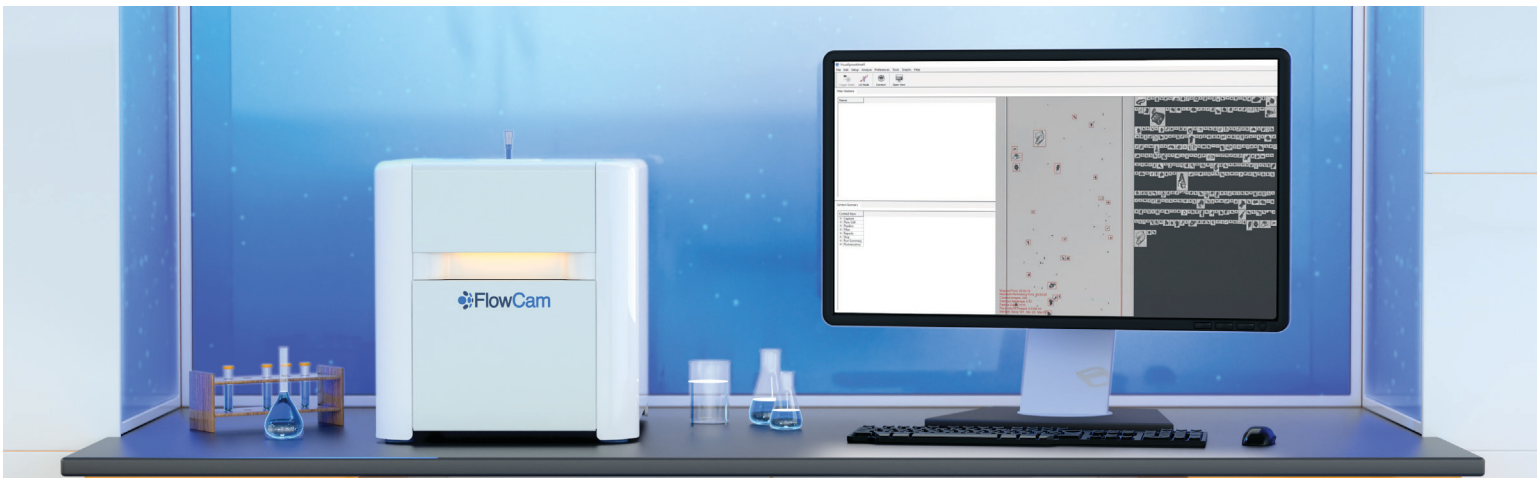
YOKOGAWA

EBOOK

The Ultimate Guide to Flow Imaging Microscopy for Protein Therapeutics

FlowCam®
Yokogawa Fluid Imaging Technologies, Inc.



YOKOGAWA FLUID IMAGING TECHNOLOGIES

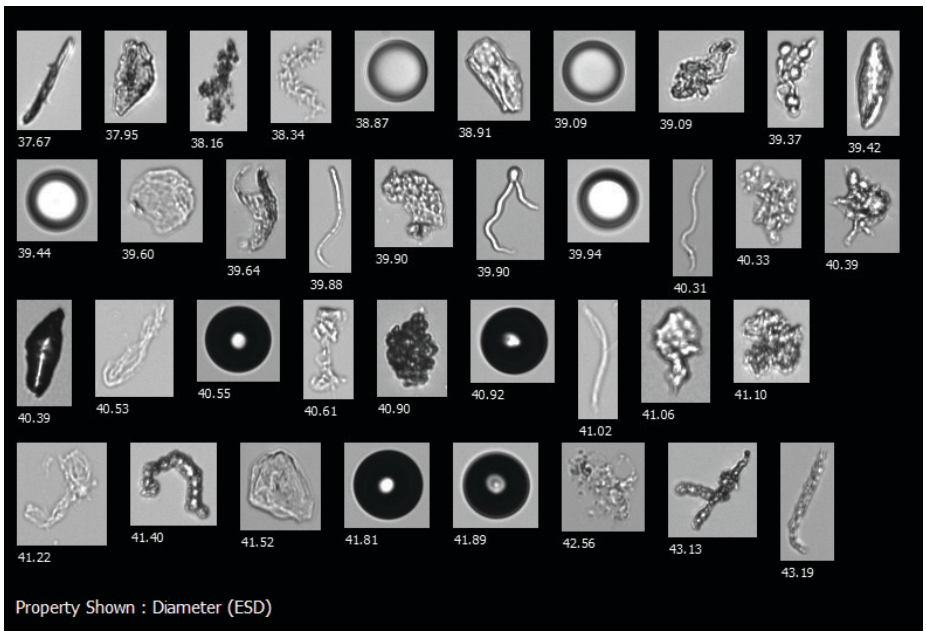
Discover a Better Way to Characterize Subvisible Particles in Biopharmaceuticals

CONTENTS

Overview

The Food & Drug Administration (FDA) strongly recommends in-depth characterization of particles in protein therapeutics and other biotherapeutics. Flow Imaging Microscopy (FIM), is increasingly being utilized because it can provide incredible insight into your formulation, allowing you to visualize your particles from 300 nm to 100 µm. Innovative instrumentation, including FlowCam Nano and FlowCam LO, allow new functionality and extend the range of imaging capabilities to the submicron size range.

In this eBook, we'll briefly review different methods available for analyzing particles, including the advantages and drawbacks of each. Then we'll take a deeper look at flow imaging microscopy (FIM) and how it works. Finally, we'll focus on how FIM helps improve characterization of active pharmaceutical ingredients (e.g. protein aggregates and cells),



A VisualSpreadsheet® collage file containing images of protein aggregates, contaminants, silicone oil droplets and other particles.

FlowCam®

CHAPTER ONE

What is Particle Analysis?

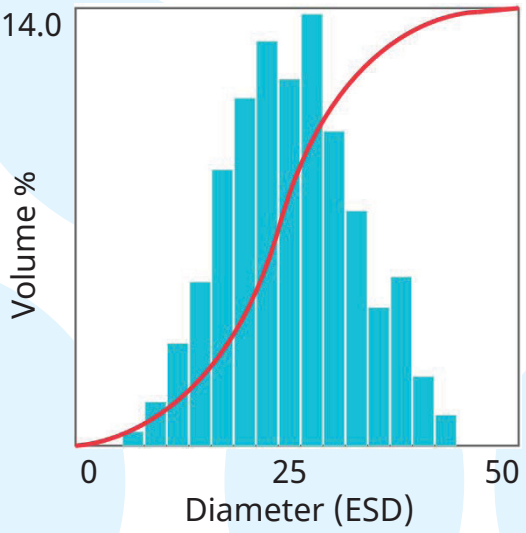
Particle analysis involves taking a sample of a substance and analyzing the individual particles that make up that sample.

Typical measurements of particles of interest include:

- Particle concentration
- Particle size distribution
- Particle count
- Particle shape

For simplicity, results of a particle analysis are typically reported graphically, with particle size plotted against some other variable. Particle size is often stated as equivalent spherical diameter (ESD), which is an estimated diameter based on measurements of the particle assuming the particle is a sphere.

A particle size distribution like that shown on the right is commonly measured and reported during particle analyses. In this graph, the particles are shown by estimated size (ESD). While estimated size distribution is considered valuable data, it only tells part of the story.



While the above graph shows particle size fairly straightforwardly, when shape and/or morphological data is needed, a more in-depth analysis is required to truly characterize a particle.

CHAPTER TWO

A History of Particle Analysis

SECTION 2.1: MICROSCOPY

The introduction of the microscope in the 1600s changed the world for scientists. For the first time, they could observe and record organisms too small to see with the naked eye.

To this day, microscopy remains the most common method for subvisible particle analysis.

ADVANTAGES OF MANUAL MICROSCOPY

The benefit of microscopes is simple: it allows you to study subvisible particles in great detail under a wide range of magnifications.

Microscopes have improved over time, allowing us to look at increasingly smaller particles, even down to the molecular level.

DRAWBACKS OF MANUAL MICROSCOPY

Using manual microscopy for particle analysis is time-consuming. Depending on the sample, it can take hours to prepare the sample, set up the slides, and measure any particles found.

It's challenging to get results that are statistically significant using manual microscopy. You can only process one small sample at a time, so it's difficult, if not impossible, to know if what you're looking at is representative of the whole.

Human factors must also be considered using microscopy. Tired eyes, interruptions, and time of day can all have an effect on the operator, and therefore the results.

"Comparing indivudual particle shape using a microscope is cumbersome and slow. It's difficult to see more than a handful of particles, and certainly not enough to get a statistically significant sample."

— Ross Clark, Distinguished Research Fellow at CP Kelco, a leading producer of specialty hydrocolliods

SECTION 2.2: VOLUMETRIC PARTICLE ANALYSIS TECHNIQUES

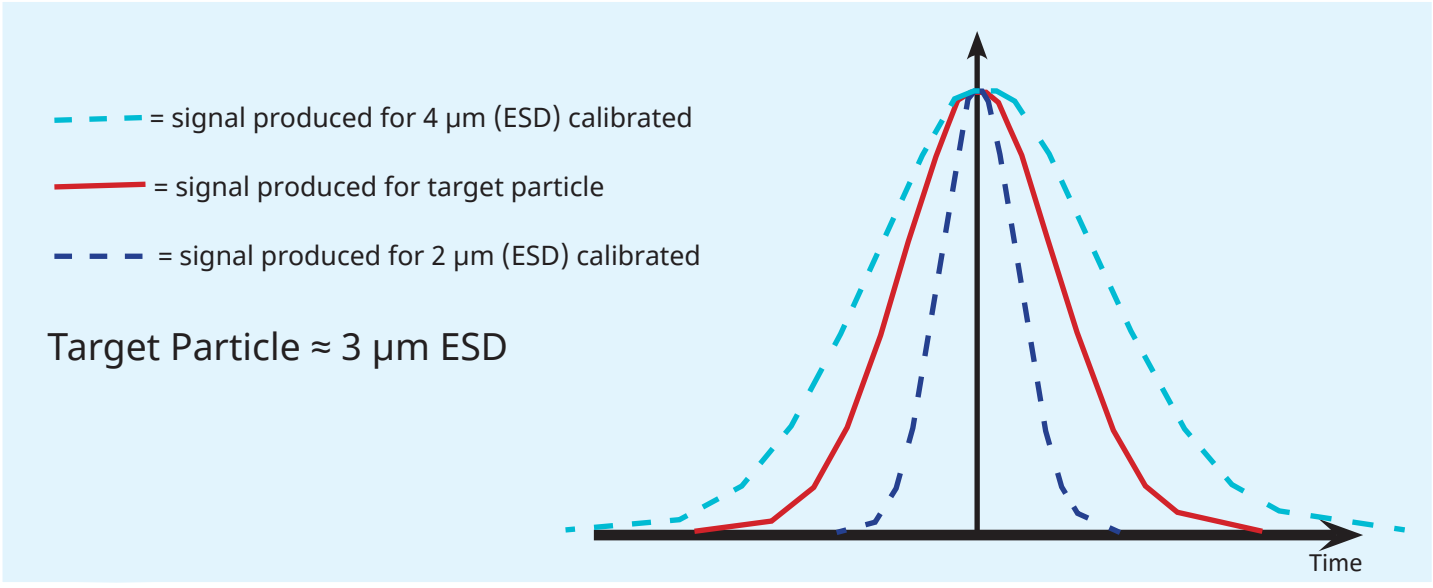
In response to the need for rapid processing of particle data, a variety of volumetric techniques have been developed. Volumetric particle analysis methods include:

- Coulter Counters
- Light Obscuration
- Laser Diffraction
- Light Scattering

These indirect techniques measure a signal that is proportional to the volume of a particle and not the actual physical dimensions of the particle.

The fundamental principle of these indirect techniques is that all particles are assumed to be spherical in shape, and the volume is converted to an equivalent spherical diameter (ESD). In these situations, it is not possible to know the actual shape of the particle, just the size distribution.

The Coulter Counter, shown here counting cells in solution, is an indirect volumetric particle analysis method.



Indirect calculation of particle size based on signal profile, where signal is proportional to volume.

ADVANTAGES OF VOLUMETRIC METHODS

Volumetric methods can rapidly count and size a statistically-significant amount of data—up to tens of thousands of particles per minute.

A particle size distribution that shows particle size versus either frequency or volume is easily created. Detailed particle statistics can be recorded for the entire distribution.

DRAWBACKS OF VOLUMETRIC METHODS

The most significant drawback to volumetric techniques is that they must assume all particles are spheres. These methods are limited to particle counting and size distribution only.

It is common for samples to be heterogeneous, containing a variety of particle types and shapes. Volumetric techniques cannot characterize different particle types in a mixture due to the assumption that all particles are spherical.

SECTION 2.3: FLOW IMAGING MICROSCOPY (FIM)

A flow imaging particle analyzer performs the following three functions all in one instrument:

- Draws a fluid sample through a microscope
- Takes digital images of the magnified particles within the fluid stream
- Characterizes the particles using a variety of measurements

ADVANTAGES OF FLOW IMAGING MICROSCOPY

FIM combines the benefits of manual microscopy with those of volumetric techniques. Microscopic particle measurements are taken from large sample volumes quickly enough to produce statistically significant results. Additionally, multiple measurements are taken for each particle, thereby providing the detailed information often needed for a thorough particle analysis.

The addition of specialized software also provides sophisticated post-processing of data to give you an in-depth analysis of your sample and a better understanding of your data.

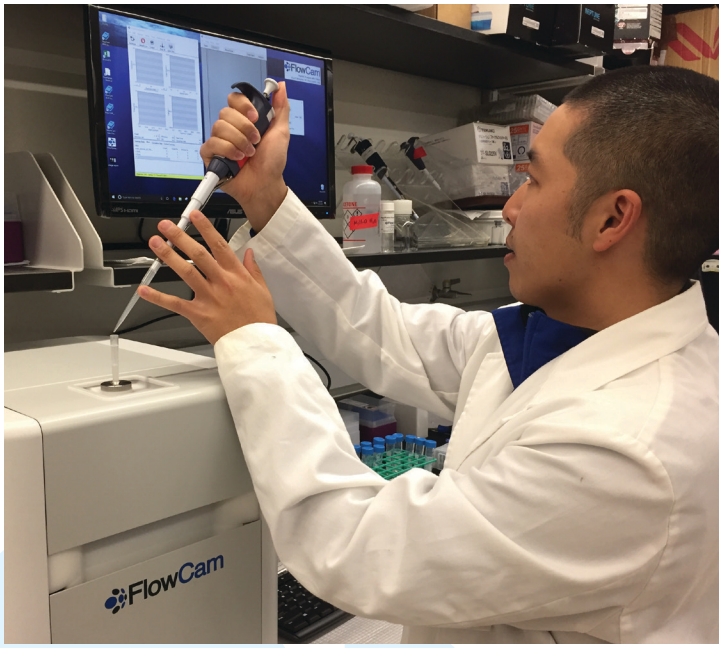
"After using light-obscuration instruments to count particles in a new parenteral product formulation, a project team at GlaxoSmithKline found that the light obscuration method yields a read-out of particles counted in the sample, but is unable to shed light on the nature of the particles. If the particles are not identified, differentiating the actual number of product-related particles can be a difficult task."

-Morrone, Greg J., and Wasfi Al-Azzam. "From Safety Snapshot: An imaging particle analyzer can give researchers a better picture of particles in parenteral formulations." *Drug Discovery & Development*, Volume 14, Issue 5.

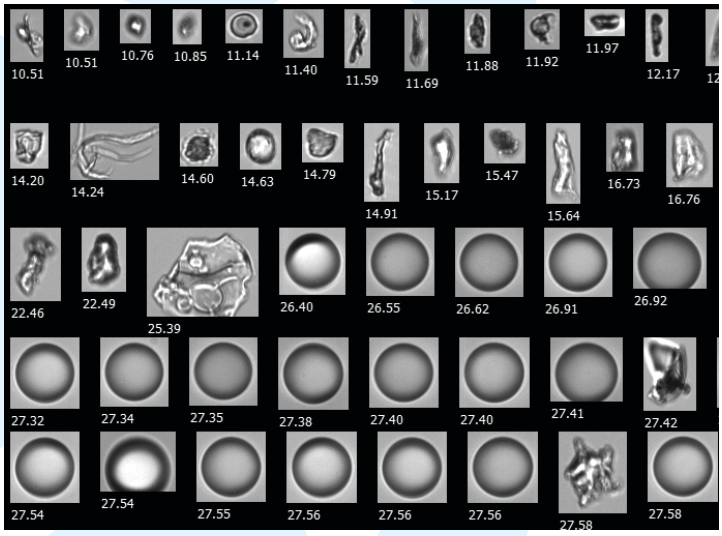
LIMITATIONS OF FLOW IMAGING MICROSCOPY

The ability of an imaging system to resolve particle details is essential for accurate measurement. The optical system and the sensor of the instrument affect its ability to size and characterize subvisible particles.

Because of this, it is important to select the correct objective lens on these types of instruments specifically for the sample you are analyzing to ensure accurate results for the particles being studied. Flow Imaging Microscopy is currently capable of counting and characterizing particles in the 300 nm - 5 mm range.



Dr. Cheng Her uses FlowCam at the University of Colorado Center for Pharmaceutical Biotechnology with Dr. John Carpenter.



A FlowCam sample of a biologic drug showing microspheres used for drug delivery alongside proteins and contaminant particles.

"Image quality is extremely important when characterizing protein aggregates. We need to be able to differentiate them from silicone oil and other contaminants in drug formulations early in the process. FlowCam allows us to do this quickly and easily."

-Dr. Jeff Schwegman, Founder and CEO
AB BioTechnologies, Bloomington, IN

CHAPTER THREE

A Closer Look at Flow Imaging Microscopy

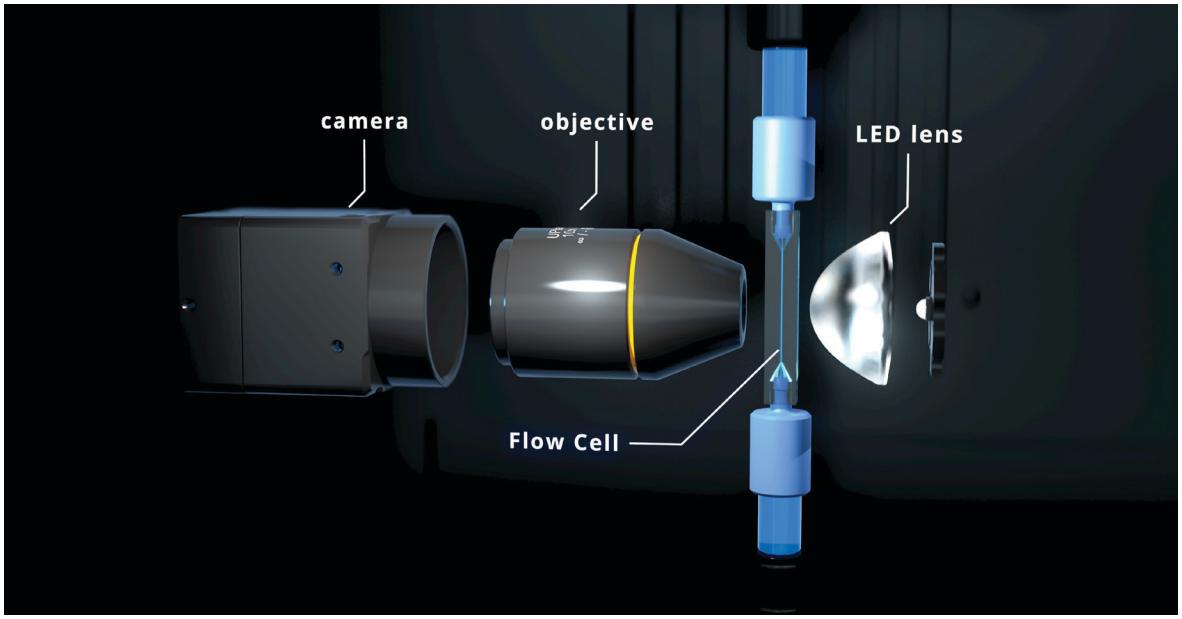
SECTION 3.1: HOW FLOW IMAGING MICROSCOPY WORKS

Flow imaging microscopy uses digital images to measure the size and shape of each particle in a sample. Essentially, the operator in classical microscopy is replaced by a computer that extracts the information from the images.

The sample containing the particles streams through the flow cell past the microscope optics. Thousands of particle images are captured per second.

To capture sharp images of moving particles, they are “frozen” in space using a strobed LED illumination source combined synchronously with a very short shutter speed.

As each frame of the camera’s field of view is captured, the software extracts the particle images from the background in real time and stores them.



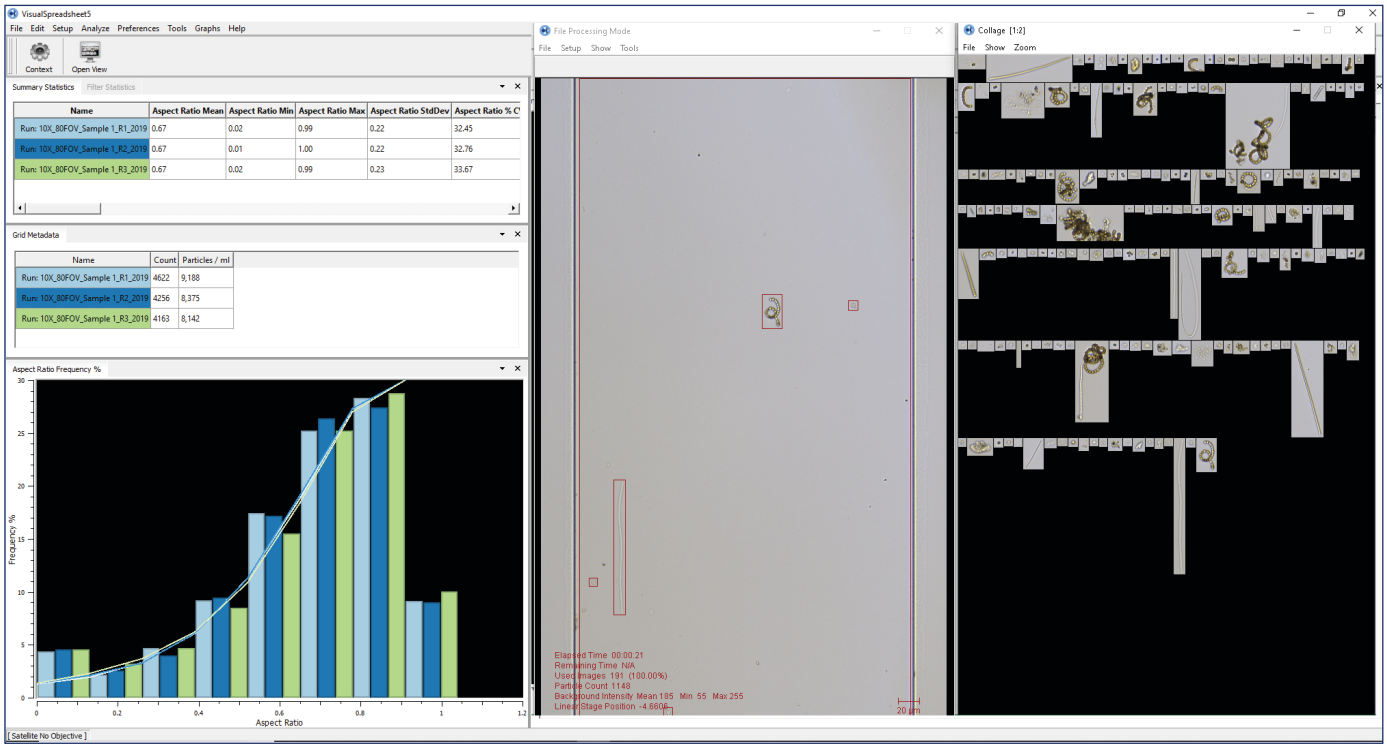
SECTION 3.2: DIRECT PARTICLE MEASUREMENTS

In an imaging-based system, particle measurements are made directly from the image of the particle. Since the system's optics are fixed and the magnification is known, distance measurements on the image can be directly converted to real distance measurements on the object.

No generalizations are made about a particle's shape. The user can also view the image to ensure that the data is being properly interpreted.

Common measurements include:

- Equivalent spherical diameter (ESD)
- Length, width, and aspect ratio
- Area and volume
- Circularity and elongation
- Edge gradient
- Intensity, average intensity, and sigma intensity
- Transparency
- and more (40+ morphological characteristics)



FlowCam screen during image capture. The middle window is the full field-of-view of camera on the flow cell. Red boxes indicate particles found. The right window is the 'collage window' of particle images that are saved and stored. The main window (left side) shows particle measurements summary graph and statistics that populate at the end of the sample run. The main window can be customized to show desired properties and display preferences.

SECTION 3.3: DATA PROCESSING - SORTING AND FILTERING MEASUREMENTS

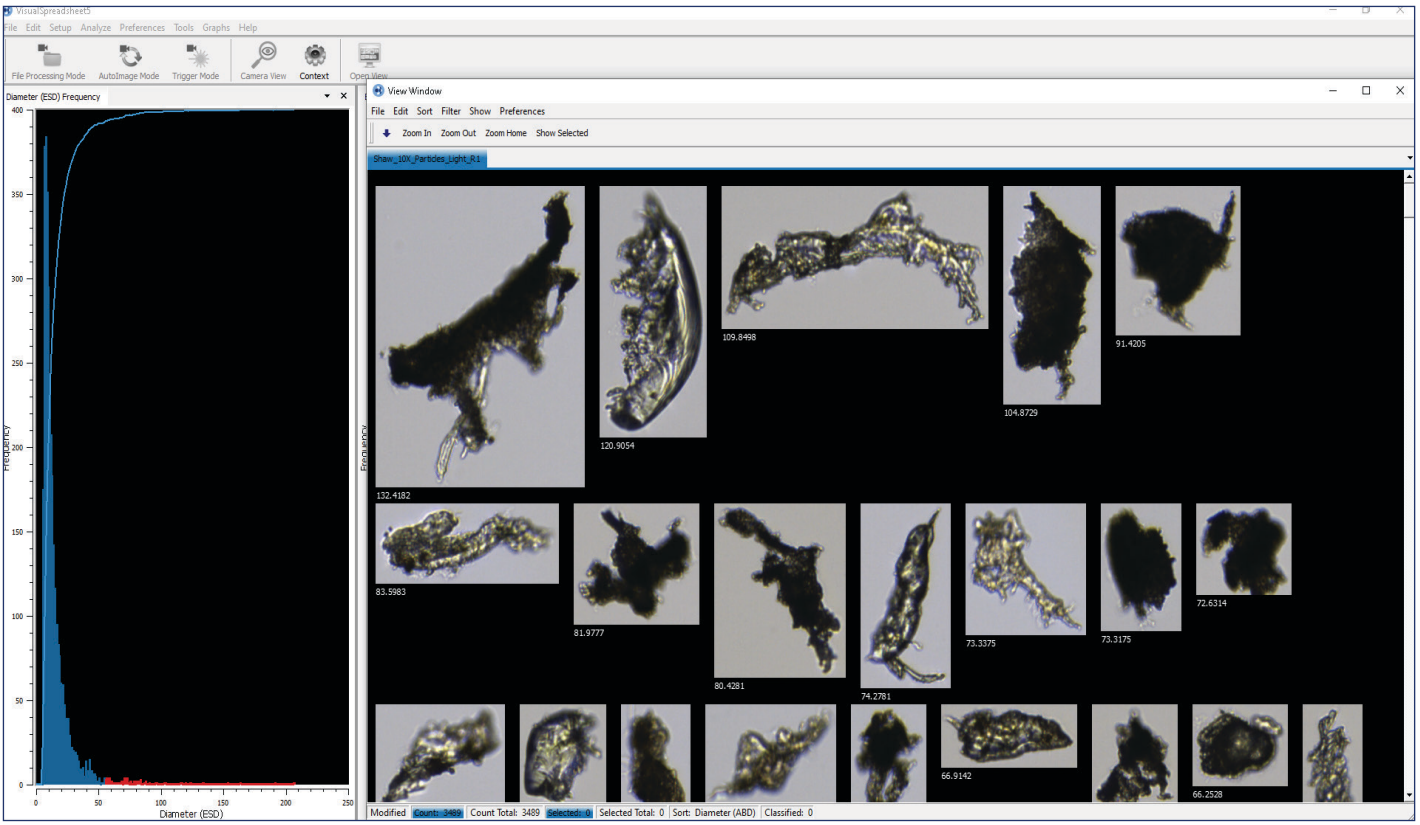
SORTING

The FlowCam system includes VisualSpreadsheet® software for both acquisition and analysis of data. VisualSpreadsheet provides the ability to sort and filter your data based on any of the measurements (or combination of measurements) acquired for the particles. The results are displayed as particle images as well as in a tabular format, and can also be exported to Excel.

The user can interact with the auto-generated scattergram to quickly select particles of interest from any of the configurable graphs.

"You can get more information from FlowCam than from any other type of instrument. Going to FlowCam with a particle problem is just the best feeling in the world because it turns data into useful information that you can use to solve a real problem."

-Dan Berdovich, Owner
Micromeasurement Laboratories, Inc.



VisualSpreadsheet interactive scattergram: only the largest particles have been selected from the histogram in the left window (red). The right window displays those particles, revealing that they are contaminants, not intrinsic particles.

FILTERING

You can also build filters based on particle properties with VisualSpreadsheet to automatically isolate particles of a particular type.

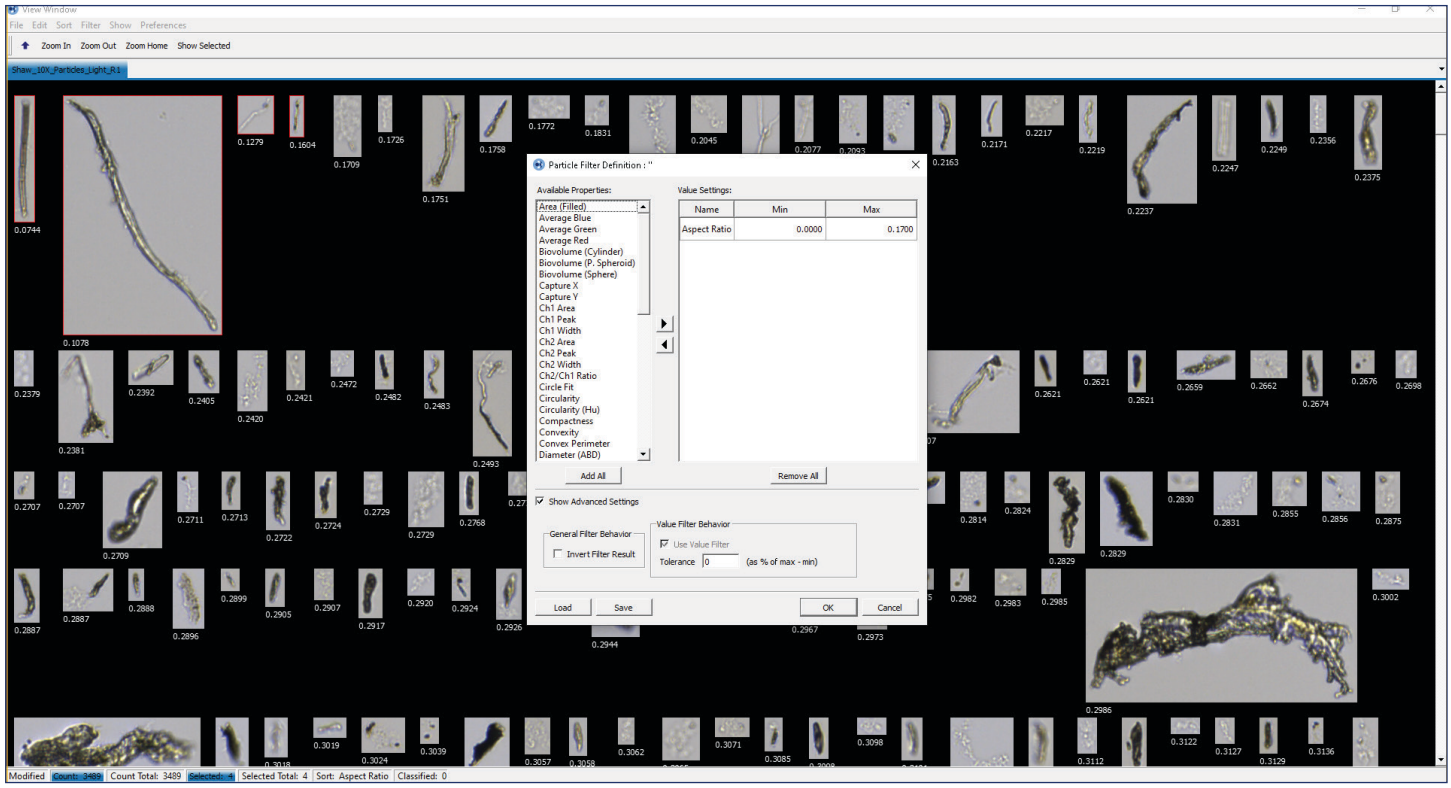
Filters can be created, saved, and applied to future runs, or in post-processing mode of past runs.

Value filters can be used to isolate particles within a specified range of any particle property. Statistical filters can be used to identify images similar to a population of user-selected images.

When analyzing protein therapeutics this is especially helpful when separating silicone oil from proteins.

"Data is one thing, but having instantaneous information is another...
FlowCam is an integral part of our screening process and enables us to quickly get the answers we need to drive the formulation development"

-Dr Jeff Schwegman, Founder and CEO
AB BioTechnologies, Bloomington, Indiana



A VisualSpreadsheet value filter was used to isolate "long and skinny" particles, limiting the display to only particles with an aspect ratio (width/length) from 0 to 0.25.

CHAPTER FOUR

Characterizing Particles in Protein Therapeutics with Flow Imaging Microscopy

SECTION 4.1: THE IMPORTANCE OF CHARACTERIZING SUBVISIBLE PARTICLES: USP <788> AND NEW FDA RECOMMENDATIONS

Particulates in parenteral drug development are a serious concern. In biopharmaceuticals the issue is compounded by reported impacts of particles such as protein aggregates on the product's efficacy, safety, and immunogenicity. To mitigate these risks, regulatory agencies recommend, if not require, companies to perform measurements to quantify and characterize these particles in biotherapeutics.

USP <788>

Characterization of sub-visible particles in parenterals was formally addressed by USP <788> in 1975. At the time of its implementation, USP <788> was primarily concerned with foreign matter, such as rubber stopper pieces and glass shards in small molecule therapeutics that might not be distributed through the blood system easily.

USP <788> states that sub-visible particles above 10 and 25 microns must be monitored and reported. Using flow imaging microscopy as an orthogonal method can help you establish the validity of your primary method (more on this in section 4.2).

NEW RECOMMENDATIONS FROM THE FDA

The FDA has recently shifted its attention to subvisible particles between 2 and 10 microns, due to an increase in issues arising from particles smaller than what the USP currently requires.

In order to inform method development and selection, risk assessment, and specification setting, the FDA now recommends characterizing the shape, type, and size distribution of all particles in this size range. In 2021, Flow Imaging Microscopy was added to USP <1788> as a strongly recommended orthogonal method to Light Obscuration. Providing particles images is recommended for any new or resubmissions.

"With FlowCam, the testing process is a whole lot easier, quicker, and more informative, in some ways, than the USP <788> testing. It's a question of taking 15 minutes compared to taking the better part of a day. This provides an estimated 10-fold savings in laboratory costs for Protein Sciences."

- David Rhodes, Formulation and Analytical Development Group, Protein Sciences, Meridan, CT

SECTION 4.2: THERAPEUTIC PROTEIN DATA ANALYSIS

Since flow imaging microscopy characterizes particles based on size and shape, you can easily differentiate between different particle types in your sample such as protein aggregates and silicone droplets. All the particles are captured as images and saved, so the results can be examined visually to ensure accuracy.

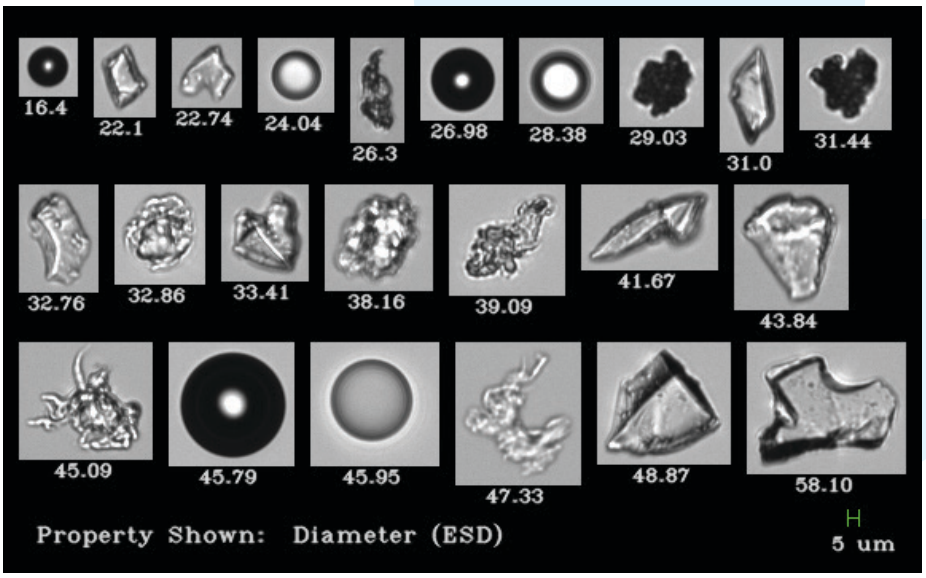
In one example, a parenteral formulation sample was analyzed using FlowCam. Basic particle filters were created for particles greater than 10 µm and greater than 25 µm.

The results showed 382 particles greater than 10 µm. When looking at the images, it was apparent that many of these particles were nominally harmless silicone droplets.

Using image analysis tools available in the base VisualSpreadsheet software, silicone droplets were isolated and eliminated from the analysis to determine a realistic number of protein aggregates.

Out of 382 particles originally detected, 195 were found to be silicone droplets, leaving 187 protein aggregates. This significant reduction is important because it could have been the difference between whether the batch was accepted or rejected in quality control.

The optional VisualAI™ software further simplifies this particle morphology analysis. VisualAI includes a powerful-yet-robust image analysis utility designed to automatically identify images of protein aggregates and silicone oil droplets in a sample and to determine the concentration of each particle type in a sample. VisualAI can also be used to flag images of some common contaminants and FlowCam artifacts such as calibration beads and air bubbles.



A VisualSpreadsheet collage file containing images of protein aggregates, glass shards, silicone oil droplets and various other intrinsic, extrinsic and inherent particles.

SECTION 4.3: FIVE IMPORTANT CONSIDERATIONS WHEN CHOOSING FLOW IMAGING MICROSCOPY

Here are five important elements that should be considered when exploring imaging particle analysis for characterization of protein therapeutics.

#1: IMAGE QUALITY

The ability of an imaging particle analysis system to properly identify and count protein aggregates is directly dependent on the quality, or sharpness, of the images. Blurry images lead to poor characterization of particles and can affect your particle size distribution.

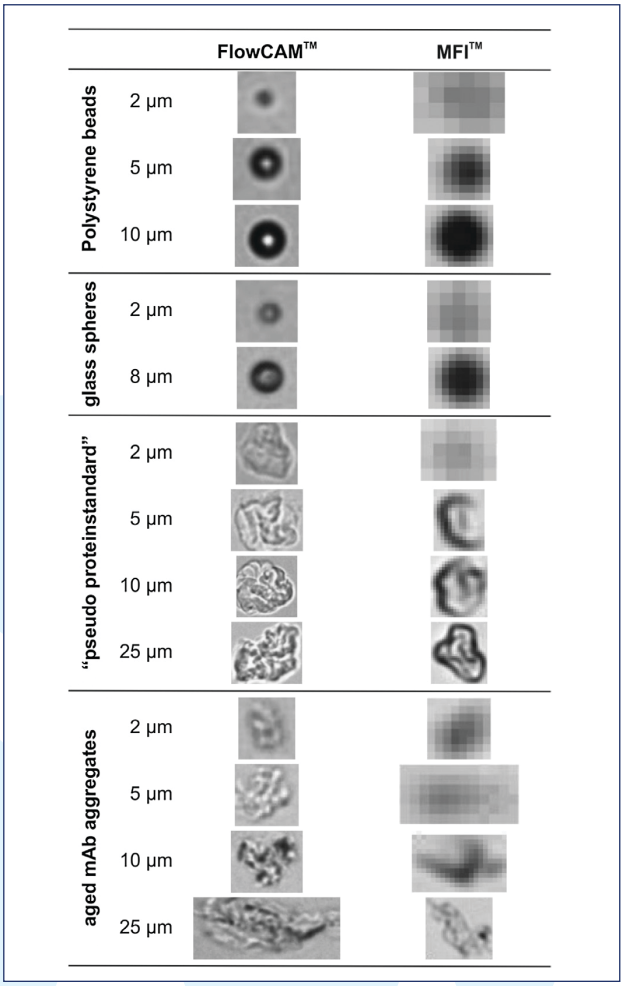
The better the overall image quality, the easier it is for the instrument's software to recognize patterns, and in turn characterize particles.

Blurry images make it difficult to distinguish protein aggregates from silicone droplets and other contaminants, especially in the 2 - 10 µm range. Poor image quality also makes it difficult for the instrument to discriminate between a protein agglomeration and several small proteins in close proximity to one another.

If particles are not characterized correctly, your size distribution may not be reliable. Smaller or faint particles (such as those with a low refractive index like proteins) may be missed entirely, lowering particle counts. Large particles may be fractionated into smaller particles - reducing the number of large particles while increasing the number of small particles.

#2: FLEXIBILITY

Not all formulations are alike – particles vary significantly from one formulation to another. Depending on the formulation, these differences may necessitate a modification to the image capture settings to ensure proper measurement. It is important to choose a system that can be configured to suit the specific formulation you are testing and achieve optimum results. This is especially true if your formulations contain stabilizers, which can affect the refractive index of the matrix.



Reprinted from European Journal of Pharmaceutical Sciences 53 (2014) 95–108, Werk, Tobias, Volkin, David B., Mahler, Hanns-Christian, Effect of solution properties on the counting and sizing of subvisible particle standards as measured by light obscuration and digital imaging methods, with permission from Elsevier.

Also, if a wide range of particles sizes is expected, an instrument may need to support multiple magnifications. For example, FlowCam 8100 provides interchangeable 2X, 4X, 10X, and 20X objectives, and utilizes fixed field of view flow cells in a variety of sizes.

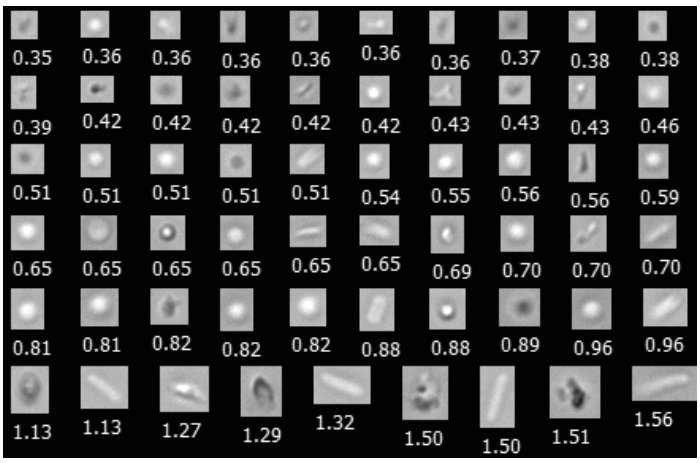
#3: INSTRUMENT SENSITIVITY

Many protein aggregates are translucent. This can make them difficult to detect with any particle analyzer because their refractive index is close to that of the matrix in which they are suspended.

In some systems, simple darker-only thresholding is used, which may cause transparent aggregates to be cut into smaller particle pieces or go undetected. This results in incorrect measurements and incorrect count or concentration calculations.

Having the ability to threshold on either darker pixels (relative to the background), lighter pixels or both simultaneously can ensure the proper characterization of protein aggregates, especially when working with high concentration formulations or those with a large amount of stabilizer.

Read more: The Benefits of Light and Dark Pixel Thresholding



#4: SAMPLE VOLUME REQUIRED

Since drug formulations can be expensive and in short supply, it is crucial to know the minimum sample volume required by your imaging particle analyzer. It is also important to understand how much sample will be needed (if any) to prime the system prior to measurement. Minimum sample volumes differ between types of imaging particle analyzers from 50 µL to 1 mL.

#5: EASE OF ANALYSIS AND DATA PROCESSING

Data is one thing – actionable information is another. Once your data is captured using a particle analysis system, it is important to have an easy way to analyze it. The quality of the software varies significantly from one type of particle analysis system to another. Choose one that allows you to efficiently interact with your data and extract information quickly and easily. Ideally with a single package for acquisition and analysis, which speeds up sample processing time.

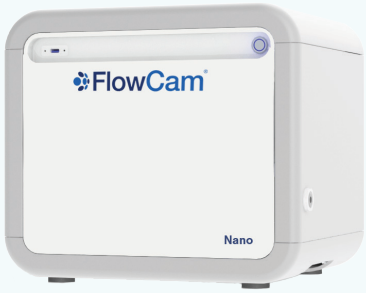
Some key software features include:

- Ability to sort and filter particle images based upon criteria you supply
- Sophisticated pattern recognition capabilities that immediately find and display all similar-type particles in a heterogeneous sample
- Support for creation of user-defined particle-type libraries to instantly enumerate concentration of specific particle types
- Satellite software for post-processing data at a remote location or sharing data with others

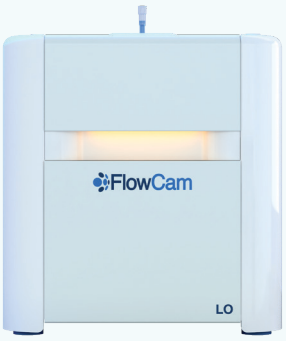
Pictured at left: A biopharmaceutical sample imaged by FlowCam Nano (particle diameter shown in µm)

Covering the Full Spectrum of Flow Imaging Technology

FlowCam 8000 Series
Particles 2 µm to 1 mm
multiple objectives



FlowCam Nano
Particles 300 nm to 2 µm



FlowCam LO
Obtain Flow Imaging and Light Obscuration data in one instrument

ALH for FlowCam™
Integrates with 8000 series and FlowCam LO for high-throughput processing

VisualSpreadsheet Software
FlowCam's image analysis software

Want to Learn More?

Visit the Resources page on our website to watch videos, download posters, papers, and more.

Don't see your specific application?
Have additional questions?
Wondering if the FlowCam will work for you?

Send an email to contact@fluidimaging.com

ABOUT YOKOGAWA FLUID IMAGING TECHNOLOGIES

Yokogawa Fluid Imaging Technologies, Inc., manufactures industry-leading particle analysis instrumentation based on digital imaging technology. Our flagship product, FlowCam, is the first automated particle analysis instrument to use digital imaging for measuring size and shape of microscopic particles in a fluid medium.

With applications in marine & freshwater research, biopharmaceutical research & development, municipal water, industrial manufacturing, and many other markets, Yokogawa Fluid Imaging Technologies leads the way in imaging particle analysis.

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