FlowCam®

The Benefits of Light and Dark Pixel Thresholding

WHAT IS THRESHOLDING?

Thresholding is a method of image segmentation used in image processing to discern the boundaries of an object from its background. Semi-transparent particles, such as protein aggregates, are often mischaracterized or even undetected by most imaging particle analyzers when thresholding is missing or improperly configured.

Dark pixel thresholding, offered by most imaging particle analyzers, fails to discern particulate matter when it is lighter than the imaged background. Utilization of both dark and light pixel thresholding enables the detection and discernment of particulates expressing ranges in opacity (opaque to transparent) and improves the particle analyzer's ability to detect, image, and analyze semi-transparent particles.

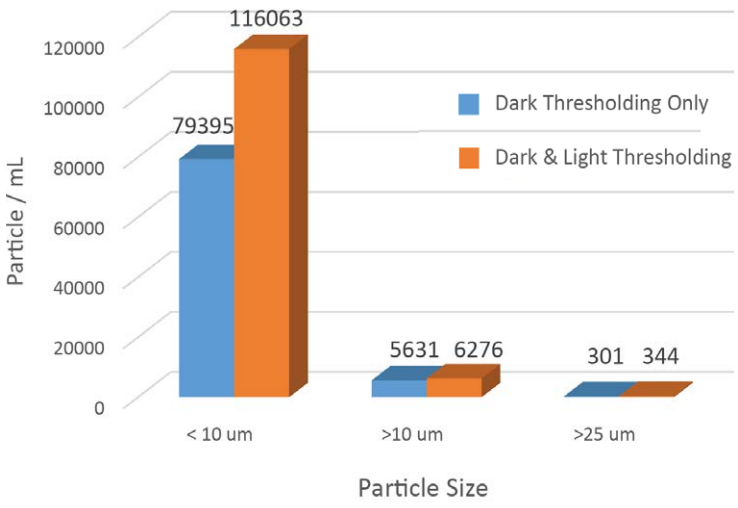

Figure 1. A protein sample was analyzed using dark pixel thresholding and both dark and light pixel thresholding. Utilization of dark and light thresholding showed an increase in particle count.

HOW DOES THRESHOLDING AFFECT INSTRUMENT SENSITIVITY?

Thresholding uses pixel intensities to differentiate the boundaries of the object from its background. Each pixel in the grayscale image is assigned an intensity value from 0 (black) to 255 (white). Color images are converted to grayscale images prior to intensity assignments. The intensity of each pixel in the image is compared to the background pixel intensity and the thresholding value. Based on these comparisons, the pixel is determined to be part of either the foreground object or the background. Dark pixel thresholding recognizes objects with pixel intensities darker than the background. Light and dark pixel thresholding recognizes objects with pixel intensities lighter and/or darker than their background. This is ideal for transparent/semi-transparent particles such as protein aggregates.

In most particle analysis instruments, the camera views opaque particles as darker than their background because the illumination source is located behind the flow cell. For this reason, most imaging particle analyzers use dark pixel thresholding only. However, dark pixel thresholding of semi-transparent particles, such as protein aggregates, often leads to fractionation into several smaller particles, resulting in inaccurate physical measurements and particle count. Use of both dark and light pixel thresholding during particle analysis recognizes the grayscale range (opaque to transparent) of semi-transparent particles, reduces fractionation, and improves particle count and morphological characterization.

Figure 2 illustrates particle analysis of a semi-transparent protein aggregate using various threshold settings. These images were obtained using FlowCam's dark and light pixel thresholding capabilities. Each green box delineates a separate particle, as identifed by FlowCam's analytical software VisualSpreadsheet, and portions identified as part of each particle are colored red.

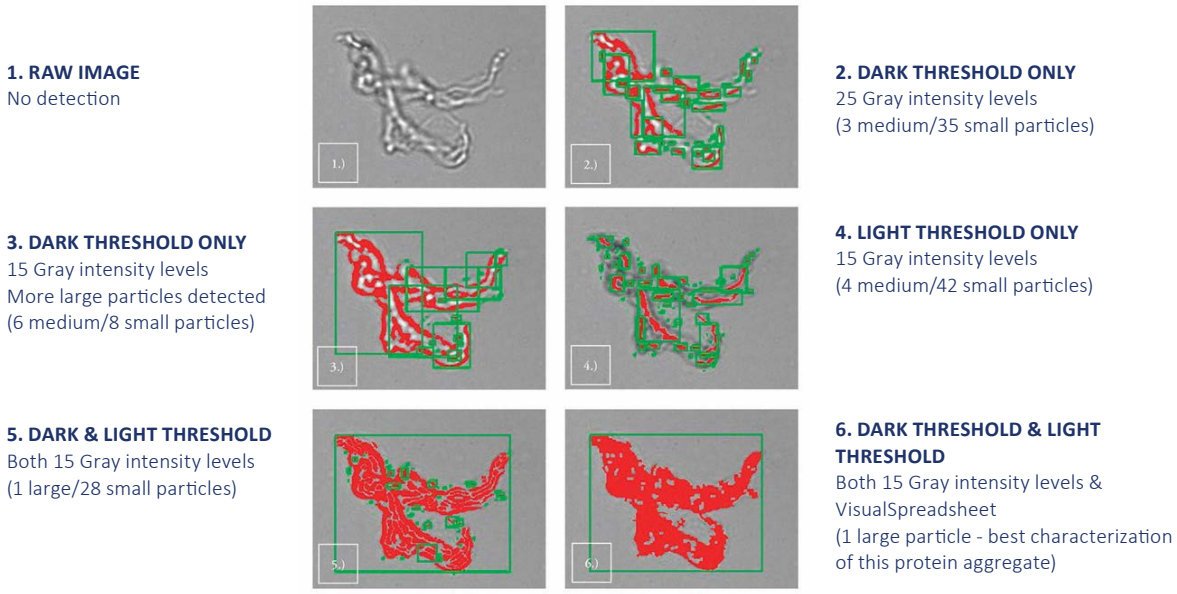

Figure 2. An example of a semi-transparent particle analyzed with FlowCam's various threshold settings. Each green box delineates a separate particle identifed by FlowCam's software VisualSpreadsheet. Portions of the image detected by the software are colored red. Data listed includes the threshold setting used, the Gray intensity levels selected, and how many separate particles the larger particle was subdivided into.

The use of only one pixel setting (either light or dark) resulted in the fractionation of the protein aggregate into as many as 46 separate particles. As image 1 shows, the protein aggregate was comprised of portions both darker and lighter than the image background. When dark pixel thresholding was used, only the portions of the protein darker than the background were recognized as particulate matter, and the protein was falsely subdivided into several smaller particles (images 2 and 3). When light pixel thresholding was used, only portions of the particle lighter than the background were recognized as particulate matter and the protein was also falsely subdivided into several smaller particles (image 4). Changes in opacity of the protein aggregate result in changes in grayscale intensity. The application of only a single type of pixel thresholding (dark or light) failed to accurately characterize a particle exhibiting both light and dark portions.

The use of both dark and light pixel settings, in conjunction with VisualSpreadsheet's neighborhood analysis feature, resulted in accurate particle characterization of the protein aggregate. When dark and light pixel thresholding was used, the particle analyzer recognized both dark and light portions of the particle as particulate matter. The filamentous nature of this protein, however, resulted in minor fractionation of the aggregate (image 5).

The software's neighborhood analysis uses a combination of close-hole iterations and distance-to-nearest-neighbor analyses to further mitigate this fractionation. Close-hole iterations fill small gaps in the segmented particle image between regions called a single particle, resulting in a less fractionated and often smoother segmented image. The distance-to-nearest-neighbor analysis then groups together particle fragments that are less than a set distance apart from each other into a single particle. By applying these additional processing steps, VisualSpreadsheet can identify the whole protein aggregate as part of a single particle (image 6).

Dark and light pixel thresholding and VisualSpreadsheet's neighborhood analysis are necessary to accurately analyze semi-transparent particles. By understanding the issues of semi-transparent particle analysis and making it easy to optimize the system to account for them, FlowCam provides accurate particle characterization, count, and concentration.