The Ultimate Guide to Flow Imaging Microscopy for Protein Therapeutics
CONTENTS

1. What is particle analysis? ................................................................. 4

2. A History of Particle Analysis .......................................................... 5
   2.1 Microscopy .................................................................................. 5
   2.2 Volumetric Particle Analysis Techniques .................................. 6
   2.3 Flow Imaging Microscopy ......................................................... 8

3. A Closer Look at Flow Imaging Microscopy ................................. 10
   3.1 How Flow Imaging Microscopy (FIM) Works ......................... 10
   3.2 Direct Particle Measurements ............................................... 11
   3.3 Data Processing: Sorting & Filtering ...................................... 12

4. Characterizing Particles in Protein Therapeutics with FIM ......... 14
   4.1 USP <788> and New FDA Regulations ................................. 14
   4.2 Therapeutic Protein Data Analysis ......................................... 16
   4.3 Five Important Considerations When Considering FIM .......... 17
The Food & Drug Administration (FDA) strongly recommends in-depth characterization of particles in protein therapeutics. Flow Imaging Microscopy (FIM), is increasingly being utilized because it can provide incredible insight to your formulation, allowing you to see your particles like never before, especially in the 2 to 10 micron range. And now with new instrumentation, Nano-Flow Imaging™ is available in the 300 nm to 10+ µm range as well.

In this e-book we’ll briefly review methods for analyzing particles as well as the advantages and drawbacks of each. Then we’ll take a deeper look at flow imaging microscopy and how it works. Finally, we’ll focus on how FIM helps improve characterization of proteins, silicone droplets, and other particles in biologic formulations.

Enjoy!
Sample analysis involves looking at a representative portion of a substance (particle) and separating it into its component parts (analysis). The basic goal is to determine the constituents of a mixture.

Typical measurements of particles that are of interest to scientists and protein formulation developers include:

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

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 value based on the volume of the particle.

While seemingly straightforward for particle size, when shape and/or morphological data is needed, a more in-depth analysis is required to truly characterize a particle.

A standard particle distribution graph is often plotted as a bell curve. 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.
CHAPTER TWO
A History of Particle Analysis

SECTION 2.1: MICROSCOPY

The introduction of the microscope in the 1600’s 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 sub-visible 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 microscopes 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 difficult to get results that are statistically significant using a microscope. 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 individual 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 sample.”

-Ross Clark, Distinguished Research Fellow at CP Kelso, a leading producer of specialty hydrocolloids
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.

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 assume that all particles are spheres. That means these methods are limited to particle counting and distribution only.

Most protein therapeutics contain a variety of particles, including proteins, silicone oil droplets, and contaminants (like glass shards) that can have a variety of shapes. Volumetric techniques are unable to characterize different particle types in a mixture due to the assumption that all particles are spheres.

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“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.”

From Safety Snapshot: An imaging particle analyzer can give researchers a better picture of particles in parenteral formulations. Written by Greg J. Morrone and Wasfi Al-Azzam. Published in Drug Discovery & Development (Vol 14 no. 5)
SECTION 2.3: FLOW IMAGING MICROSCOPY

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

- Examines a fluid under 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 images quickly enough to produce statistically significant results. Additionally, many different 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 a more in-depth analysis of your sample and a better understanding of your data.

“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. The FlowCam allows us to do this quickly and easily.”

-Dr. Jeff Schwegman, Founder and CEO, AB BioTechnologies, Bloomington, IN
Dan Berdovich uses the FlowCam particle analysis system at his contract laboratory - Micro Measurement Laboratories, Inc. where he leads a team that specializes in particulate matter testing, particle identification, method validation, and visual inspection standards development for pharmaceutical and biotechnology companies worldwide.

LIMITATIONS OF FLOW IMAGING MICROSCOPY

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

Because of this, counting should be limited to particles having an ESD of 1 μm and greater, and particle characterization (i.e. shape) should be limited to particles having an ESD of 300 nm and greater.

It is important to optimize the settings on these types of instruments specifically for the sample you are analyzing to ensure accurate results.

“You can get more information from the FlowCam than from any other type of instrument. Going to the 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 of Micromeasurement Laboratories, Inc.
SECTION 3.1: HOW FLOW IMAGING MICROSCOPY WORKS

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

The sample containing the particles streams by the microscope optics in the flow cell (which replaces the microscope slide), and thousands of particle images are captured per second.

In order to freeze the moving particles in space, a strobed illumination source is combined synchronously with a very short shutter speed in the camera.

As each frame of the field of view is captured, the software, in real-time, extracts the particle images from the background and stores them.
SECTION 3.2: DIRECT PARTICLE MEASUREMENTS

In an imaging-based system, particle measurements are made directly from an 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 assumptions are made about a particle’s shape, while multiple measurements are made for each particle. Plus, you can 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

FlowCam screen during image capture. Upper right window is full field-of-view of camera on the flow cell. Red boxes indicate particles found. Lower right window is the ‘collage window’ of particle images that are saved and stored. Main window (left side) shows particle measurements summary graph and statistics saved.
SECTION 3.3: DATA PROCESSING - SORTING AND FILTERING MEASUREMENTS

SORTING

The FlowCam system includes VisualSpreadsheet software, which 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.

You can interact with the scattergram created to quickly select particles of interest from any of the configurable graphs.

VisualSpreadsheet interactive scattergram feature: 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 aggregates rather than large, single particles.
FILTERING

You can also build filters with VisualSpreadsheet to isolate particles of a particular type in the sample automatically.

Multiple filters can be created, saved, and reused, which allow you to separate a sample into its component parts based upon particle properties.

One filter type is a value filter. For example, you can choose particles within a specific size range that are long and skinny. The other type is a statistical filter, where you can click on several images that are of a particular type, and the software finds images similar to those selected.

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

"Data is one thing, but having instantaneous information is another... The 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

VisualSpreadsheet filtering was used to isolate “long and skinny” particles. A filter was applied to limit the display to only particles with an aspect ratio (width/length) from 0 to 0.25. These images are shown on the right.
Particulates in parenteral drug development are a serious issue. In biopharmaceuticals the issue is compounded by reported impacts of protein aggregates and particles on the product’s efficacy, safety, and immunogenicity. Therefore strengthening requirements for the in-depth characterization of the identity and quantity of particles in protein therapeutics is currently being discussed throughout the industry and regulatory agencies.

**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 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. While the compendial methods are light obscuration and microscopy, as previously mentioned in Chapter 2, both of these methods have limitations that may impact your results.

Using flow imaging microscopy as an orthogonal method can help you establish the validity of your primary method (more on this in section 2).

**NEW RECOMMENDATIONS FROM THE FDA**

Based on increasing awareness of issues that may arise from particles smaller than what USP <788> currently includes, the FDA is now requesting that sub-visible particles between 2 – 10 microns be characterized to inform method development and selection, risk assessment, and specification setting. This includes characterizing the particle’s shape, type (i.e. protein, silicone oil, etc), and size distribution. Providing particle images is also suggested.

Flow imaging microscopy is uniquely suited to address these new recommendations.

"With the 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 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 this example, a parenteral formulation sample is analyzed by the FlowCam. Basic particle filters are created for particles greater than 10 µm and greater than 25 µm.

The below results show particles greater than 10 µm - a total of 382 particles were found. When looking at the images, it is apparent that many of these particles are actually silicone droplets. Silicone droplets are introduced during the filling process, but are not harmful, so they are allowable.

Using VisualSpreadsheet®, 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.

FlowCam results for a protein therapeutic sample filtered to show particles greater than 10 microns - a significant portion of which are silicone oil droplets.
#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 single protein amalgamation from 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.

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#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.

Also, if a wide range of particles sizes is expected, an instrument may need to support multiple magnifications. For example, the FlowCam® 8000 provides interchangeable 2X, 4X, 10X, and 20X objectives, and can utilize both disposable flow cells and 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 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.

Learn more in our poster, The Importance of Thresholding in Imaging Analysis of Protein Aggregates. Click link above or go to http://hub.am/1oI46Yv

#4: SAMPLE VOLUME REQUIRED

Since drug formulations can be expensive and in short supply, it is crucial to know what is 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.

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.**

**Want to Learn More?**

The benefits of flow imaging particle analysis are examined in Protein Aggregation and Emerging Tools to Support Development and Characterization, a recent presentation by Gilead’s Danny K. Chou, PharmD, PhD.

[Download Presentation >>](http://hub.am/1mRstWW)

You can also find more resources, including videos, articles, and technical posters on our website.

[Go to FlowCam® Protein Aggregate Application >>](#)

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**Covering the Full Spectrum of Flow Imaging Technology**

- **FlowCam 8000 Series**
  - 2 µm to 5 mm
- **FlowCam ALH**
  - Integrates with 8000 series for hi-throughput processing
- **FlowCam Nano**
  - 300 nm to 10+ µm
ABOUT FLUID IMAGING TECHNOLOGIES

Fluid Imaging Technologies, Inc., manufactures industry-leading particle analysis instrumentation based on digital imaging technology. Our flagship product, the 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, chemicals, oil & gas, biofuels, and many other markets, Fluid Imaging Technologies leads the way in imaging particle analysis.