YOKOGAWA FLUID IMAGING TECHNOLOGIES E-BOOK

The Ultimate Guide to Flow Imaging Microscopy

FlowCam®
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5. A Suite of Flow Imaging Microscopes
OVERVIEW

In this e-book, 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 is an advantageous technology for analysis of algae, protein therapeutics, the microencapsulation process, heterogeneous samples, and particle uniformity in column packing materials.
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 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.
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 difficult 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 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.

Target Particle ≈ 3 µm ESD


The Coulter Counter, shown here counting cells in solution, is an indirect volumetric particle analysis method.
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 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 of all particles being spherical.

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, 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.”

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)
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.

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.
SECTION 3.1: HOW FLOW IMAGING MICROSCOPY WORKS

Flow imaging microscopy uses digital image 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 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 illumination source combined synchronously with a very short shutter speed.

As each frame of the camera’s 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 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. 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

“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

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, and can also be exported to Excel.

You 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 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.

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 based on particle properties with VisualSpreadsheet to isolate particles of a particular type in the sample automatically.

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 by a specified size range of a certain particle property. Statistical filters can be used to identify images similar to a population of user-selected images.

“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 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 µm.
CHAPTER FOUR
Flow Imaging Microscopy Applications Overview

SECTION 4.1: IDENTIFICATION OF AQUATIC MICROORGANISMS

SECTION 4.2: DETECTION OF PROTEIN AGGREGATES IN PARENTERAL DRUG FORMULATIONS

SECTION 4.3: MICROENCAPSULATION PROCESS ANALYSIS

SECTION 4.4: PARTICLE DIFFERENTIATION IN A HETEROGENEOUS SAMPLE — WASH WATER

SECTION 4.5: OUTLIER CHARACTERIZATION — COLUMN PACKING MATERIAL QUALITY CONTROL
SECTION 4.1: IDENTIFICATION OF AQUATIC MICROORGANISMS

The unique advantage of FlowCam® is that it relies primarily on morphological and grayscale discrimination of algal cells as opposed to spectral discrimination typical of a flow cytometer.

With the fluorescence capabilities of FlowCam, spectral data can be used along with the morphological data to further discriminate algal cells. This overcomes a significant limitation of flow cytometers, which only provide relative particle size, usually expressed as ESD.

In this example, a sample of mixed algae cultures was analyzed with the FlowCam in “trigger mode”, meaning an image was only captured when a fluorescence event occurred. In a sparse sample, typical of ocean or lake water, trigger mode ensures that all particles of interest (living algae) are captured, while non-fluorescing particles such as detritus or sediment pass without being captured.

Using user-defined libraries of algal cell images, the statistical pattern recognition of VisualSpreadsheet® automatically classified the different types of microorganisms found in the sample.

Even though this sample contained green algae of a similar size, subtle differences in morphology were successfully used to separate them into the appropriate taxa. This ability to isolate different species is something a flow cytometer could not achieve - they would all be just green algae.
Particulates in parenteral drug development have always been a serious issue. In biopharmaceuticals the issue is compounded by reported impacts of aggregates and particles on the product’s efficacy, safety, and immunogenicity. FDA regulations strongly recommend in-depth characterization of the identity and quantity of particles in protein therapeutics.

In this example, a parenteral formulation sample was analyzed by the FlowCam®. A total of 382 particles greater than 10 µm were found. When reviewing the images, it was apparent that many of these particles were actually silicone oil droplets. Silicone droplets are introduced during the filling process but are not harmful, so they are allowable.

Using VisualSpreadsheet®, silicone oil droplets were eliminated from the statistical analysis to determine a realistic number of particles of concern, like protein aggregates.

Out of the 382 particles originally detected, 195 were found to be silicone droplets, leaving 187 that are actual protein aggregates. This represented a significant change in the number of particles found that are greater than 10 µm.

This was important because it could have been the difference between whether the batch was accepted or rejected in quality control.
SECTION 4.3: MICROENCAPSULATION PROCESS ANALYSIS

Microencapsulation is a process by which small amounts of a substance (an active ingredient) are packaged inside a second substance to shield the active ingredient from the surrounding environment. The process is used extensively for delivering particles in a wide range of applications, from pharmaceuticals to foods to detergents.

Flow Imaging Microscopy provides unique insight into the microencapsulation process. While studying the effects of temperature, concentration, pH or other variables that affect the process, you can monitor capsule formation in real time, optimizing your process and ensuring quality.

In this example, the FlowCam was used to monitor coacervate formation in a test vat as the sample cooled under constant agitation.

Samples were collected and analyzed every 15-30 minutes. Visual examination of particle images and statistical pattern recognition analysis confirmed that at $t_0 + 39$ minutes the most clean coacervates were formed. After this point, the gelatin began to attach itself to the capsule walls, causing agglomeration and eventually disintegration of the coacervates.

The FlowCam yields tremendous insight into the process of coacervate formation, and can be an indispensable tool for microencapsulation research & development and quality control applications.
The FlowCam excels at analyzing heterogeneous samples, where multiple particle types are present. In these situations, imaging particle analysis and pattern recognition techniques can provide an automated method for characterizing the types and quantities of particles present.

In this example, a wash water sample from a manufacturing process for electronic devices was analyzed. These devices are washed to remove traces of fibers, metals and plastics from the manufacturing process. It is important that the wash water contains less than a certain number of each of these particle types, as too many particles could cause failures. Additionally, the types and quantity of particles present in the wash water serves as an indicator for any problems arising in the production process.

Analysis of this sample revealed a diversity of particle types: long, skinny fiber particles, semi-transparent metal shavings, and more opaque plastic particles.

A library for each particle type was built based on particle characteristics, and each subsequent run was automatically filtered into the different particle types. The corresponding volume percent, particles/mL, and PPM was simultaneously calculated.

The FlowCam allows the user to quickly determine if there is an issue with their manufacturing process in real-time and make the necessary adjustments.
SECTION 4.5: OUTLIER CHARACTERIZATION - COLUMN PACKING MATERIAL QUALITY CONTROL

Digital image analysis with FlowCam provides critical size and shape information on column packing materials. This allows for tighter column density control, and in turn, better control of column performance. It also helps in the tracing of the damaged (non-spherical) particles that are often present in different lots of packing material.

In this example, a polymer resin sample was analyzed by the FlowCam. The frequency and volume distributions shows a typical Gaussian size distribution with a mean equivalent spherical diameter (ESD) of 60.80 µm (upper two graphs). The aspect ratio scattergram (lower left graph), however, indicates that the particles are not uniform in shape.

Using VisualSpreadsheet, acceptable, spherical particles were separated from unacceptable, irregularly-shaped particles. Libraries were created for each particle type.

Even though all of the particles had similar ESDs, particle images were necessary to determine the quality of the particles present and assess whether they could be misshapen beads, pieces of broken beads, or contamination.

In this case, the FlowCam characterized 9,261 particles automatically in a little over a minute, and was used to determine if this batch of polymer resin met the necessary quality specifications.
5.0 Covering the Full Spectrum of Flow Imaging Technology

FlowCam 8000 Series
Particles 2 µm to 1 mm
multiple objectives

FlowCam Cyano
Distinguish between
cyanobacteria and other algae

FlowCam Nano
Particles 300 nm to 10 µm

FlowCam Macro
Particles 300 µm to 5 mm

FlowCam 5000
Particles 2 µm to 1 mm
single objective

FlowCam ALH
Integrates with 8000 series for
high-throughput processing

VisualSpreadsheet Software
FlowCam’s image analysis software

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

Send us an email to contact@fluidimaging.com and we can arrange to run one of your samples for free.
ABOUT YOKOGAWA FLUID IMAGING TECHNOLOGIES
Yokogawa 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, Yokogawa Fluid Imaging Technologies leads the way in imaging particle analysis.