Designation: E3060 – 16

Standard Guide for Subvisible Particle Measurement in Biopharmaceutical Manufacturing Using Dynamic (Flow) Imaging Microscopy[1]

This standard is issued under the fixed designation E3060; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 Biotherapeutic drugs and vaccines are susceptible to inherent protein aggregate formation which may change over the product shelf life. Intrinsic particles, including excipients, silicone oil, and other particles from the process, container/closures, equipment or delivery devices, and extrinsic particles which originate from sources outside of the contained process, may also be present. Monitoring and identifying the source of the subvisible particles throughout the product life cycle (from initial characterization and formulation through finished product expiry) can optimize product development, process design, improve process control, improve the manufacturing process, and ensure lot-to-lot consistency.

1.2 Understanding the nature of particles and their source is a key to the ability to take actions to adjust the manufacturing process to ensure final product quality. Dynamic imaging microscopy is a useful technique for particle analysis and characterization (proteinaceous and other types) during product development, in-process and commercial release with a sensitive detection and characterization of subvisible particles at ≥2 and ≤100 micrometers (although smaller and larger particles may also be reported if data are available). In this technique brightfield illumination is used to capture images either directly in a process stream, or as a continuous sample stream passes through a flow cell positioned in the field of view of an imaging system. An algorithm performs a particle detection routine. This process is a key step during dynamic imaging. The digital particle images in the sample are processed by image morphology analysis software that quantifies the particles in size, count, and other morphological parameters. Dynamic imaging particle analyzers can produce direct determinations of the particle count per unit volume (that is, particle concentration), as a function of particle size by dividing the particle count by the volume of imaged fluid (see Appendix X1).

1.3 This guide will describe best practices and considerations in applying dynamic imaging to identification of potential sources and causes of particles during biomanufacturing. These results can be used to monitor these particles and where possible, to adjust the manufacturing process to avoid their formation. This guide will also address the fundamental principles of dynamic imaging analysis including image analysis methods, sample preparation, instrument calibration and verification and data reporting.

1.4 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E2589 Terminology Relating to Nonsieving Methods of Powder Characterization

2.2 *ISO Standards:*[3]

ISO 2859 Sampling Procedures for Inspection by Attributes

ISO 8871 Elastomeric Parts for Parenterals and for Devices for Pharmaceutical Use

ISO 9276-6 Representation of Results of Particle Size Analysis Part 6: Descriptive and Quantitative Representation of Particle Shape and Morphology

2.3 *Other Standards:*

ANSI/ASQ Z1.4-2003 Sampling Procedures and Tables for Inspection by Attributes[3]

ASME BPE-2014 Bioprocessing Equipment[4]

[1] This guide is under the jurisdiction of ASTM Committee E55 on Manufacture of Pharmaceutical and Biopharmaceutical Products and is the direct responsibility of Subcommittee E55.03 on General Pharmaceutical Standards.

Current edition approved June 1, 2016. Published June 2016. DOI: 10.1520/E3060-16.

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.

[4] Available from American Society of Mechanical Engineers (ASME), ASME International Headquarters, Two Park Ave., New York, NY 10016-5990, http://www.asme.org.

BS 6001-1:1999+A1:2011 Sampling procedures for inspection by attributes. Sampling schemes indexed by acceptance quality limit (AQL) for lot-by-lot inspection[5]
USP <787> Subvisible Particulate Matter in Therapeutic Protein Injections[6]
USP <788> Particulate Matter in Injections[6]
USP <1663> Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems[6]
USP <1664> Assessment of Drug Product Leachables Associated with Pharmaceutical Packaging Delivery Systems[6]
USP <1787> Subvisible Particulate Matter in Therapeutic Protein Injections[6]

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in this standard, refer to Terminology E2589.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *brightfield illumination, n*—a method of providing light into a measurement space whereby the illuminated objects are located between the light source and the viewing receiver.

3.2.2 *circularity, n*—degree to which a particle (or its projection area) is similar to a circle.

3.2.3 *cumulative particle size distribution, n*—a representation, as a table, graph, or mathematical function, that gives the total fraction or concentration of particles greater than or less than a set of specified size values.

3.2.3.1 *Discussion*—Cumulative particle size distributions may be expressed as either mass, volume, area, number, or concentration values.

3.2.4 *depth of field, n*—depth of field is the distance between the nearest and farthest objects that are in acceptably sharp focus in an image.

3.2.5 *dynamic imaging, n*—particle size and shape analysis using computer image analysis techniques on instantaneously captured still frame projected images of particles in motion (also referred to as *flow imaging*, *flow microscopy*, *direct imaging*).

3.2.6 *equivalent diameter, n*—the diameter of a sphere or circle that is equal to the measured diameter obtained by a particle sizing instrument.

3.2.6.1 *Discussion*—For dynamic imaging, the reported diameter is based on the projected area of a measured particle.

3.2.7 *extrinsic particle, n*—a particle introduced from sources that are foreign or external to the manufacturing process.

3.2.8 *Feret diameter, F, n*—apparent diameter of an object determined from the distance between two parallel tangents on opposite sides of a binary object.

3.2.8.1 *Discussion*—There are an infinite number of Feret's diameters; the maximum and the minimum Feret's find most use within imaging.

3.2.9 *field of view, n*—the two dimensional, lateral extent of the imaged area.

3.2.10 *frequency distribution, n*—a representation, as a table, graph, or mathematical function, that gives the frequency or count of values within a set of specified intervals.

3.2.11 *inherent particle, n*—a particle made entirely of components of the formulated drug product or its manufacturing intermediate, arising from the product itself.

3.2.12 *intrinsic particle, n*—a particle composed of materials that the product or intermediate contacts or is mixed with during the manufacturing process or during storage in primary packaging components.

3.2.13 *particle size distribution (PSD), n*—a frequency or volume distribution of the concentration of particles versus particle size.

3.2.13.1 *Discussion*—Dynamic imaging particle analyzers of use to the biopharmaceutical industry report the PSD as the concentration of particles per unit volume within specified size ranges, where the size is most commonly the equivalent diameter but may be another morphological size attribute. See Appendix X1.

3.2.14 *subvisible particle, n*—a particle with a measured equivalent diameter within the approximate range 1 µm to 100 µm.

NOTE 1—When it is necessary to specify an exact size range, the range should be defined explicitly rather than by such adjectives as subvisible.

3.2.14.1 *Discussion*—The term particle may be used to designate any self-contained object that is optically distinguishable from the background image, including liquid droplets and gas-phase bubbles.

3.2.14.2 *Discussion*—The 100 µm upper limit is based on the historical definition of subvisible particle as used in the field of drug inspection. Particles of 20 µm or smaller of sufficient optical contrast are readily visible under bright illumination, especially when present in numerous quantity.

3.2.15 *threshold, n*—the minimum quantitative change in intensity (of either positive or negative sign) from the background pixel value for a pixel to be identified as a possible particle.

3.2.16 *volume distribution, n*—a frequency distribution that gives the distribution of particle volume as a function of particle size.

4. Significance and Use

4.1 This guide will encompass considerations for manufacturers regarding sources and potential causes of subvisible particles in biomanufacturing operations and the use of dynamic imaging particle analyzers as a suggested common method to monitor them. The guide will address the following components of particle analysis using dynamic imaging microscopy: fundamental principles, operation, image analysis methods, sample handling, instrument calibration, and data reporting.

[5] Available from British Standards Institution (BSI), 389 Chiswick High Rd., London W4 4AL, U.K., http://www.bsigroup.com.

[6] Available from U.S. Pharmacopeial Convention (USP), 12601 Twinbrook Pkwy., Rockville, MD 20852-1790, http://www.usp.org.

5. Types of Particles

5.1 USP <1787> defines three subcategories of particles related to their source or nature. When combined with appropriate strategies for characterizing particle types, this categorization scheme provides a framework for assessing the root cause and acceptable concentrations of different types of particles.

5.1.1 Inherent particles are related to the product formulation (for example, chemical and physical properties and concentration of the Active Pharmaceutical Ingredient (API) proteins, excipients, API solid suspensions, emulsions, adjuvant aluminum salts added to vaccines). Packaging of the product and external stresses (including temperature, mechanical shock or movement, light exposure, and interaction with liquid/solid and liquid/air interfaces) can all have substantial impact on the concentration and characteristics of protein aggregates. Protein aggregates may change over time, in both concentration and characteristics, and some levels of protein degradation or related aggregation, or both, may be expected. Inherent particles must be well characterized and monitored over the product shelf-life.

5.1.2 Intrinsic particles include product contact materials from the manufacturing process or primary packaging components (that is, silicone oil, glass, stainless steel, rubber closure, polymer tubing, semi-solid silicone lubricant, process related fibers, etc.). This category also includes stability-indicating particles found predominantly during development or stability studies (formulation degradation, container closure-related, glass delamination, stopper degradation, etc.). The presence of intrinsic particle types must be minimized, and if they are stability-indicating, they should be eliminated whenever possible.

5.1.3 Extrinsic particles comprise any particles not sourced from the manufacturing process or product contact materials including particles of a biological source (that is, external environmental fibers, hair, airborne particles, etc.). Extrinsic particle types should be a rare occurrence and eliminated.

6. Sources of Particles

6.1 Subvisible particles may be generated by a number of sources during the manufacturing process. In analyzing the risk of particle generation introduced by various process steps, it is useful to understand the sensitivity of the drug product or substance to a variety of stresses known to promote particle formation.

6.2 *Sources of Inherent Particles:*

6.2.1 Stresses which may cause inherent particle changes may include:

6.2.1.1 Interaction with interfaces or other particles.

(1) Increased interfacial transport resulting from agitation, stirring, etc.

(2) Interfacial adsorption: both liquid/vapor and liquid/solid

(3) Nucleation on other particles

(4) Trace metals and other molecules promoting oxidation and aggregation

6.2.1.2 Chemical environment.

(1) Formulation, which may promote or hinder particle generation

(2) Excipients

(3) Impurities

6.2.1.3 Physical environment.

(1) Vibration

(2) Mechanical shock

(3) Cavitation

(4) Temperature and humidity

(5) Environment—contamination

(6) Intense light exposure

6.2.2 The count and characteristics of the particles formed as a result of these stresses will vary in general with the duration of the stress and subsequent storage time and conditions.

6.3 *Sources of Intrinsic Particles:*

6.3.1 Intrinsic particles may be formed when materials in contact with drug substance or product are stressed, such as the shedding of particles by pumps used in fill and finish operations. In other cases, the stresses may be minimal, but the materials are not verified to be sufficiently particle free; an example would be the shedding of particles from a filter. As with inherent particles, the creation of particles depends both on the duration of particular stresses and the time of storage.

6.4 Combinations of particular stresses may arise in different process steps during manufacturing operations, including:

6.4.1 Formulation,

6.4.2 Sterilization,

6.4.3 Storage: conditions, time of storage, and choice of container,

6.4.4 Transport,

6.4.5 pH adjustments,

6.4.6 Viral Inactivation Steps,

6.4.7 UF/DF,

6.4.8 Container or closure siliconization, which may promote aggregation of proteins,

6.4.9 Freeze-thaw,

6.4.10 Mixing, and

6.4.11 Fill/Finish.

6.5 Components in the manufacturing process may contribute particles directly (for example, polymer particles shed by a single use system component or other flexible system components), or may contribute to increased particle load indirectly (for example, protein adsorption and subsequent desorption as a particle from a hydrophobic polymer surface). The use of components and filters requires the development of compatibility profiles with the product and solutions to assure leachable substances are not a concern as discussed in USP <1663> and USP <1664>. The therapeutically active drug substance (small or large molecule) would have to be shown not to bind to the filter system as evidence by loss of potency or any indications of API degradation. Process steps may either increase or decrease particle concentrations, or a combination thereof. For example, filtration will remove inherent particles but may introduce intrinsic particles shed from the filtration media or even promote further growth in inherent particles by nucleating interfacial growth of protein aggregates. ISO 8871

is a guide to the compatibility of rubber or elastomeric components for most aspects of stopper performance testing. In addition, many protein solutions or drug formulation impurities can interact with medical grade silicone used to lubricate the container, closure or plunger, and result in increased protein aggregate formation over time in the absence of surfactant. Also, residual tungsten from the manufacturing of syringe barrels with staked cannulas has been implicated in protein aggregation and particle formation. Pumps are another common source of particles and should be inspected frequently for indicators of wear or particulate generation. Piston pumps can generate stainless steel particles, peristaltic pumps can cause spallation or abrasion of the inner tubing wall and generate polymeric particles, and diaphragm pumps can generate rubber diaphragm particles over time. Close attention to pump maintenance is recommended.

7. Baseline Monitoring During the Manufacturing Process

7.1 Biopharmaceutical manufacturers should establish baselines for particle levels at key steps in the manufacturing process to evaluate the effects of component changes, process changes and stability on the product. Baseline data should be in place to assess and understand how these changes impact the particle formation during and after the manufacturing process. Particle baselines may be developed during:

7.1.1 Formulation Development

7.1.2 Clinical Lot Manufacturing

7.1.3 Routine Manufacturing

7.2 Testing should be conducted at time of release and at the conclusion of shelf life in order to assess the formation and change in distribution of subvisible particles over time. Particle data should be collected according to size in the following categories: 2–5 µm, 5–10 µm, 10–25 µm, 25–50 µm and 50–100 µm (Options for reporting these data are given in Section 13). Changes in quantities or distribution of subvisible particles should be investigated to identify root cause. Manufacturers may consider particle contributions from other process steps and studies, including:

7.2.1 Scale-Up,

7.2.2 Freeze/Thaw studies,

7.2.3 Development stability studies,

7.2.4 Container/Closure studies, and

7.2.5 Transport/Storage studies.

7.3 Monitoring should also be considered during key manufacturing operations, in particular:

7.3.1 Sterilization,

7.3.2 Filling,

7.3.3 Container/Closure supplies and use,

7.3.4 Marketed Product Stability studies,

7.3.5 Manufacturing Site changes, and

7.3.6 Manufacturing Device process changes.

7.4 Once the baselines are available, significant deviations from the baseline should be noted and particles should be characterized if possible. This characterization may help identify root cause. Studies should be undertaken to address the sources or adjust the process, or both, to minimize their formation. In addition, the contribution of particles from the external environment during the manufacturing process, particularly during filling operations, should be evaluated, understood and minimized.

7.5 As part of the baseline characterization, it is desirable to identify the dominant subpopulations of particle types. One useful approach is to generate samples with particles of known composition and known mechanism of generation. From these samples, images representing different categories of particle types can be used to generate parameters for filtering of the images to categorize them, based on assessment of risk. Image distinction may be straightforward for some common uniform particle types such as silicone oil, whereas distinguishing rare particles such as extrinsic fibers from fibrous protein particles is difficult. Image analysis is a rapid means of identifying particle types, but care in interpretation of images is necessary, especially for irregularly shaped particles. Shape information (for example, aspect ratio, circularity, etc.) and image intensity analysis measurements (for example, average intensity, intensity differences, etc.) may also be included. Accurate morphological analysis may not be possible for particles below 5 µm, depending on the instrument used. Because dynamic imaging does not provide direct chemical information, the specificity of image analysis, especially (but not only) for small particles, cannot equal the specificity of microspectroscopy techniques. While use of Fourier Transform Infrared spectrometry (FTIR) or Raman microspectroscopy and Scanning Electron Microscopy-Energy Dispersive Spectroscopy (SEM-EDS) methods can identify particle types with greater confidence than dynamic image analysis, these methods have greatly reduced throughput and have limitations on minimum particle size or composition. SEM-EDS gives basic elemental composition of both organic and inorganic particles as small as 100 nm, but the method is not appropriate for fragile and highly hydrated protein particles, or similar particles. FTIR and Raman are generally limited to particle sizes greater than ≈10 µm, with greatly reduced throughput and less chemical specificity near the low end of the size range. Positive identification of particles below ≈10 µm is impractical and in some cases, not possible. When investigating deviations from process control, dynamic image analysis and investigation by spectroscopic or other chemically specific methods may be warranted.

7.6 Dynamic image analysis provides a highly sensitive method for measuring the particle size and counting the number of particles. Typical limits of detection for dynamic image analysis correspond to very low volume fractions of particles. For example, 200 particles per milliliter at a diameter of 5 µm is equivalent to a volume fraction of only 10^{-8}. As a result, for many common particle types, detection of particles is possible at concentrations far below levels that would impact product quality.

7.7 From the perspective of risk analysis, particles may be categorized as:

7.7.1 Particles that may be present in the final drug product and represent a potentially significant risk to safety or efficacy (for example, aggregated protein, foreign material),

7.7.2 Particles with low intrinsic risk (for example, silicone oil), and

7.7.3 Particles of unknown composition.

8. Apparatus

8.1 *Principles of Measurement:*

8.1.1 Dynamic image analysis is a particle analysis technique using light microscopy to examine microscopic particles in a moving fluid. Basic instruments are identical to a standard light microscope, with the difference being that in a Dynamic Image Particle Analyzer the sample fluid is imaged dynamically, while in motion, as opposed to the sample being imaged statically as it is (stationary) in light microscopy. The primary benefit to dynamic image particle analysis is that since the fluid is being imaged dynamically, larger numbers of particles can be imaged, stored and measured in a short period of time. The larger number of particles analyzed yields much higher levels of statistical confidence versus static microscopy.

8.2 *Basic Hardware Configuration:*

8.2.1 Two distinct configuration types for flow imaging systems are designated here: *(1)* stand-alone instrument using a sample obtained from a batch and *(2)* in-line configurations whereby a probe containing the system components is inserted into a process vessel or pipe. While this document will concentrate on the stand-alone type of system, since it is the most common (largely because samples are usually drawn from the final drug product in its packaged form), the basic techniques are very similar for the in-line type of technology with the exception that no "sample handling" is involved. Dynamic Image Particle Analyzers (see Fig. 1) consist of 3 basic components: fluidics, optics and electronics:

8.2.2 The optics are essentially microscope components, while the electronics consist of the image sensor (camera) and supporting electronics required to obtain and process the digital images of the particles. The fluidic system consists of sample introduction fittings, tubing, a flow cell and a pump. In some systems, samples are introduced into the flow cell by a robotic fluid sampler. The pump can be either peristaltic or syringe type, and may be controlled by the system computer. The fluidics flow is generally as follows: the pump (typically located downstream of the flow cell), pulls sample fluid from the sample introduction fittings through the flow cell and out into waste (the sample can be recirculated back to introduction if desired, but generally it only passes through once so that every particle is only imaged once).

8.3 *Flow Cell/Fluidics:*

8.3.1 In stand-alone instruments, the flow cell is a critical system component as it must restrict the position of the particles perpendicular to the microscope's optical axis in order to keep them in reasonable focus (within the depth of field). The flow cell itself is typically a rectangular piece of material (typically quartz glass) through which the sample is actually imaged. See Fig. 2.

8.3.2 The flow cell is typically designated by the depth as shown in Fig. 2. Different types and configurations of flow cells may be available. In some of these, the width of the flow cell may be greater than the actual camera field of view or illuminated area, while in other configurations the flow width may be restricted to stay inside of the camera field of view or illuminated area. In either case, the manufacturer must properly calculate the volume of sample being imaged in order to get valid concentration figures. Because the optical depth of field (area perpendicular to the optical axis in sharp focus in the object space) decreases with increasing magnification, it is important to match flow cell depth to optical magnification in the system. While most systems have only a single magnification/flow cell size available, some systems do offer

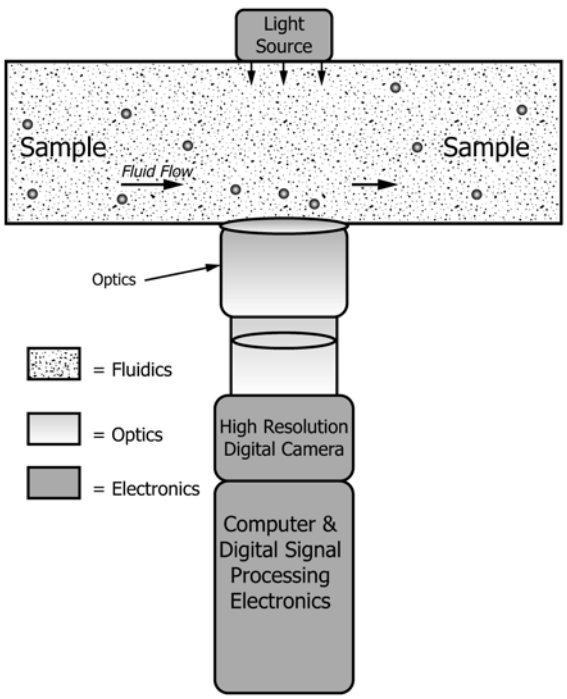


FIG. 1 Components of a Dynamic Imaging Particle Analyzer

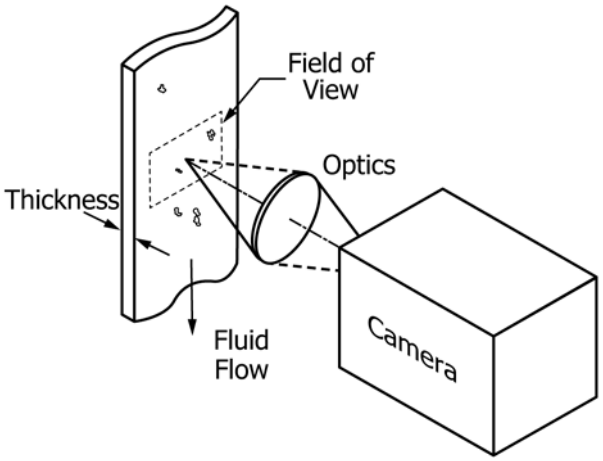


FIG. 2 Flow Cell

different magnifications; in these systems it is critical that the manufacturer's recommended flow cell size is matched to the magnification.

8.4 *In-line Illumination:*

8.4.1 The flow cell is typically designated by the volume of space, within the interior of the cell, where particles are imaged. This relies to a great degree on the width, height and depth of the camera view, and may be further restricted by the cell parameters, but also is influenced by the threshold values selected which define a particle from its background. The manufacturer must properly calculate the volume of sample being imaged in order to get valid concentration figures. Some systems use a different arrangement to hydrodynamically focus the particles relative to the optics, usually referred to as a sheath flow system. A sheath flow system uses a wide tube of glass through which a tunnel of fluid (sheath fluid) is created for the sample to pass through in the center by varying the velocity and density of the two fluids so that they do not mix. The net result is that the sample particles pass through the imaging zone in a very narrow stream (typically in single file), and thus remain in sharp focus. These systems often have very small measured imaged volume, and concentration determination may have increased uncertainty. Effects of the sheath flow interaction with the product sample are unknown.

8.4.2 Since dynamic imaging systems use light microscopy, the minimum particle size which can be counted is set to approximately 1 μm, or larger for morphological determinations. This is due to diffraction effects and camera pixel size, which create hard limits on the minimum size. The maximum size particle that can be measured is typically going to be restricted by flow cell depth: particles larger than the flow cell depth may cause clogging of the flow cell, which is to be avoided. In the case of biopharmaceuticals, where the particles (particularly protein aggregates) may be pliable, some particles larger than the flow cell depth may be seen.

8.5 *Illumination:*

8.5.1 As particles pass through the flow cell, they are illuminated most commonly from behind ("back-lit", although some systems may use "front lighting") by a light source, typically a modulated source. The light source is "strobed" (typically at a synchronous interval) in order to capture a blur-free image of the particles as they flow through the cell. Most commonly, the transmitted or reflected light is collected and focused without alteration by filters or polarizers, which is termed brightfield illumination. Some types of particles, such as glass shards, may be challenging to identify with brightfield illumination. Alternate illumination types (for example, darkfield) may provide improved sensitivity in principle, but these alternate illumination schemes may be technically challenging to implement in commercial dynamic imaging particle analyzers.

8.5.2 For in-line systems, all of the above descriptions apply, with the exception that the imaging system is now integral to a pipe or vessel and views the sample therein. The cell, as defined above, still exists for the in-line installation. The illumination configurations available should be the same for the iin-line instrument as for the lab instrument in order to allow for the comparison of images and data from both analyses.

9. Image Processing

9.1 In all imaging systems, the stages of image processing in general can be defined as follows:

9.1.1 *Step 1*—Acquisition of raw gray-scale (or color) image (full camera field of view).

9.1.2 *Step 2*—Reduction of gray-scale or color image to a binary image where each pixel can only have one of two values: particle or not particle.

9.1.3 *Step 3*—Grouping of contiguous particle pixels to form "isolated objects" (individual particles).

9.1.4 *Step 4*—Once each particle is isolated in the binary image, measurements are made and stored for each particle. Measurements may be related to particle morphology or image intensity, as well as particle size.

9.2 *Thresholding and Pixel Grouping ("particle detection"):*

9.2.1 Step 2 above, where the original gray-scale image is converted into a binary image, is one of the most critical steps in the process, and can sometimes produce different results depending on the method used. The most common method used is gray-scale thresholding: in this process, a threshold is chosen. Pixels with an intensity above this threshold are classified as "particle" while those below the threshold are classified as "not particle". Threshold settings may be fixed by the manufacturer or adjustable. It should be noted that for a mixture of either particle types or sizes that there is no unique threshold that will distinguish the edge of a particle in an identical manner. Note also that the measured particle size depends on the illumination conditions, and as a result the illuminating light level needs careful control. Since most dynamic imaging systems are back-lit, particles will typically have a darker value than the background due to the fact that the particle will obstruct or refract, or both, the light from the illumination source. As a result, most gray-scale methods involve setting a threshold darker than the background; if the intensity value for that pixel is equal to or greater than the threshold value (darker) when compared to the background, then the pixel is considered to be "particle". The background

value for each pixel in the camera array is the value obtained when no particles are present, typically derived by averaging the pixel value over several frames as the fluid is moving through the flow cell. In general, each manufacturer will either pre-set the threshold method/values or supply recommended settings to use. The system must be set up and calibrated per the manufacturer's recommendations. It is, however, important to understand what thresholding method and values are used because they can affect particle measurements dramatically. The lower size limit for particle detection depends both on the optical resolution of the system and on the pixel size of the camera; this limit is approximately 1 μm for typical dynamic imaging particle analyzers.

9.2.2 In Step 3, once every pixel in the original image has been classified as "particle" or "not particle" by the thresholding process, contiguous groups of pixels classified as "particle" are grouped together to form objects. In this case each stand-alone object is an actual particle in the original image, consisting of one or more touching pixels classified in the binary image as "particle" pixels. This process, referred to as "particle detection", can be simple (for example, pixels that are touching each other in the binary image), or complex (proprietary algorithms).

9.2.3 The thresholding and particle detection processes also become important when looking at how concentrated a sample can be when being imaged. There is an upper limit for measurement where particles are too crowded together to be separated and counted individually. In a sample with a high particle concentration, two particles in close proximity to each other in the camera image may be interpreted as a single particle. While there are some algorithms available for "de-clumping" or "de-aggregating" particles, their use is typically limited to particles of known shape, and generally the analyses are too computationally expensive to work in a real time environment. Separation of particles by the use of image analysis software should not be used as particles may be genuinely aggregated. Commercial dynamic imaging systems in common use for biopharmaceutical applications conform to this recommendation.

9.2.4 Unfortunately, there is no way to predict a specific concentration number that would become a limit, as it is sample dependent. For instance, incorrect interpretation of two overlapping particles as a single particle is more likely, at a fixed particle concentration, if the sample contains large particles than if the sample consists of uniformly small particles. For this reason, the only way to determine if a sample is too concentrated to analyze correctly is to try running it to see if there are any overlap issues. This can also be achieved by using standard particles of known sizes, estimating the coincidence concentration levels per size and in mixtures. Additionally, with biologics, high protein concentrations can sometimes cause artifacts known as Schlieren Lines, so care must be taken to ensure these artifacts are not introduced. High protein concentration may also produce high background and therefore impact thresholding, accuracy of sizing, and counting.

9.3 *Image Analysis:*

9.3.1 Once the particle detection is complete and binary particle images have been produced, the software will then make measurements for each particle to be stored and associated with each corresponding particle. The actual measurements made on each particle may vary by system, but typically include equivalent diameter, Feret diameter, aspect ratio (breadth/length) and other morphological measurements like circularity, area, etc. For many dynamic imaging particle analyzers, equivalent diameter is calculated from the projected area of a particle (with any holes filled first) as if that area formed a circle, and in equation form is:

$$\text{Projected Equivalent Particle Diameter} = \sqrt{(\text{area} \ / 0.785)}$$

9.3.2 Some systems provide the option to fill or not fill holes in the binary image, or to use alternative definitions of the equivalent diameter, such as averaging the Feret's diameter over multiple angular orientations.

9.3.3 Most systems also make and record intensity-based measurements such as average intensity and intensity variation for each particle as well. It should be noted that these intensity-based measurements are made from the original gray-scale (or color) image as now defined by the binary particle outline. It should be further noted that such measurements are 2-dimensional ratios when the particles are actually 3-dimensional.

9.3.4 Once the images and measurements are gathered, the resultant data is stored, typically in spreadsheet or database form. Most systems will also save at least some (if not all) of the original particle images so that they can be viewed/reviewed after analysis, providing visual proof of the measurements. Data may be post processed to produce detailed statistics about the population(s) contained in the sample. Typical outputs here might be particle size distributions (PSDs) based on number or converted to another parameter such as volume or particle shape distributions.

9.4 *Use of Filters to Create Subpopulations:*

9.4.1 Since dynamic imaging systems produce multiple measurements for each particle, the output data can be used to identify subpopulations of the original sample. Distinguishing subpopulations has varying success based upon the uniqueness of image attributes collected for a particular subpopulation. Digital filters can be defined using any particle measurement or group of measurements applied to the spreadsheet data. Simple size filters are frequently used to quantify subpopulations by size, such as particles ≥10 μm and particles ≥25 μm.

9.5 *Image Characterization:*

9.5.1 Digital filters can be used simply to bin data by parameter values, as is the case in size filters, and can also be made specific enough to create subpopulations that classify particles by nominal particle type. The success of particle classification depends on the degree of parameter differences (for example, circularity, image intensity) between the images of different particle types. In practice, the best filters will combine both morphological and image intensity criteria, and will be verified for the particular instrument and optical set up in use. At the present state of the art, filters are often not effective at classifying heterogeneous particles (for example,

protein aggregates associated with silicone oil droplets), particles or droplets of unusual morphology (for example, doublets of silicone oil), particles of different types but similar morphology (for example, fiber contaminants versus large, fibrous protein particles), and particles or droplets at sizes at or below the optical resolution of the instrument. Digital filters are most useful in distinguishing irregular solids (predominantly aggregated protein in most cases) from liquid droplets or gas bubbles at diameters >5 μm.

9.5.2 Particle classification should not be inferred directly from measurements of test samples with heterogeneous particle populations. Instead, samples containing known particle types at the size of interest should be produced and then measured. For example, silicone oil droplets may be created by sonication of silicone oil in a detergent solution, or protein particles may be created from a variety of physical or chemical stresses. From measurements of these known particles, image parameter values characteristic of this particle type may be obtained. Combinations of several different image parameters may be used for image identification in subsequent measurements by application of an appropriate software filter on the measured particles. Most filters are value filters where a range of values for specific measurements are defined and only particles meeting those requirements are counted in that subpopulation. Development and verification of digital filters is still under development, and application of digital filters to multiple particle types in routine measurements remains a challenge.

9.5.3 Although dynamic imaging analysis is a useful tool to monitor the concentration of particles in a process, with high sensitivity and reasonably fast throughput, investigations of the root cause of particle formation will require additional methods to confirm particle identification. Dynamic imaging alone is insufficient to confidently identify particle type.

9.6 *Reference Library:*

9.6.1 Filters for creating subpopulations and particle classification may be stored simply as a set of conditions (value or statistical), but they can also be stored as a reference library of images. This may be useful especially for classification, as the library can be used as a visual aid for the operator in either verifying particle types classified by the filters or as a reference set of images for operator training. A trained operator can often distinguish unusual or heterogeneous particles with more success than a digital filter, although manual inspection of images is tedious. A library may be used simply as a set of reference images, but it can also be used as the basis from which values or statistics are generated for filter creation.

10. Sampling

10.1 Sample handling will depend on the nature of the particles to be measured, the particle size range, and the particle concentration. Procedures and recommendations for sample selection, preparation and handling of drug products have been covered in the United States Pharmacopeia, specifically USP <787>, USP <788>, and the informational chapter USP <1788>. Although USP chapters are not mandatory for manufacturing processes within the scope of the present Guide, and manufacturing processes are outside the scope of USP chapters below number <1000>, the technical methods discussed within these USP chapters may be useful in developing appropriate methods.

10.2 *Sampling Plans:*

10.2.1 Sampling plans should be based on considerations of the purpose of the testing, product volume, particle numbers historically found in comparison to applicable limits, PSD, and variability of particle counts between units. Guidance on sampling plans is available in defined sampling standards such as BS 6001-1:1999+A1:2011; ISO 2859 (suffix -1 through -5); or ANSI/ASQC-Z 1.4. USP <787> and <788> may also provide useful guidance.

10.2.2 When sampling from bulk solution consideration for homogeneity of the samples is very important. Samples of bulk solution must take into account the possibility of stratification within the vessel or container. This should be covered by multiple samplings taken at top, middle and bottom of the container volume if the sample in the container has not been homogenized prior to sampling.

10.2.3 In all cases, the validity of conclusions regarding particulates requires an assessment of the statistical significance of the measurement results, including both the variability of results from different samples and the overall number of measured particles of interest.

10.3 *Sample and Instrument Preparation:*

10.3.1 The limit of quantification of particles by dynamic imaging is generally limited by the cleanliness of the instrument and the care taken in sample introduction. Prior to use, the instrument should be cleaned according to manufacturer's instructions. Procedures to introduce samples into the instrument while maintaining sample cleanliness will vary from instrument to instrument. Potential sources of extraneous particles and methods to minimize these particles include:

10.3.1.1 *Airborne particles*—Minimize the time that samples are exposed to room air, or place the instrument in a laminar flow cabinet.

10.3.1.2 *Test sample containers*—Prepare the test specimens by removing the outer closures but not the sealing closure. Rinse the exteriors of the containers with filtered particle-free water.

10.3.1.3 *Abrasion products*—Particles may be created when two solid surfaces slide against each other, especially if they are dry. Minimize this effect by wetting Luer fittings prior to engagement and by not tightening plastic vial closures more than necessary.

10.3.1.4 *Flow cell and tubing*—Particles may be left behind by prior samples. Clean according to manufacturer's recommendations. If necessary, replace flow cell and tubing.

10.3.1.5 *Secondary sample containers (for example, vials or syringes) vials and sample handling equipment*—Use only vial types that have been verified to have acceptably low introduction of particles. Vials, pipet tips, and other sample handling equipment may require rinsing or cleaning prior to use.

10.3.1.6 In order to verify the cleanliness of the sample containers two types of system controls are recommended: a Blank Test (environmental/procedural) and System Suitability Verification. See USP general information chapter <1788>.

10.3.1.7 When analyzing in the process line system components must meet ASME BPE requirements for bio process equipment. This construction will ensure that the particle size analyzer is designed and manufactured of the same material, process and finish used in the rest of the piping, vessels and instrumentation. This eliminates risk of an inline probe causing contamination issues. Introduction of the probe into the process stream must also be done in such a way as to avoid contamination from the outside environment. The ability to obtain continual data from an in-line instrument reduces the handling error and gaps in readings from a batch sample setup. There is no concern for contamination from other sources since the only particles that will be read are due to the process itself. In addition a better understanding of the source of the subvisible particle can be obtained, and determined if they are due to protein agglomeration, bioburden, rouging or equipment failure (gasket breakdown, pump, etc.). A typical in line system is shown in Fig. 3.

10.4 *Sample Dilution:*

10.4.1 Sample dilution is discouraged except where absolutely necessary, for example, when high concentration causes particle overlap in the camera view. After dilution, the composition of the sample is changed and hence particle distribution might not be representative. Dilution is allowed as long as the diluent (typically the formulation buffer) and methods are demonstrated to be appropriate and the smallest level of dilution that allows for reproducible testing is used. Under certain circumstances dilution of samples may be required to obtain reliable results. This may be the case with high-concentration products or high-viscosity products, or both, that cannot be drawn into the instrument properly; and cases where the high concentration results in very small differences in refractive index between the solution and the protein aggregates, etc. In situations where sample dilution is conducted, the underlying rationale for the dilution as well as the suitability of the selected dilution scheme including choice of diluent must be demonstrated. Suitability of the dilution scheme and of the diluent may be assessed by demonstrating consistency of results over a dilution range. Note that the particle values of the buffer should not be subtracted from the sample particle values.

10.5 *Sample Degassing:*

10.5.1 Eliminating gas bubbles is a key step, especially for proteinaceous products that readily entrain gas. Two methods are recommended: either allowing the product fluid to stand under ambient pressure or applying a gentle (for example, 75 Torr) vacuum. A degassing method should be developed which shows the least impact on the sample particulates. Other methods may be used when demonstrated to be suitable. USP <787> provides recommended procedures for degassing therapeutic proteins by vacuum degassing. Sonication should be avoided for proteinaceous products.

10.6 *Sample Mixing:*

10.6.1 Care should be taken to minimize agitation and other stresses to the preparations (for liquids or reconstituted powders) that may accelerate the development of unintentional protein nucleation or aggregation and to prevent introduction of air bubbles into the preparation under examination. Once the samples have been degassed, they must be remixed gently to suspend all particles by mixing the contents of the sample gently but thoroughly by an appropriate means, for example, swirling or rotating of the container.

10.7 *Instrument Priming:*

10.7.1 Buffers, dilute solutions, and water all have a refractive index close to that of pure water. However, when a sample with high protein or excipient concentration is measured, the instrument must be primed, according to manufacturer's recommendations, with a fluid of the same refractive index as the sample. When the cell and priming liquid have different viscosities, the volume of priming liquid may increase. Proper priming of the instrument may be assessed by running blank samples. If there is mixing of liquids of different refractive index, patterns of light and dark wavy lines or regions will appear in the cell (Schlieren lines), and these lines will be interpreted as long, irregular particles.

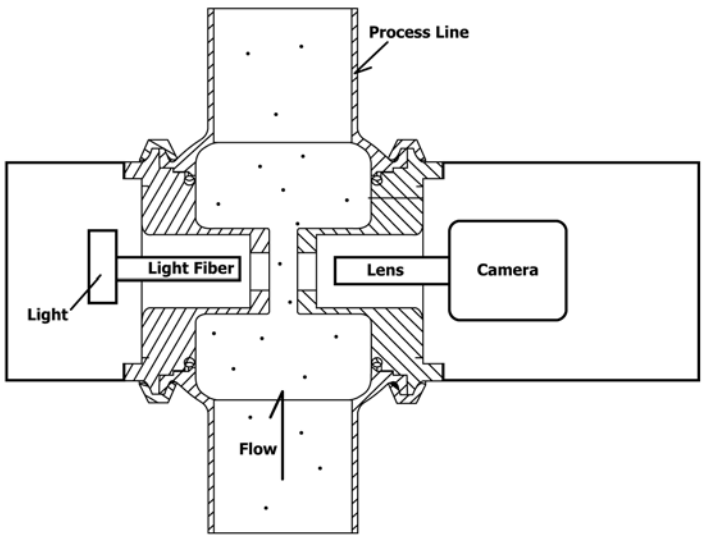


FIG. 3 In Line System

10.7.2 In addition to ensuring that the cell is filled with liquid of the same refractive index as the test sample, priming also contributes to the flushing of previous samples from the cell. The user should verify that the priming method is sufficient to reduce the particle counts from the previous sample to acceptable levels. Proper priming and flushing is especially important when measuring a sample with a particle concentration much less than that of the previous sample. Conversely, minimal priming is needed when measuring the same sample repeatedly. The limit of quantitation for flow imaging is often limited by the particle count of particles from previously run samples.

10.8 *Sedimentation:*

10.8.1 When a sample is loaded into a particle counting system, the particles begin settling as soon as the convection velocity of the fluid falls to a value near the sedimentation velocity. With time, the bottom of the sample will have a higher proportion of particles than the top of the sample, potentially leading to inaccurate particle counts.

10.8.2 The sedimentation velocity for spheres is proportional to the density difference between the particle and its matrix fluid and to the square of the particle diameter, and inversely proportional to the viscosity of the medium within which the particle resides. As a result, large, high density particles sediment much faster than smaller, low density particles. The table below gives the time for solid spheres of a given size and composition to settle by 1 cm (a value large enough to significantly perturb counts in typical particle counters) in a liquid of viscosity 1 cP (1 mPa.s), which reflects the viscosity of water at 20°C. Sedimentation times will increase in proportion to any increase in sample viscosity. See Table 1.

10.8.3 There are four ways of reducing the effects of sedimentation:

10.8.3.1 *Method 1*—Stir the sample continuously; however, this can again change particle properties, dissolve large agglomerates or generate new particles. This is practical only with large-volume samples and counters equipped with stirrers.

10.8.3.2 *Method 2*—Perform the measurement rapidly. If the measurement is completed quickly enough, the sedimentation will not affect the particle density, especially if the sampling system is configured to draw out fluid from the middle of the sample.

TABLE 1 Sedimentation Times

Material Diameter, (μm)	Polystyrene	Silica	Soda-lime glass	Protein particle, compact[A]
	Time to sediment 1 cm, (min)			
1	5429	287	191	815
2	1357	72	48	204
5	217	11	7.7	33
10	54	2.9	1.9	8
20	14	0.7	0.5	2.0
40	3.4	0.2	0.1	0.5
70	1.1	0.1	0.0	0.2

[A] Stated times are minimum times; actual times will be longer depending upon particle packing density.

10.8.3.3 *Method 3*—Measure the entire sample. Sedimentation only causes a redistribution of particles, and not an actual change in the particle numbers. If the entire sedimented sample is measured, the total number of particles will be the same as for the unsedimented sample.

10.8.3.4 *Method 4*—Increase the viscosity of the matrix fluid. This method is not recommended due to potential sample interactions.

10.8.4 In practice, Methods 1, 2, and 3 are often applicable for Dynamic Imaging Particle Analyzers.

10.9 *Final Mixing and Sample Introduction:*

10.9.1 Samples should be mixed prior to introduction into the instrument to promote sample homogeneity and reduce particle segregation by settling. When possible, mixing should be sufficient to guarantee a uniform particle concentration throughout the sample while not introducing any alterations to the PSD. A typical mixing protocol is to gently tip the vial back and forth 10 times. For some delicate samples, even minimal stirring will cause changes in the PSD. The sample mixing protocol should be verified to be suitable for the particular samples being tested.

10.9.2 Sample introduction may be by pipet for manual systems or by fluidics for robotic instruments. The sample introduction process should be verified to confirm that no extraneous particles are introduced.

10.10 *Robotic Sampling:*

10.10.1 If robotic sampling is used it should be qualified for the application and proven to not introduce additional variables. Some samples may not be amenable to automation (for example, samples that are highly viscous, sensitive to stirring, or that are especially adherent to the fluidics components). Refer to the manufacturer's instructions for guidance.

11. Traceability

11.1 To obtain an accurate PSD reporting the distribution of particle concentration versus particle size, three primary quantities must be determined accurately: particle diameter, particle count, and the volume of sample that is imaged (see Appendix X1). For dynamic imaging instruments, the accuracy of particle count and the imaged volume are not assessed independently. Instead, particle concentration standards are used, thus reducing the primary traceability requirements to two independent quantities: particle equivalent diameter and particle concentration. USP <1788> gives methods for both initial verification and routine checks of dynamic imaging instruments. These methods provide an assessment of both accuracy and precision of PSD measurements. The remainder of this section presents a general overview of methods to achieve accurate results.

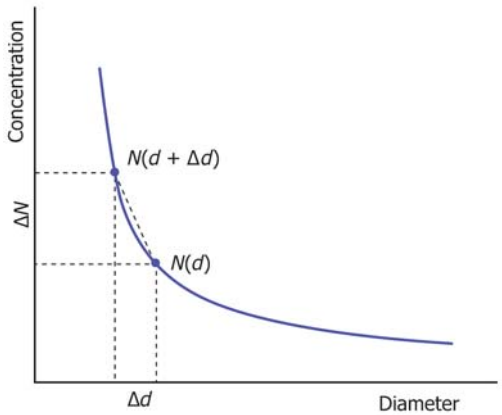
11.2 Particle concentration is determined by dividing the count of imaged particles (generally within specified size ranges) by the volume of imaged sample. The volume of imaged sample equals the number of image frames times the volume of liquid imaged for each frame. Direct measurement of the flow-cell thickness is difficult, so it is not generally practical to determine the imaged sample volume by dimensional measurements. Instead, the accuracy of particle concentration is assessed by measuring commercial or user-prepared

bead suspensions of known bead concentration. Regular use of particle concentration standards is also useful to confirm that the flow path through the instrument is not blocked by agglomerated particles.

11.3 Accuracy of particle diameter is especially important in determining the PSD of polydisperse particle suspensions, such as protein aggregates, where the particle count rises rapidly with a decrease in diameter. If the Dynamic Imaging Particle Analyzer reports diameters that are incorrect, the evaluation of which particles to include in a specified size bin will also be incorrect, as shown in Fig. 4.

11.4 In principle, particle diameter accuracy may be assessed by measuring beads of known diameter in the instrument, for a set of diameter values spanning the range of interest. These standard materials are usually particles that are spherical in nature when the actual particles being tested may not be this shape. In practice, the indicated diameter depends on the morphology and optical contrast of the particles of interest. Commercial diameter standards with a morphology and optical contrast typical of protein particles are under development, but are not yet available. Once such standards are available, they should be used to understand the biases of dynamic imaging analysis instruments. The reported equivalent diameter is inferred from a single image, so the reported diameter will vary for a non-spherical object depending on its orientation in the flow field. The development of algorithms to partially compensate for particle alignment is an active research topic. Additional challenges that are active research areas are how to correct the instrument diameter readings when the measured particles are highly heterogeneous in optical properties (for example, silicone oil and protein particles) and how to adjust the optical contrast of a standard to match the reduced optical contrast of protein particles in high-concentration solutions.

11.5 Instrument flow rate does not enter into the algorithm for determining particle concentration, and thus minor variations of flow rate do not cause significant variations in particle concentration. However, a flow rate that is too small will result in particles being counted multiple times during passage through the imaged volume, as well as excessively long run times, and a flow rate that is too high can cause reduced sampling efficiency and blurred images. Calibration of flow rate should be performed according to the manufacturer's recommendations to assure proper sampling of the test fluid.



NOTE 1—The curved line represents a typical PSD for a polydisperse particle suspension. The error in concentration is given by $\Delta N = -m\Delta d$, where m is the slope of the diagonal dashed line.

FIG. 4 Relationship Between a Diameter Error Δd and a Concentration Error ΔN

12. Troubleshooting

12.1 Problems in dynamic imaging can be divided into two categories: instrumental issues and sample issues. Common instrumental problems, along with recommended solutions, include:

12.1.1 *Improper focus,* which will result in incorrect particle diameters and differences in the measured morphological and intensity parameters (for example, circularity, intensity distribution, etc.). This problem will be indicated by a change in diameter readings PSL (polystyrene latex) beads, relative to the diameter obtained when the instrument was properly adjusted. Refocus the instrument according to manufacturer's recommendations.

12.1.2 *Large number of false positive particle counts*—False counts can result from intermixing of fluids of different refractive index, or from movement of a cell with dirty windows. For refractive index issues, prime the system with a larger volume of liquid with the same refractive index as the sample, or with the sample itself. Special care needs to be taken when there is the possibility of intermixing of fluids of different viscosities. For vibration issues, clean the cell, check that it is securely mounted, and minimize environmental vibrations.

12.1.3 *Gross misalignment of the light source,* which will cause a variation in the light intensity across the field of view. Adjust the light source according to manufacturer's recommendations.

12.1.4 *Inaccurate counts,* as indicated by a PSL bead suspension of known count as compared to manufacturer's qualified limits. First, ensure that the beads are fully dispersed by following the bead manufacturer's recommendations or by vigorously agitating the sample for 10 s and then sonicating for 20 s. Check for partial blockage of the cell (see below). If inaccurate counts are repeatable after bead resuspension, and for multiple bottles of PSL beads, change the flow cell and refocus.

12.2 Common sample problems, along with recommended solutions, are:

12.2.1 *A blocked cell,* which will cause a reduction in particle counts, especially of large particles. Backflush the cell or replace. In cases where it is known that much larger particles exist, it may be appropriate to pre-filter the sample to remove these larger particles but this in itself may be problematic. Samples with many large particles blocking cells cannot be accurately measured.

12.2.2 *High counts in blanks*—Replace polymer tubing or fittings upstream of the flow cell, or clean thoroughly with manufacturer-recommended cleaning solution. Wipe off and rinse with particle free water any mating fittings.

12.2.3 *Air bubbles*—Large air bubbles may occur during measurements of viscous samples if there is a loose connection. Tighten connections or run a reduced flow rate. Large

numbers of small air bubbles are a consequence of an inadequately degassed sample. Refer to the methods in USP <787>.

12.2.4 *High refractive index*—A high protein or excipient concentration will result in decreased particle contrast and possibly increased sample opalescence and viscosity. Of these effects, the decreased contrast is most serious, leading to erroneously small reported diameters. Although this general effect is well established, there is not yet consensus on how to correct for the reduced diameters. If the sample is opalescent, additional experiments may need to be performed in order to understand the influence on quantification. Ensure that it remains largely transparent for path lengths of the thickness of the flow cell. Viscosity increases do not directly affect the measurement provided that the flow rate is sufficiently small to avoid bubble creation by cavitation. Reduced flow rate is only feasible if the image acquisition rate can be reduced proportionately. The system flow rate should be verified when working with high viscosity samples (refer to manufacturer instructions).

12.2.5 *Lack of repeatability*—Sample counts may vary due to particle sedimentation, or changes in the PSD on mixing. The solutions to these problems are contradictory: reduction of sedimentation effects requires sample mixing, while stability of the PSD may require avoiding excess mixing. Systematically vary the stirring protocol, and evaluate the repeatability for each protocol.

12.3 There are several common quality assurance techniques applicable to dynamic imaging that are related to the instrument and the sample preparation. It is important to monitor repeatability and intermediate precision, run blanks and run standards of known concentration.

12.4 Some manufacturers implement proprietary algorithms to identify particles that are stuck on a surface. There is an alternative method that can identify partially blocked cells and particles that are stuck on a surface. Assume that the variable x measures the distance across the field of view transverse to the flow direction, and $N_t(x)$ is the cumulative number of particles captured for transverse pixel values between 0 and x. If the particles are randomly distributed, as they should be, then a plot of $N_t(x)/N$, where N is the total number of particles measured, will be a straight line. Steps in the line indicate stuck particles. Smooth deviations from a straight line are indicative of a clog or partial clog in the flow cell.

13. Data Reporting

13.1 Data reports may contain both quantitative information (generally the PSD) and qualitative information (particle images or observed attributes). Instruments may be optimized differently either to obtain high-quality images or to obtain accurate counts.

13.2 The particle size distribution (PSD) is most commonly reported as the particle concentration (particles per unit volume) as a function of the equivalent particle diameter. There are three common forms of the PSD:

13.2.1 *Cumulative PSD*—A histogram giving the total, or cumulative, number of particles per unit volume of equivalent diameter greater than or equal to the diameter d, plotted or tabulated as a function of d. Cumulative PSDs may be readily compared across instruments with different diameter bins and do not change magnitude with different diameter bins (However, each instrument may calculate the equivalent diameter on different basis, leading to differences in particle concentration). Another major advantage is that the cumulative PSD reports total particle loads of particles of interest above a specified size. A disadvantage is that the cumulative PSD does not readily identify the diameter region where most particles occur.

13.2.2 *Distributive PSD*—A histogram giving the differential number of particles per unit volume of diameter greater than or equal to the equivalent diameter d, but less than $d + b$, where b is the bin width. This quantity may also be plotted or tabulated as a function of d. To prevent misinterpretation, the width b should be fixed for all values of d on a given histogram. A distributive PSD is useful to identify the diameter regions where most particles occur.

13.2.3 *Binned PSD (a variant of distributive PSD)*—A histogram or table giving the number of particles per unit volume of diameter greater than or equal to the diameter d_i, but less than diameter d_{i+1}+1, for a small set of diameter values $\{d_1, d_2, \ldots d_N\}$. A binned PSD is a useful way to reduce a large data set to a small set of numbers.

13.3 Regardless of its form, the PSD will depend on how the diameter is inferred from the particle image, and the data report should clearly state or reference how the diameter was determined.

13.4 Report may also include summaries of subpopulations found through filtering or other methods used to characterize specific particle types such as silicone droplets or artifacts such as air bubbles.

13.5 In addition to the PSD, data reports may include or reference the following information:

13.5.1 Sample volume and preparation,

13.5.2 Liquid used for instrument priming and background measurement,

13.5.3 Instrument model, magnification settings and other pertinent parameters,

13.5.4 Imaged volume, or illumination zone if no flow cell is used,

13.5.5 The definition of diameter used in analyzing and reporting the data, and

13.5.6 Identification of any algorithms used to categorize particle subpopulations.

13.6 Other particle attributes may be reported as a function of particle diameter, including the aspect ratio, circularity, or Feret diameter. Reporting these parameters can be useful to monitor either the change of a particle attribute (for example, a needle-like crystal becoming shorter) or a change in the relative concentrations of two or more different particle types.

13.7 For PSD plotted over a wide range of diameters or particle concentrations, use of logarithmic axis scales helps the reader visualize particle concentrations over the full reported range.

13.8 Inclusion of representative images is optional and often helpful in classifying the particle. Images should cover a representative size range, and the report should specify if any image sorting took place prior to image selection.

13.9 A variety of other plots are available in commercial dynamic imaging analysis software. These plots are useful primarily in data analysis, categorization of particles into different subpopulations, or troubleshooting poor performance. Examples are plots of particle intensity, circularity, or aspect ratio versus diameter, to separate spherical air or oil droplets from irregularly shaped particles. Report may include potential next steps to improve process design and process improvement opportunities.

13.10 Additional data reporting considerations should include compliance to 21 CFR part 11 electronic record storage and retrieval, final testing-release and stability testing to end of shelf-life. The PSD profile typically will change over time, data can be evaluated over a statistically significant number of lots to develop an action level or limit that would be required for acceptance at time of release for distribution. Specific lots placed into the product stability program can be evaluated at selected intervals to measure any change in the PSD profile over time. The end of shelf-life data is useful in developing an expected overall action level or limit at the time of expiration. Action levels or limits can be derived from data analysis using the mean and three standard deviations or other statistical methods. The data should also be reviewed periodically for both positive and negative trends in relation to product and or batch specific performance.

13.11 U.S. FDA has requested that protein drug manufacturers characterize and quantify the inherent product proteins in the less than 10 µm range. Based on instrument resolution and sensitivity the range typically evaluated is 2–10 µm. Other methods are available to characterize inherent particle populations below 2 µm. Sub-visible particles in the 0.1–2 µm range could be qualitatively assessed. This is in addition to the requirements for USP <787> subvisible particle counting greater than 10 µm and greater than 25 µm. In addition to the subvisible PSD, the expected inherent particle population extending into the visible range >100 µm should be well known.

14. Keywords

14.1 biologics; biopharmaceutical; diameter; direct imaging; droplet; dynamic imaging; flow imaging; flow microscopy; parenteral; particle; particle size distribution; protein; protein aggregates; subvisible particle

APPENDIX

(Nonmandatory Information)

X1. SINGLE PARTICLE VERSUS ENSEMBLE DETECTION TECHNIQUES

X1.1 Particle size analysis techniques can be divided into two families: those techniques that rely either on the detection and counting of single particles, and those that simultaneously measure an ensemble of particles. Single particle measurement techniques include both dynamic imaging and techniques in which particles are counted as they flow through a small orifice (for example, electrical sensing zone, light obscuration, and flow cytometry). Dynamic imaging particle analyzers infer particle size from the projected geometrical cross section of a particle. Other types of particle analyzers infer particle size from different particle properties (for example, displaced volume for electrical sensing zone techniques, optical cross section for light obscuration and the light scattering channels of flow microscopy.)

X1.2 Single particle techniques determine particle count directly, by summing the counts of individual particles. When the count is divided by the instrumental determination of the imaged volume of the sample, the particle concentration (counts per unit volume) is obtained. The PSD thus obtained is an absolute determination of the particle concentration for stated size intervals, provided that all particles are counted, and that the fluid volume and particle size determination are correct. Note that the imaged volume of the sample is the sum of the fluid volume within the field of view of each microscopic image, and not the total volume of sample that passes through the flow cell.

X1.3 Ensemble techniques, in contrast, obtain a PSD from analysis of a measured distribution (for example, the angular dependence of scattered light). For most ensemble instruments, the reported PSD gives the relative number or frequency of particles as a function of size. This approach works well when the mass or volume of the particles can be measured independently (for example, one could measure the PSD of a measured mass of a powder form of a pharmaceutical ingredient) but has limited utility in assessing the prevalence of particle impurities in biopharmaceutical samples. Some ensemble instruments are capable of reporting the absolute particle concentration, but such values depend on assumptions of the particle type and properties, and these assumptions should be verified as appropriate for the particle suspensions being measured. Accurate deconvolution of the experimental signal to obtain a PSD may be difficult when the particle sizes cover a wide size range (for example, a factor of ten or more).

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