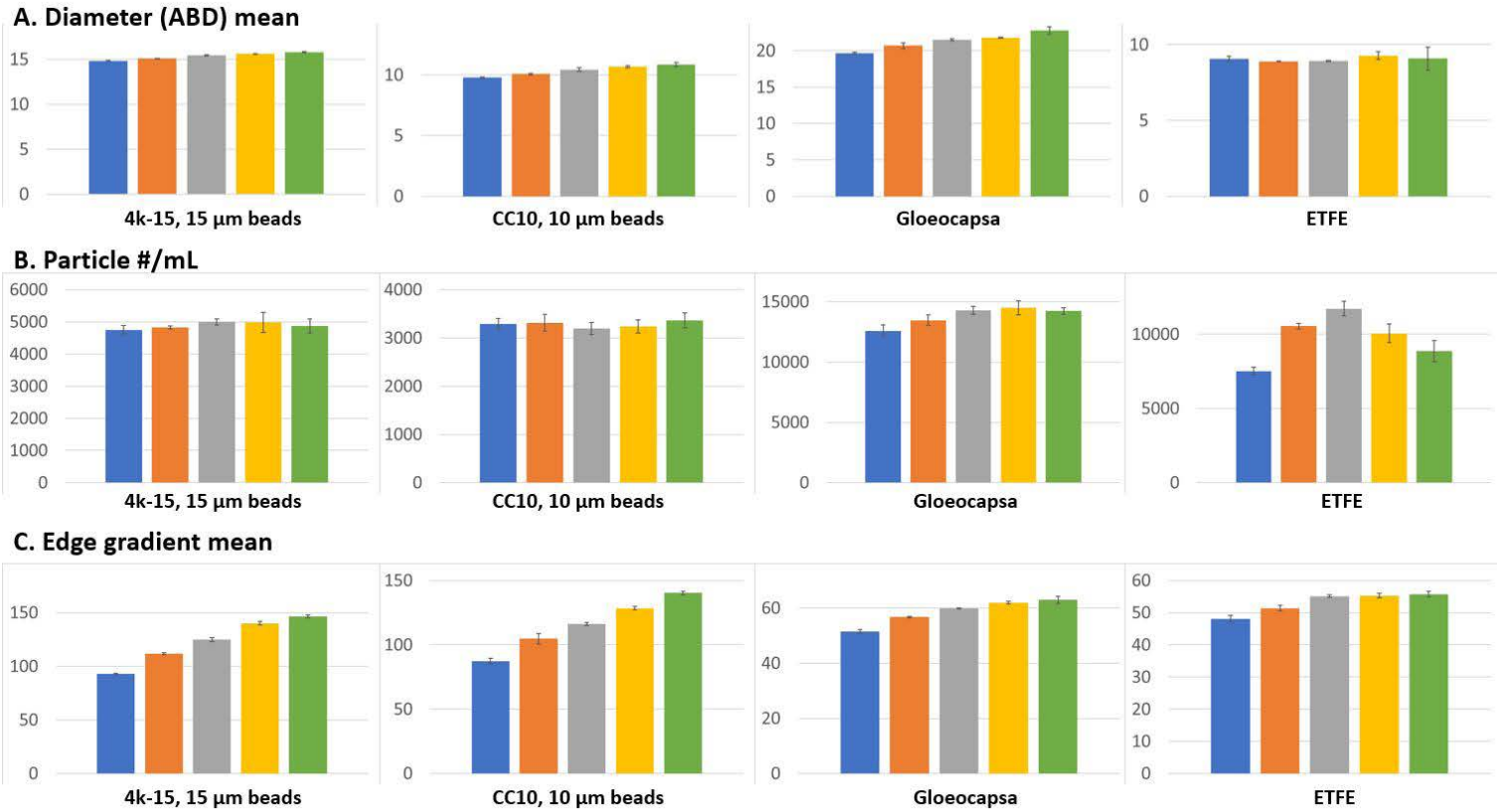

Figure 2. FlowCam (color camera) quantitative metrics of particle samples at various background intensities

Particle samples from left to right: 4K-15, 15 µm beads; CC10, 10 µm beads, *Gloeocapsa*, (phytoplankton), and ETFE, (protein surrogate). Background intensity mean (BIM) values are denoted by color: blue, 120 BIM; orange, 140 BIM; gray, 160 BIM; yellow, 180 BIM; green, 200 BIM. Diameter average values as calculated by area-based diameter (ABD) mean of triplicate runs, **A**. Particle #/mL average values of triplicate runs, **B**. Edge gradient mean average values of triplicate runs, **C**.

FlowCam color camera	Sample	Diameter (ABD) mean (std dev, n=3)	Particle #/mL (std dev, n=3)	Edge Gradient Mean (std dev, n=3)
4k-15 ~5,000 P/mL	120 BIM	14.84 (0.05)	4754.25 (134.89)	92.76 (0.87)
	140 BIM	15.11 (0.01)	4829.08 (42.23)	111.84 (0.90)
	160 BIM	15.47 (0.04)	4995.46 (92.57)	124.86 (1.90)
	180 BIM	15.59 (0.02)	4979.41 (313.79)	140.09 (1.69)
	200 BIM	15.8 (0.05)	4869.36 (222.76)	146.44 (1.39)
% Difference across BIMs:		2%	2%	15%
CC10 3,000 P/mL	120 BIM	9.77 (0.07)	3293.69 (107.00)	87.39 (2.03)
	140 BIM	10.08 (0.06)	3311.95 (171.70)	104.71 (4.17)
	160 BIM	10.45 (0.14)	3194.96 (122.21)	116.21 (1.25)
	180 BIM	10.67 (0.09)	3239.19 (133.95)	128.43 (1.36)
	200 BIM	10.86 (0.17)	3365.56 (155.33)	140.42 (1.35)
% Difference across BIMs:		4%	2%	15%
GLOEO	120 BIM	19.65 (0.17)	12589.85 (475.76)	51.47 (0.69)
	140 BIM	20.69 (0.36)	13467.43 (433.99)	56.76 (0.29)
	160 BIM	21.51 (0.13)	14285.19 (322.05)	59.87 (0.19)
	180 BIM	21.77 (0.07)	14502.19 (575.66)	61.95 (0.45)
	200 BIM	22.78 (0.51)	14237.47 (276.32)	63.05 (1.27)
% Difference across BIMs:		5%	5%	7%
ETFE	120 BIM	9.06 (0.17)	7521.14 (257.85)	48.1 (1.05)
	140 BIM	8.9 (0.02)	10544.41 (188.71)	51.37 (0.84)
	160 BIM	8.93 (0.04)	11722.88 (492.75)	55.13 (0.41)
	180 BIM	9.28 (0.27)	10066.71 (640.04)	55.29 (0.69)
	200 BIM	9.08 (0.77)	8859.17 (708.44)	55.77 (0.84)
% Difference across BIMs:		2%	14%	6%

Table 2. FlowCam (color camera) quantitative metrics of particle samples at various background intensity mean (BIM) levels

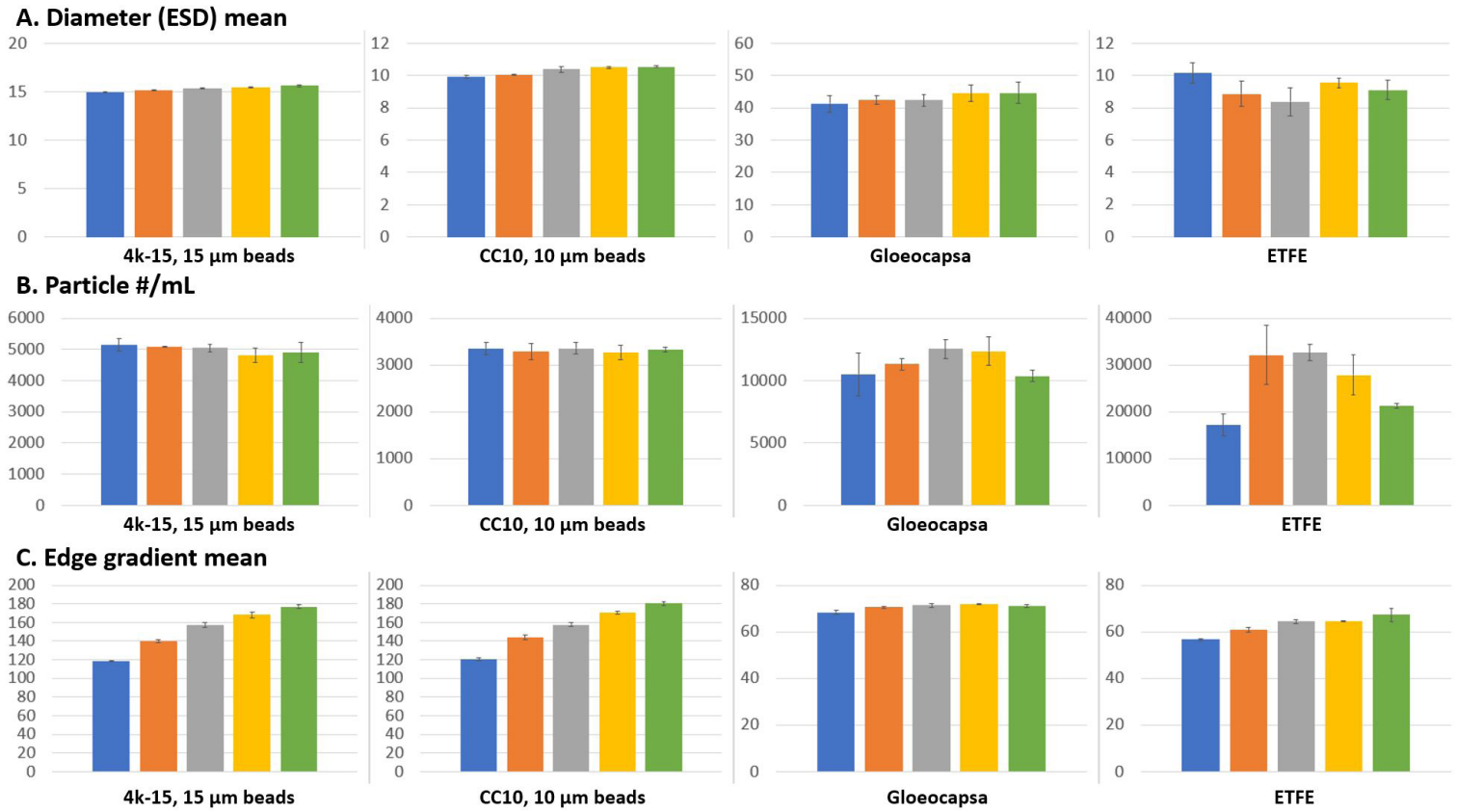

Figure 3. FlowCam (black and white camera) quantitative metrics of particle samples at various background intensities

Particle samples from left to right: 4K-15, 15 µm beads; CC10, 10 µm beads, *Gloeocapsa*, (phytoplankton), and ETFE, (protein surrogate). Background intensity mean (BIM) values are denoted by color: blue, 120 BIM; orange, 140 BIM; gray, 160 BIM; yellow, 180 BIM; green, 200 BIM. Diameter average values as calculated by equivalent spherical diameter (ESD) mean of triplicate runs, **A**. Particle #/mL average values of triplicate runs, **B**. Edge gradient mean average values of triplicate runs, **C**.

FlowCam B&W camera	Sample	Diameter (ESD) mean (std dev, n=3)	Particle #/mL (std dev, n=3)	Edge Gradient Mean (std dev, n=3)
4k-15 ~5,000 P/mL	120 BIM	14.98 (0.01)	5138.52 (207.20)	118.27 (0.49)
	140 BIM	15.19 (0.06)	5071.76 (12.91)	139.72 (1.9)
	160 BIM	15.36 (0.02)	5040.51 (127.73)	157.05 (2.66)
	180 BIM	15.48 (0.05)	4811.34 (230.66)	168.02 (2.69)
	200 BIM	15.64 (0.09)	5462.28 (295.14)	176.72 (3.77)
% Difference across BIMs:		2%	4%	13%
CC10 3,000 P/mL	120 BIM	9.95 (0.10)	3353.6 (127.73)	120.51 (1.71)
	140 BIM	10.06 (0.02)	3282.95 (168.74)	143.98 (2.87)
	160 BIM	10.4 (0.17)	3355.49 (129.06)	157.56 (2.17)
	180 BIM	10.52 (0.06)	3272.92 (151.84)	170.47 (1.14)
	200 BIM	10.56 (0.08)	3397.56 (187.95)	181.43 (3.04)
% Difference across BIMs:		3%	2%	13%
GLOEO	120 BIM	41.32 (2.65)	10494.94 (1685.65)	68.21 (0.82)
	140 BIM	42.51 (1.30)	11313.51 (449.80)	70.47 (0.31)
	160 BIM	42.39 (1.73)	12534.17 (767.64)	71.44 (0.82)
	180 BIM	44.57 (2.66)	12354.12 (1122.10)	71.96 (0.08)
	200 BIM	44.72 (3.17)	10377.18 (470.48)	71.09 (0.49)
% Difference across BIMs:		3%	8%	2%
ETFE	120 BIM	10.17 (0.61)	17095.11 (2355.28)	56.68 (0.40)
	140 BIM	8.88 (0.79)	32127.08 (6275.47)	60.85 (0.96)
	160 BIM	8.38 (0.87)	32629.14 (1774.39)	64.37 (0.80)
	180 BIM	9.55 (0.28)	27874.88 (4305.37)	64.36 (0.21)
	200 BIM	9.12 (0.62)	21212.91 (515.01)	67.3 (2.88)
% Difference across BIMs:		7%	21%	6%

Table 3. FlowCam (black and white camera) quantitative metrics of particle samples at various background intensity mean (BIM) levels

An increase in the edge gradient mean values as background intensity values increased was observed with both instruments for all sample materials, but was most pronounced with the 4K-15 and CC-10 bead samples at 15% and 13% difference across BIMs for FlowCam color and black and white cameras, respectively (Figure 2C and Figure 3C; Tables 2 and Table 3). In contrast, edge gradient changes for *Gloeocapsa* and ETFE with BIM were more subtle; the difference in values was between 6-7% on the color camera-equipped unit and was within 2-6% on the black and white camera-equipped unit. The increase in edge gradient with background intensity observation is expected with bead-like samples since the brighter background at higher BIM values generally increases the measured edge gradient. The increased edge gradient effect is dampened for *Gloeocapsa* and ETFE particles as higher BIMs will consonantly impact background brightness and the brightness of these transparent particles.

Particle Imaging Results

Images representative of the average particle diameter value for each material (Figure 4 and Figure 6) and images of particles among the highest diameter sizes for phytoplankton (*Gloeocapsa*) and protein (ETFE) (Figure 5 and Figure 7) at the lowest (120) and highest (200) background intensity levels were selected for illustrative purposes. Edge trace and binary overlay details were used to qualitatively illustrate image quality changes with changes in background intensity.

Representative particle images of each sample material from both FlowCam instruments, shown in Figures 4-7, illustrate the effect background intensity can have on particle imaging analysis. Lower background intensity values yield different edge tracing and binary overlay outputs compared to higher background intensity values. As best shown in the *Gloeocapsa* images, differences in edge tracing and binary overlay can result in more of the particle getting "missed" and bigger holes getting defined at the lower or higher background intensity levels. It should be noted that within each sample type, the same capture context settings were used across all BIM measurements and that these settings were not necessarily optimized for extreme background intensities. Adjusting these settings to account for each background intensity will likely yield more consistent segmentation across BIM values.

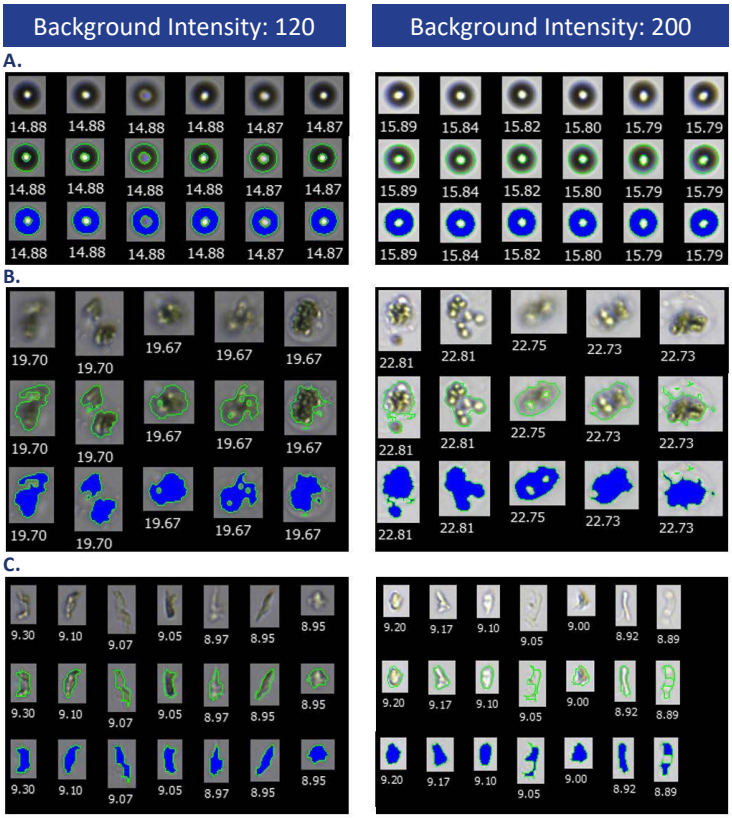

Figure 4. FlowCam (color camera) particle images at background intensity 120 (left) and 200 (right) of the following materials: 4K-15 beads, **A**; Phytoplankton, *Gloeocapsa*, **B**; Protein, ETFE protein surrogate, **C**. Particles sorted by Area Based Diameter (ABD). Edge trace is shown in green; binary overlay is shown in blue. The diameter ABD value is noted below each image. Representative images of average-size particles for each sample material are shown.

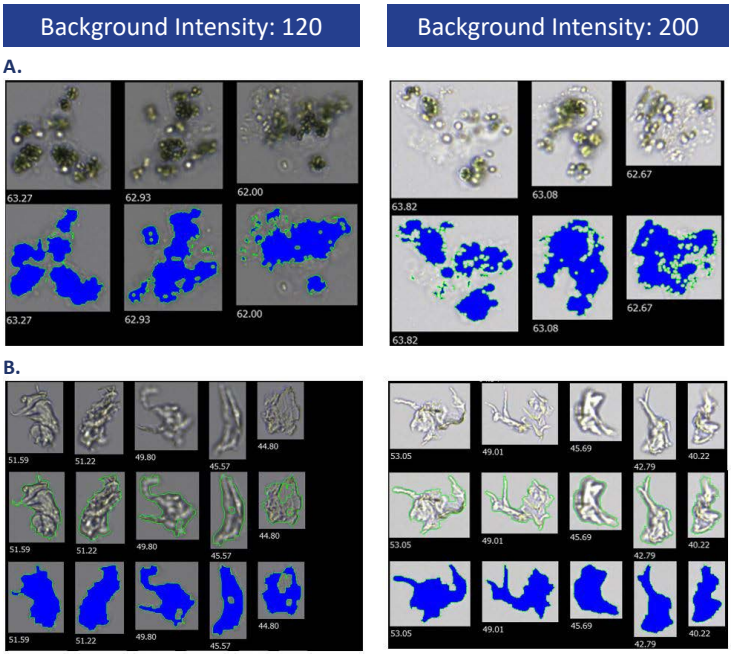

Figure 5. FlowCam (color camera) particle images at background intensity 120 (left) and 200 (right) of the following materials: Phytoplankton, *Gloeocapsa* **A**; Protein, ETFE protein surrogate, **B**. Edge trace is shown in green; binary overlay is shown in blue. The diameter ABD value is noted below each image. Representative images of larger particles are shown.

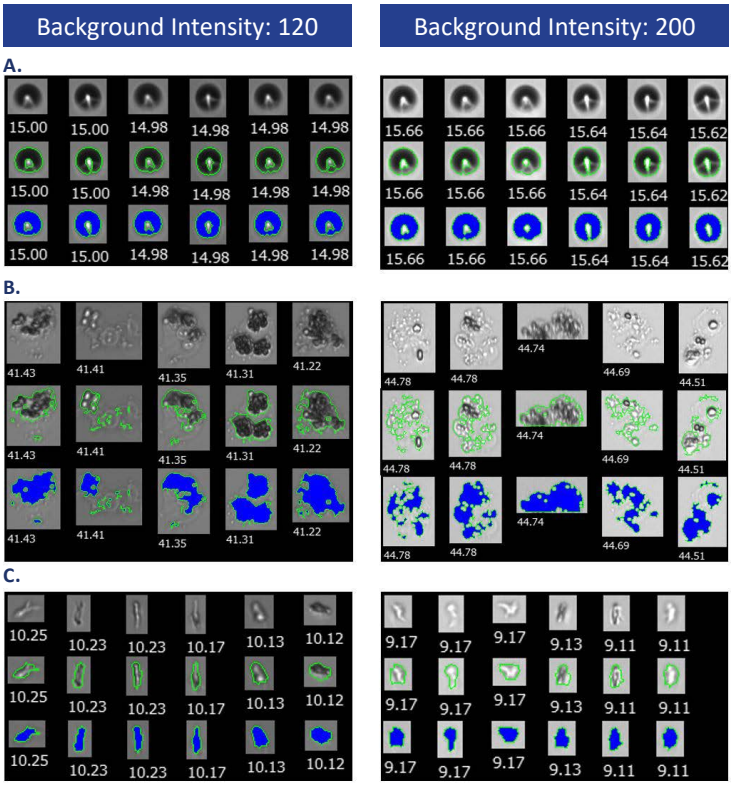

Figure 6. FlowCam (black and white camera) particle images at background intensity 120 (left) and 200 (right) of the following materials: 4K-15 beads, **A**; Phytoplankton, *Gloeocapsa*, **B**; Protein, ETFE protein surrogate, **C**. Particles were sorted by Equivalent Spherical Diameter (ESD). Edge trace is shown in green; binary overlay is shown in blue. The diameter ESD value is noted below each image. Representative images of average-size particles for each sample material are shown.

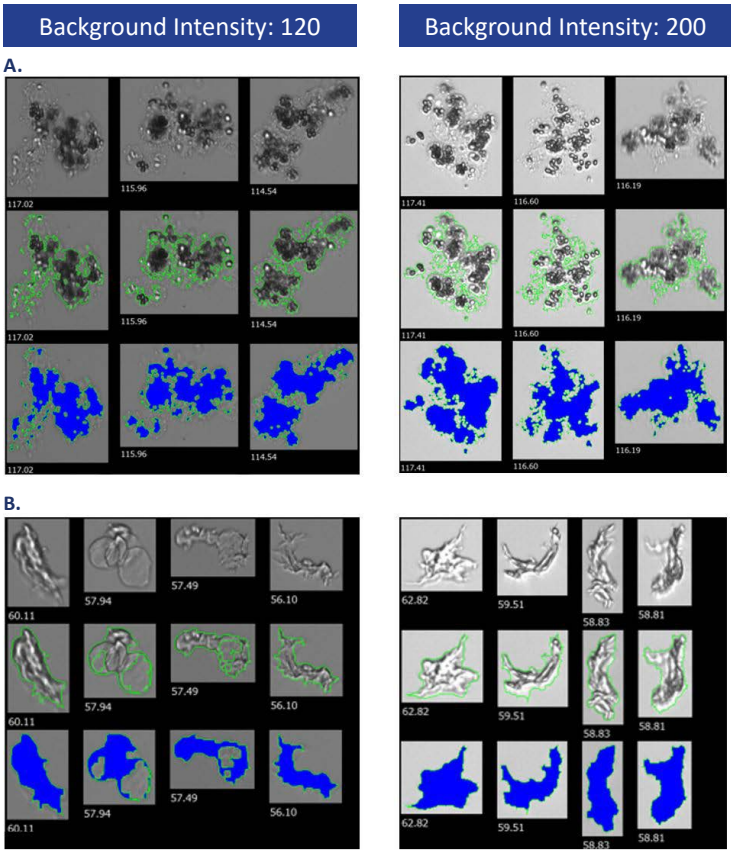

Figure 7. FlowCam (black and white camera) particle images at background intensity 120 (left) and 200 (right) of the following materials: Phytoplankton, *Gloeocapsa* **A**; Protein, ETFE protein surrogate, **B**. Edge trace shown in green; binary overlay shown in blue. The diameter ESD value is noted below each image. Representative images of larger particles are shown.

CONCLUSION

Particle imaging analysis with FlowCam color and FlowCam black and white cameras is robust across a range of background intensity settings[2]. The best yield on quantitative metrics, especially for counting particles <10 µm, can be achieved at 160 BIM, a value in the middle of the range of BIM values tested (120-200), and within the recommended range of BIM values.

While the performance of FlowCam is robust to different background intensity values, these results indicate that background intensity changes can impact particle data. The results shown in Figures 2 and 3 (and Tables 2 and 3) indicate that BIM can impact edge gradient values reported by the instrument. Figures 2-7 show that different BIM values can also subtly influence particle sizing at fixed context settings. It is therefore recommended that once an optimal background intensity level and the associated capture settings are determined, these values should be used consistently to avoid particle detection and characterization bias.

REFERENCES

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https://info.fluidimaging.com/thresholding-whitepaper-download

2. Color vs. Black and White: How to Choose a FlowCam Camera
https://info.fluidimaging.com/color-or-bw-choose-flowcam-camera