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▲⟨1788.3⟩ FLOW IMAGING METHOD FOR THE DETERMINATION OF SUBVISIBLE PARTICULATE MATTER

1. INTRODUCTION

Flow imaging (FI) is useful for an approximate particle range from 2 to 100 µm, depending on the instrument and its configuration, covering particle sizes from the accepted subvisible size range to hundreds of micrometers (see *Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨1787⟩ and *Methods for the Determination of Subvisible Particulate Matter* ⟨1788⟩). Specialized instruments or configurations can cover expanded size ranges. FI instruments provides both individual microscopic images of observed particles and reports of particle size distribution (PSD) and particle concentration at selected size thresholds. Values of morphological parameters and sample images corresponding to the stated size bins or thresholds may be added to the report. Particle size can be reported as an equivalent circular diameter [similar to light obscuration (LO)] or based on Feret's diameter, which captures some information about the shape. Algorithms categorizing particles are usually based on optical density and morphological parameters such as circularity, allowing them to differentiate to some degree between elongated fibers, dense symmetrical air bubbles, circular silicone oil droplets, and amorphous protein particles, etc.

This is an essential characterization method that is often used as an orthogonal method to the compendial LO and membrane microscope (MM) methods, especially during development. This method is described, along with other orthogonal techniques, in ⟨1787⟩. Because of its increasing use and importance as a technique to size, count, and bin by morphology, more details on how to optimize the application of this technology are provided here.

2. TEST APPARATUS

2.1 Principles of Operation

The principles of operation and the strengths and weaknesses of FI are discussed in ⟨1787⟩. Briefly, the apparatus is a liquid-borne particle counting and imaging system using a sample-feeding device to deliver controlled aliquots of liquid for analysis. The samples flow through an

optical cell positioned between a light source and camera, allowing the capture of a digital stream of particle images present in the sample.

Image analysis of the captured image frames can provide necessary information. Fundamentally, information may be obtained about particle size and number within a size range from about 2 to 100 µm (exact size range and larger size ranges will depend on the instrument, cell size, and magnification). Other parameters such as shape, contrast, and transparency of the particles allow for the evaluation of the particle population.

FI combines the benefits of in situ observation, as in LO, with the ability to qualitatively evaluate some characteristics of the particles, as in MM. Air bubbles and immiscible liquids are detected because imaging is performed on flowing liquid samples. With software algorithms, these common artifacts may be discriminated from "real" particles. The effectiveness of particle discrimination is a function of magnification, resolution, software algorithms, and particle properties.

Therefore, product fluids with air bubbles and immiscible liquid droplets (e.g., silicone oil), which are not discriminated from other types of particles when using LO analysis, are imaged by FI and can be discriminated through software processing. It is the responsibility of those performing the test to ensure that the operating parameters of the instrumentation meet the required accuracy and precision, that artifacts and interferences observed in certain products and preparation methods are eliminated or accommodated, and that the algorithm is assigning particle type correctly.

High-resolution optics have a small depth of field relative to optical configurations with lower resolution. Thus, for typical instruments where the particle suspension travels through a flow cell of approximately 100-µm thickness, there is a tradeoff between ensuring that all particles remain in focus versus obtaining high-resolution images. It may be appropriate to obtain high-quality images with different hardware settings than typically required to obtain an accurate particle count.

FI instruments vary in the flexibility and range of adjustment of instrument hardware. Some instruments will have user-selectable flow cells, objectives, and illumination. Other instruments will have predominantly fixed settings. It is the user's responsibility to determine if the optical configuration of a particular instrument, whether fixed or variable, serves the stated purpose of the measurement.

It is important to note that for compendial applications, the ultimate goal is that the imaging instrument accurately size and count the particles present. FI enables classification of particles according to particle image properties (e.g., aspect ratio, brightness, transparency, and circularity), which can assist the user in understanding the nature of particle subpopulations and in diagnosing possible particle count interferences (e.g., as air bubbles). Establishing an image library will aid in the categorization of particulate unknowns and could be part of risk assessment. The instruments that are available range from manual calibration and standardization systems to sophisticated hardware- and software-based standardization systems. Thus, it is not possible to specify the exact methods to be followed for instrument standardization, and it is necessary to emphasize the required end result of a standardization procedure rather than a specific method for obtaining this result. This chapter is intended to emphasize the criteria that must be met by a system rather than specific methods to be used in their determination. It is the responsibility of the user to apply the various methods of standardization applicable to a specific instrument. Indeed, no single method will apply to all experiments. Factors such as the desired range of detection, formulation characteristics, available sample volumes, and nature of the particulate matter are typical considerations for the method used. Validation concepts for a limits test in *Validation of Compendial Procedures* ⟨1225⟩ provide sufficient guidance for these purposes.

3. INSTRUMENTATION STANDARDIZATION TEST

3.1 Concentration Calibration

The particle concentration reported by a FI instrument is based on the total number of particles measured, within a specified range of diameters, divided by the total volume of fluid that has been imaged. Each camera image records particles within a volume equal to the imaged area multiplied by the interior thickness of the flow cell. Consequently, the imaged sample volume requires accurate knowledge of the flow cell thickness but does not depend directly on the liquid flow rate through the cell. Because direct measurement of flow cell inner dimension is difficult for some types of flow cell, the thickness is often inferred by measuring the particle concentration of a suspension of microspheres of certified concentration. Calibration procedures are given in the *Appendix*.

3.2 Sample Flow Rate

The flow rate through the cell should be consistent with the manufacturer's recommendations in order to ensure that the instrument is functioning properly. This may be accomplished by weighing the amount of liquid passed through the system at a specified rate, using a calibrated stopwatch to measure the elapsed time. Instruments may be operated accurately over a range of flow rates. Perform *7. Test Procedure* at the same flow rate as that selected for calibration of the instrument.

3.3 Diameter Calibration

Refer to ⟨1788⟩ for general guidance on diameter calibration. A microscopic particle image depicts the projected area of a particle. From this area, a variety of effective diameters may be obtained, depending on the instrument model and software settings. Instruments should be calibrated so that the reported effective diameter for polystyrene (PS) microspheres equals the actual microsphere diameter. Calibration procedures are given in the *Appendix*. Calibration of an instrument with PS microspheres may result in incorrect reported diameters for particles with low optical contrast. Refer to *Diameter Bias* for guidance in this circumstance.

3.4 Sensor Performance or Resolution

The particle size resolution of the instrumental particle counter is dependent upon the instrument model, flow cell, and hardware and software settings. Poor particle size resolution will lead to broadening of the PSD, which can lead to errors in reported particle concentrations. A procedure for determining instrument resolution is given in the *Appendix*.

3.5 Qualification Considerations

Qualification of the instrument should be based on frequency of use, maintenance schedule and accuracy requirements. Specific qualification considerations are similar to those for LO. Critical operational criteria consist of the following:

- *Integrated microscopy and fluid handling*—The fluidics system, microscope, and instrument control should be designed to provide repeatable imaging of particles, in a known fluid volume for each frame, at a controlled sample flow rate.
- *Instrument dynamic range*—The dynamic range of the instrument used (i.e., the range of particle sizes that can be accurately sized and counted) must include the smallest particle size to be enumerated in the product.
- *Automated particle identification*—Particles should be identified with image threshold settings suitable for the detection of protein aggregates or other species of interest. The system should support classification of particles by size, morphology, and image intensity parameters. Agreement with visual analysis of the images should be verified by a skilled operator.

Reliable and accurate measurements require attention to several stages in the measurement process. Key quality activities include:

- Initial instrument qualification
- Method development
- Periodic operational checks
- Routine running of blanks and inspection of data
- Instrument maintenance

INITIAL QUALIFICATION

All methods should be qualified for performance, considering the guidance in ⟨1225⟩. Specifically, volume accuracy, standards for particle size, particle count accuracy, and a determination of image resolution should be included in qualification.

METHOD DEVELOPMENT

Adsorption: Compared to PS microspheres and other inert particles, some drug substances (e.g., proteins) are much more likely to adsorb onto the walls of the fluidic system. The degree of adsorption can vary with the nature of the formulation, the nature of the particulate matter, and the active pharmaceutical ingredient (API). To assess adsorption, run different prime or rinse volumes and check for variations in particle concentration. Run buffer blanks after the completion of a run and observe the particle counts in the blanks as a function of blank volume.

Diameter bias: Estimates of diameter bias may be corrected by adding an appropriate correction (which may be positive or negative) to the measured diameter of each particle, then recalculating the PSD. Diameter bias may be assessed by measuring monodisperse particles of a known size or polydisperse particles of known PSD provided that the chosen particles have optical properties similar to those in the test sample. Refer to ⟨1788⟩, *11.3 Validation Considerations for All Techniques* for further guidance. Consult manufacturer's recommendations for additional information and specific guidance.

The apparent diameter of a particle is affected by the following:

- The choice of threshold
- The degree to which the particle is out of focus
- Diffraction effects
- The ability of the instrument software to identify adjacent, low-contrast regions as a single particle
- Alignment of elongated or platelike particles in the flowing fluid

If the incorrect diameter is reported, PSD and particle concentration for affected size bins will be inaccurate. Concentration errors are most prevalent for small particles near the lower limit of the instrument's resolution and those that are difficult for the instrument to resolve (e.g., glass lamellae or protein aggregates in high-concentration formulations).

Opalescence: Particles may be obscured in samples that are highly opalescent. To assess the visibility of particles, spike microspheres of known diameter and concentration into a test sample, measure the microsphere diameter and concentration, and compare to the diameter and concentration expected. The choice of microsphere for this analysis should have a similar refractive index as the particles of interest in the test sample (e.g., silica microspheres could be used to mimic lower-contrast protein aggregates; PS could be used to mimic higher-contrast particles).

Viscosity: The low volumetric flow rate of FI instruments makes them generally insensitive to typical fluid viscosities. Confirm that no bubbles are introduced on priming or running at standard speed. For systems that use a peristaltic pump, confirm that the delivered volume is correct if the sample viscosity differs substantially from the viscosity of water. Reduce volumetric flow rates if necessary.

Stuck particles: Stuck particles may be observed during the actual data collection or in post-collection analysis. If particles or air bubbles become temporarily affixed in the detection zone of the sample cell, the data around the affixed particle should be eliminated using software recognition to avoid overcounting particles; if this is not possible, the entire data set should be discarded and the sample cell should be changed.

Schlieren effects: Inhomogeneities in solvent refractive index due to mixing (typically when water or buffer is mixed with a high-concentration protein suspension or cold solvent) lead to localized variance in the background intensity, which can impact analysis. Schlieren effects are often more pronounced when the sample viscosity is high relative to buffer or water. These inhomogeneities should be removed from the flow channel before the system background is recorded or data is collected. Priming with a sufficient volume of the

sample to be measured, or with an equivalent solution, to reach the equivalent refractive index and viscosity of the test samples prior to its injection can remove such artifacts.

Particle classification: After data has been acquired, many FI systems enable the sorting of images by size, image intensity attributes, and morphological image attributes. This type of classification can be very useful in distinguishing silicone oil or air bubbles from other types of particles. However, care must be taken to ensure that the classification algorithms work as intended. Studies of different types of particles can be used to generate reference libraries, and additional measurements combining known populations of particles can be used to confirm the performance of the algorithms. As mentioned above, the "binning" should be verified by a subject matter expert to ensure the algorithm differentiates populations as expected.

Software settings: Instruments will differ in the degree to which software settings can be altered. The manufacturer should be consulted for recommended instrument settings for the type of samples being investigated. Common settings may include:

- Prime and sample volumes
- Flow rate through the cell
- Illumination intensity
- Particle identification threshold
- Image processing settings (e.g., closing holes in the binary image)

Hardware setup: Instruments will differ in the degree to which hardware settings can be altered. The manufacturer should be consulted for recommended instrument settings for the type of samples being investigated. Common settings may include:

- Magnification
- Collimating lens
- Cell inner dimensions
- Sample delivery and mixing

There is a tradeoff between high optical resolution (obtained using high-magnification objectives with a high-numerical aperture) and large depth of field (obtained using objectives with low-numerical aperture). Large depth of field is necessary for accurate particle counts, but high resolution may be necessary for detection of low-contrast particles and accurate classification. Care must be taken that the choice of magnification and cell is compatible with the desired purpose of the test. For an instrument where sheath flow is used, throughput is less, but all of the particles are within the depth of field.

Fouling: Test samples with high particle loads or with particle sizes approaching the flow cell thickness may cause fouling of the cell entrance. Often, the flow path will only be partially clogged such that the liquid will continue to flow through the cell and small-size particles will still be observed. Evidence of partial fouling is typically a reduction in the observed concentration of larger size particles, a variation in count rate during a run, or visual observation of contaminants near the entrance of the flow cell. If fouling is observed, it may be helpful to backflush the flow cell to remove the clog, and then reconfirm the count accuracy using larger-size PS microspheres (e.g., 25 µm). For the best result, alternate liquid and air in the fluid line when flushing to change samples or to clean the flow cell.

SYSTEM SUITABILITY

Instrument configuration: Verify appropriate instrument hardware and software settings and record them as appropriate (see *Analytical Instrument Qualification* ⟨1058⟩).

Blanks: Run filtered water as needed throughout the day to ensure cleanliness of the system. The measured concentration for a well-cleaned system is typically <60 particles ≥5 µm per milliliter. Some debris inside the instrument may be loosened and introduced into the count when exposed to surfactant solutions. Additional runs with filtered buffer or filtered test sample may be useful to confirm that carryover from the previous sample is acceptably low.

Prior to testing blanks, degas according to the guidance in ⟨1788⟩.

Size and concentration standards: It is prudent to verify system performance often according to the guidance in ⟨1788⟩. Failure to reproduce the size and/or count reference values can often be traced to a clogged or dirty flow cell, compromised tubing or pump, or other hardware problems. Errors in measured concentration may be due to partial clogs of the flow cell, especially for particles near the upper size limit. Refer to *Fouling* in the previous section for guidance. If counts are below the certified value or deviate statistically from prior readings, backflush, clean the cell, and repeat the concentration measurement.

PRACTICAL MAINTENANCE

Routine flushing: Maintain system cleanliness by flushing with water or filtered formulation buffer between samples. Users may wish to introduce small slugs of air to assist in removing any trapped particulates from the flow channel between samples. Samples with particles that adhere preferentially to the flow cell may require rinsing with 70% isopropanol and/or surfactant solution to remove. The system should be flushed between samples to ensure background levels do not significantly affect the counts.

Short- and long-term storage: For short-term storage, the system should be cleaned as recommended by the manufacturer, rinsed with copious amounts of purified water, and kept filled with water to limit the accumulation of material or growth of organisms between routine uses. Long-term storage may necessitate the removal of all solvent from the FI system, possibly with the use of dry nitrogen to drive all water from the flow path.

4. TEST ENVIRONMENT

Specimens, sampling and specimen-preparation equipment (e.g., pipet tips, vials), and the FI instrument itself must be cleaned to the extent that the level of particles added by testing has a negligible effect on the outcome of the test. For FI instruments, the predominant sources of contaminating particles are often the fluidics components that come into direct contact with the test specimen. When cleaning these components, it may be necessary to disconnect tubing lines, etc., to permit cleaning of tubing joints or other crevices that can harbor particles.

See *⟨1788⟩, 12. Test Environment and Preparation*.

5. PARTICLE COUNTING ACCURACY (SYSTEM SUITABILITY)

The particle counting accuracy (system suitability) should follow the process described in *⟨1788⟩, 13. Instrument Standardization Tests*, using one of three standards: 1) USP Particle Count Set RS (a dilution may be used if deemed necessary); 2) commercial preparation of standard calibrator spheres of nominal diameter 5–30 µm, certified by the manufacturer; or 3) a laboratory-prepared suspension of standard calibrator spheres having a nominal diameter of 5–30 µm. (Refer to *3.1 Concentration Calibration* for additional information.) Spheres with diameters <5 µm are harder to distinguish from environmental or sample-bottle debris. Spheres with diameters >30 µm are more prone to measurement errors due to sedimentation.

Stir continuously throughout the analysis, if possible. In systems without continuous stirring, the use of standards with a nominal diameter of 5–10 µm is recommended. Perform five counts on samples of the suspension. Obtain the mean cumulative particle count per milliliter for the main singlet particle peak, excluding the first run. The lower and upper diameter limits for delineation of the peak may be set by consulting the FI instrument vendor and any available information from the standard manufacturer.

Thorough mixing is imperative. Analysis must be conducted in a timely manner to avoid count bias due to the settling of dense species but in a careful manner to avoid generating new particles or breaking existing ones.

5.1 Interpretation

The instrument meets the requirements for *5. Particle Counting Accuracy (System Suitability)* if the count obtained within the specified diameter limits conforms to the values that accompany the USP Particle Count Set RS or other certificate of particle concentration. If the instrument does not meet the requirements for *5. Particle Counting Accuracy (System Suitability)*, repeat the procedure. If the results of the second test are within the limits given above, the instrument meets the requirements of the test. If on the second attempt the system does not meet the requirements of the test, determine and correct the source of the failures, and retest the instrument.

6. BLANK TEST

Refer to ⟨1788⟩ for guidance on blank handling.

Conduct a blank test (perform after the *5. Particle Counting Accuracy (System Suitability)* test to ensure that any microspheres have been cleared from the system). The blank test for the FI method ensures that the instrument, diluent, glassware, and environment are suitable. Failure of a blank consisting of nominally particle-free water is most often a reflection of contamination in the flow cell or in the fluidics upstream of the flow cell. The acceptance criteria of the blank may be set using the desired statistical precision based on the expected sample counts and the historical background levels observed with clean instruments. Obtain the mean cumulative particle concentration. The concentration obtained should be below a predetermined cutoff specified by the method developed in *3. Instrumentation Standardization Test*.

7. TEST PROCEDURE

7.1 Product Determination

Because the volumes for FI are small, the nominal volume of the product is not important. The analysis will reveal the nature of the particles present in the product and will be used to support the counts from the other techniques.

Deliver the aliquot into the sampler and count particles according to the developed method. Commercial FI instruments will generate reports of the PSD or of the number of particles above stated diameters, as determined by software settings and the specified method. On the basis of the volume of fluid imaged, the software will normalize the particle counts so that results are reported as particle concentrations as well as particle counts.

8. FLOW IMAGING CALCULATIONS

FI instruments typically report particle load as a number concentration of particles per milliliter. For determination of the particles per container, multiply the average particle concentration (in units of particles per milliliter) by the liquid volume of the full container (in milliliters):

Particles per container = average particles/mL × nominal product volume (mL).

9. CONSIDERATIONS OF CONCENTRATION LIMITS BY FLOW IMAGING

For properly conducted measurements, particle concentrations as obtained by FI will be more accurate than those obtained by LO for particles with low optical contrast (such as aggregated proteins). However, LO remains an important tool for assessment of particle load because:

- LO instruments are highly reliable and repeatable
- Regulatory requirements for non-US markets may require the use of LO
- There is a long history and significant accumulated experience with LO testing of pharmaceutical products, and the historical values of measured particle concentration are readily compared to contemporary LO measurements

The correlation between FI and LO results will be repeatable if the measured particles have relatively high optical contrast (i.e., a refractive index difference between the particle and the matrix liquid >0.15), or if the nature of the particles in the test samples is similar to that of the samples used to establish the correlation in the drug-characterization phase of development. PS particle standards give equivalent PSD profiles with FI and LO. However, very different particle counts may be obtained with natural particle populations, especially those with protein or other semisolid particles.

APPENDIX

Methods for FI Instrument Verification

CONCENTRATION CALIBRATION

The particle concentration reported by an FI instrument may be checked by measurement of commercial PS concentration standards. The particle suspensions should have a nominal microsphere concentration between 3,000 and 10,000 mL^{-1}, with a stated uncertainty of the certified value of 10% or better in an aqueous media. The PSD should be largely monodisperse with a mean diameter of either 5 or 10 µm. Microsphere suspensions should be thoroughly mixed prior to use by following the manufacturer's instructions. Perform a preliminary measurement of the PSD to identify the measured diameter of the main peak. Adjust the measurement protocol to establish diameter limits corresponding to the lower and upper bounds of the main peak. Manufacturer's guidance on the diameter limits corresponding to the certified value may be useful for this purpose.

Instrument hardware should be adjusted so that each run records at least 1100 counts in the main peak (to obtain a relative standard deviation from Poisson count variance of ≤3%). If the instrument cannot be so adjusted to achieve this count, the results of multiple runs may be combined to obtain sufficient counts.

DIAMETER CALIBRATION

Background: Calibration and adjustment of FI instruments to give correct diameter readings are typically performed at the manufacturer's factory or by field technicians, using proprietary methods. The tests below give recommendations for instrument verification suitable for implementation by manufacturers or field technicians.

Users, in general, will implement the less complex system suitability tests discussed in *5. Particle Counting Accuracy*, which suffice to demonstrate continued reliability of instrument performance.

Manual method: Calibrate the instrument with a minimum of three calibrators, such as near-monosize PS microspheres having diameters of approximately 2, 5, 10, 15, and 25 µm, in an aqueous, particle-free vehicle.[1] The calibrator spheres must have a mean diameter within 5% of the nominal diameters and be standardized against materials traceable to National Institute of Standards and Technology (NIST) standard reference materials. The microsphere suspensions should have a nominal microsphere concentration between 3,000 and 10,000 mL^{-1}, in an aqueous media. The PSD should be largely monodisperse. Microsphere suspensions should be thoroughly mixed prior to use by following the manufacturer's instructions. Perform a preliminary measurement of the PSD to identify the measured diameter of the main peak. Adjust the measurement protocol to establish diameter limits corresponding to the lower and upper bounds of the main microsphere peak. Manufacturer's guidance on the diameter limits corresponding to the certified value may be useful for this purpose. The range between lower and upper bounds is intended to include all single spheres, taking into account the standard deviation of the spheres and the instrument resolution while excluding noise and aggregates of spheres. Determine the mean of the measured particle diameters, and compare it to the stated mean diameter of the calibrator spheres.

Automated method: The calibration (size response) curve may be determined for the instrument by the use of validated software routines offered by instrument vendors; these may be included as part of the instrument software or used in conjunction with a microcomputer interfaced with the counter. The use of these automated methods is appropriate if the vendor supplies written certification that the software provides an appropriate response curve and if the automated calibration is validated as necessary (see also ⟨1058⟩.

METHOD FOR DETERMINING INSTRUMENT RESOLUTION

Determine the resolution of the particle counter for 10-µm particles, using the 10-µm calibrator spheres. Other particle diameters can be used when justified. The relative standard deviation of the size distribution of the standard particles used should be NMT 5%. Run the microsphere suspension through the system to obtain at least 1000 counts in the main microsphere peak. Filter the data to remove any particles with circularity <0.85. the data to obtain the PSD with bin sizes NMT 0.25 µm. Identify a range of particle bins that are symmetric about the bin that has the maximum count and a sufficiently broad range of diameters such that the lowest- and highest-diameter bin have <1% of the count of the bin with the maximum count. Compute the standard deviation of the diameters of the filtered data corresponding to the range identified by data binning. The instrument resolution may be calculated from the observed deviation using the formula for percent resolution given in *Light Obscuration Method for the Determination of Subvisible Particulate Matter* ⟨1788.1⟩, *Appendix*.▲ (USP 1-May-2021)

[1] ASTM standard F658-00a provides useful discussions pertaining to calibration procedures applying near-monosize latex spheres.

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