USP Particle Count Standards with Aura and Particle Vue 3.1

Introduction

There is a need for an accurate and reproducible method to count bead counting standards for all membrane microscopy methods. Counting microsphere particle standards on membranes is problematic due to their significant aggregation upon filtration, a problem that does not commonly occur with real biopharmaceutical particles.

In this technical note, we present a validated methodology for accurately counting United States Pharmacopeia (USP) microsphere count standards by seamlessly calculating the cumulative area of all the microspheres in a sample and dividing that value by the area of a particle singlet. This new approach allows users to validate absolute counting with their Aura™ instruments in accordance with USP guidelines using validated counting reagents.

Methods

Preparation and Measurement

We validated this approach using a thorough inter-user (3), inter-instrument (4), and inter-bottle (6) methodology. First, a single Aura membrane plate was backgrounded on four separate Aura systems. USP counting beads measuring 10 µm (Thermo CC10-PK Count Cal Standards) specified at 3000 beads/mL ±10%, were used. Each bead bottle was brought to room temperature and then shaken for 30 seconds by each individual user, before each user pipetted 50 µL of standard into 8 different wells. The membrane plate was dried and then imaged on each of the four Aura instruments.

Data Analysis

The beads were counted using two orthogonal approaches.

1. Automated: Beads were counted manually from the Backgrounded Membrane Imaging (BMI) whole membrane brightfield images to ensure the pipetting precision and count standard quality for each of the three users
2. Automated by software: Calculated using the following equation:

$$Counts = \frac{\sum_i Area_i}{Area_{th}}$$

where $Area_i$ is the area for each cluster of beads (aggregated or not) and $Area_{th}$ is the theoretical area of a bead singlet. This area calculation was performed directly using the Expression Engine in Particle Vue software version 3.1 as shown in Figure 1 using the Action **Sum** for the Item **Area**.

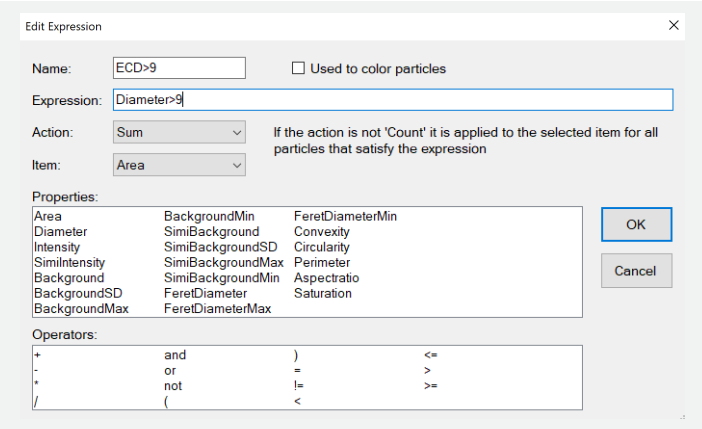
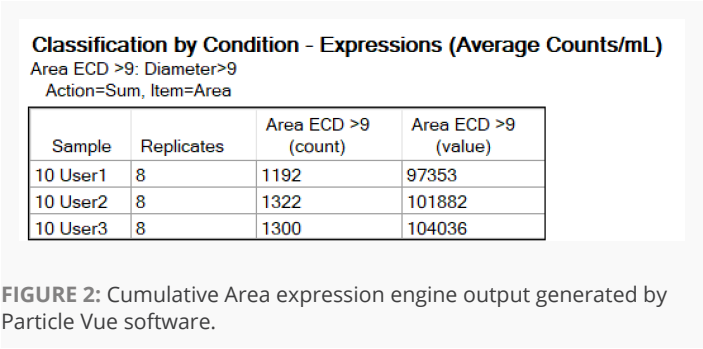


FIGURE 1: Cumulative Area Calculation analysis performed using the expression engine in Particle Vue software.



Classification by Condition - Expressions (Average Counts/mL)
Area ECD >9: Diameter>9
Action=Sum, Item=Area

Sample	Replicates	Area ECD >9 (count)	Area ECD >9 (value)
10 User1	8	1192	97353
10 User2	8	1322	101882
10 User3	8	1300	104036

FIGURE 2: Cumulative Area expression engine output generated by Particle Vue software.

This expression only evaluates particles whose Equivalent Circular Diameter (ECD) is >9 µm for the 10 µm beads in order to filter broken beads and other contaminants in the bead vials. The output from the expression engine can be found in the experimental reports section, on the expressions tab (Figure 2).

The values in the "Area ECD >9 (value)" column represent the total area of all particles with an equivalent circular diameter (ECD) >9 µm as shown in Figure 2. Dividing this number by the area of a single 10 µm bead, using the average diameter specified by the vendor (e.g. 78.7 μm^2 for a 10.01 µm bead) will result in the number of beads present in all wells for a single user or condition. Simple math allows users to convert this data into average counts/mL. The following sample calculation was performed for User 1 using the equations below:

$$Area = \pi * \frac{10.01^2}{4} = 78.7\ \mu m^2$$

$$\frac{97353}{78.7} = \frac{1237\ total\ beads\ on\ the\ membrane}{for\ 8\ wells\ for\ User\ 1}$$

$$\frac{1237\ beads}{8\ wells} = \frac{154.62\ beads}{well}$$

$$\frac{154.62\ beads}{well} * \frac{1\ well}{50\ \mu L} * \frac{1000\ \mu L}{mL} = \frac{3092.4\ beads}{mL}$$

For each bead type, this calculated cumulative area was then divided by the area of a 10 µm bead (specified at 10.01 µm by Thermo) to result in the total number of singlet beads for each bead type.

Results

Manual counting revealed that all users were precise in their pipetting and that all 6 vials were prepped within the ±10% concentration margin of error (Figure 3a). Additionally, manual counts displayed consistency across all four Aura systems, proving inter-machine imaging reliability. The output of the expression engine, here named "Bead Sum Area", was divided by the area of the 10.01 µm (78.7 μm^2) bead, using a simple Microsoft® Excel® table. The data (Figure 3b) show that using this methodology produces accurate and robust absolute particle counting cross all four Aura systems, all users, and all bottles by using validated USP count standards. This methodology counted an average of 3225 ±10% of the 10.01 µm beads/mL between all users and machines. These numbers match the expected counts for these standards.

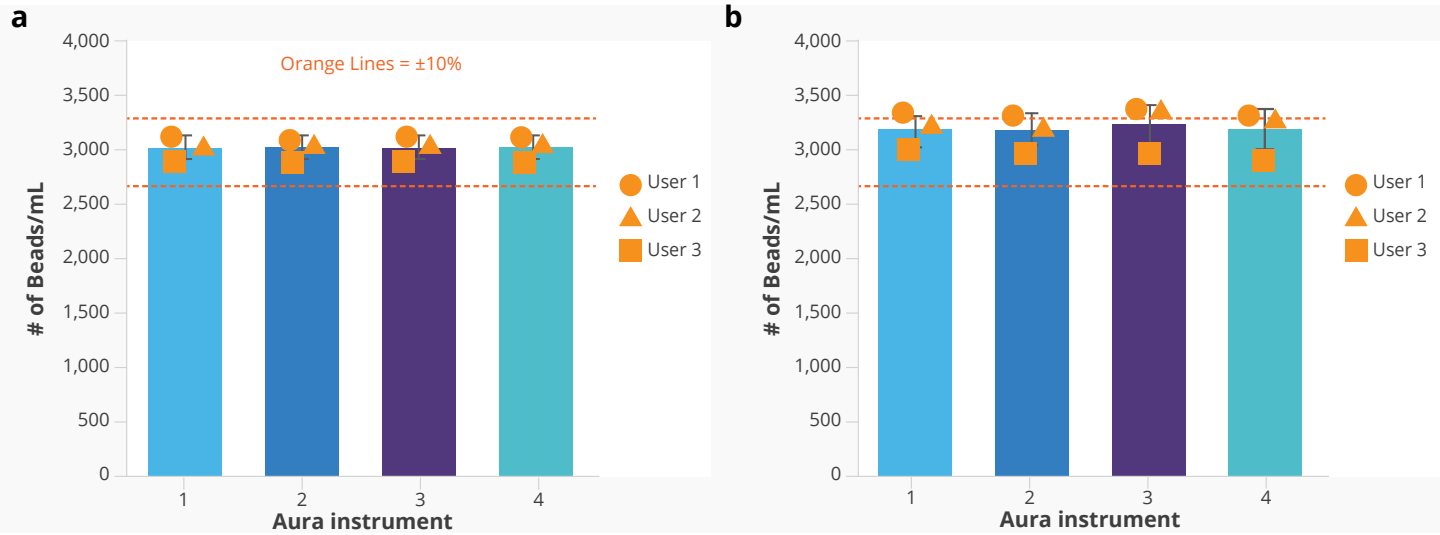


FIGURE 3: Inter-instrument and Inter-user counting 10 µm count standards. The data shown is an average of the data collected by 3 individual users. (a) Manual counting of beads using the BMI membrane image. (b) Bead counts calculated with an area-based approach using the expression engine in Particle Vue software v3.1. All counts are within the error specifications defined on the bottle (±10%)

Discussion

The data in Figure 3 shows that using the expression engine area method in Particle Vue software version 3.1 the Aura performs reliable, reproducible absolute particle counting even when accounting for inter-instrument and inter-user variability. Manual counting confirms that beads were formulated as specified, provide an orthogonal verification method, and demonstrates that 50 µL samples are enough for accurate absolute counting even if concentrations of 3000 particles/mL mean that only 150 particles are on the membrane. Furthermore, the method is accurate using the listed sizes of the counting standards and is an additional level of confirmation of the precision of the instrument. Interestingly, this method essentially served as a surrogate mass measurement: By calculating the total amount of material (integrating across all particle areas), it shows that there is very valuable information of the sample by looking beyond traditional particle counts and size. Variability in counting totals of these USP bead standards is dependent on instrument accuracy, pipetting accuracy, inter-bottle variability, and intra-sample variability. We have shown here that manual counts are within ±1% between instruments, however single users can display a high degree of variance within multiple pipetted wells. Additionally, each bottle of beads is not homogeneous and is not always consistent with other batches and lots. For these reasons we recommend allowing ±15% variability from the expect count standard of 3000 beads/mL. Users should reliably produce counts between 2550 and 3450 beads/mL for 10 µm beads with multiple users and between multiple instruments.

On rare occasions, users may experience count totals from the area counting method that do not match the manual counts. This is largely due to the heterogeneity of the bead bottles, bead degradation during storage, and the presence of broken beads and contaminants in the samples which may result in overcounting. Fortunately, we present software data filtration methodologies can deal with imperfect bottles, whose imperfections are shown in Figure 4, with lots of particles measuring below 10 µm and displaying SIMI signatures well below what the bead clusters show.

We used Side Illumination Intensity (SIMI) as an additional data filter to improve data analysis. SIMI is a surrogate measurement for particle height, with objects that protrude out of plane showing greater side angle scattering. Because many dust and debris particles are not as tall as the USP count standard beads, they display significantly less SIMI intensity. Setting a threshold to eliminate particles with an intensity <15 removes flat dust from the analysis. The expression shown in Figure 5 below will perform analysis exactly as above and discard any particles with average

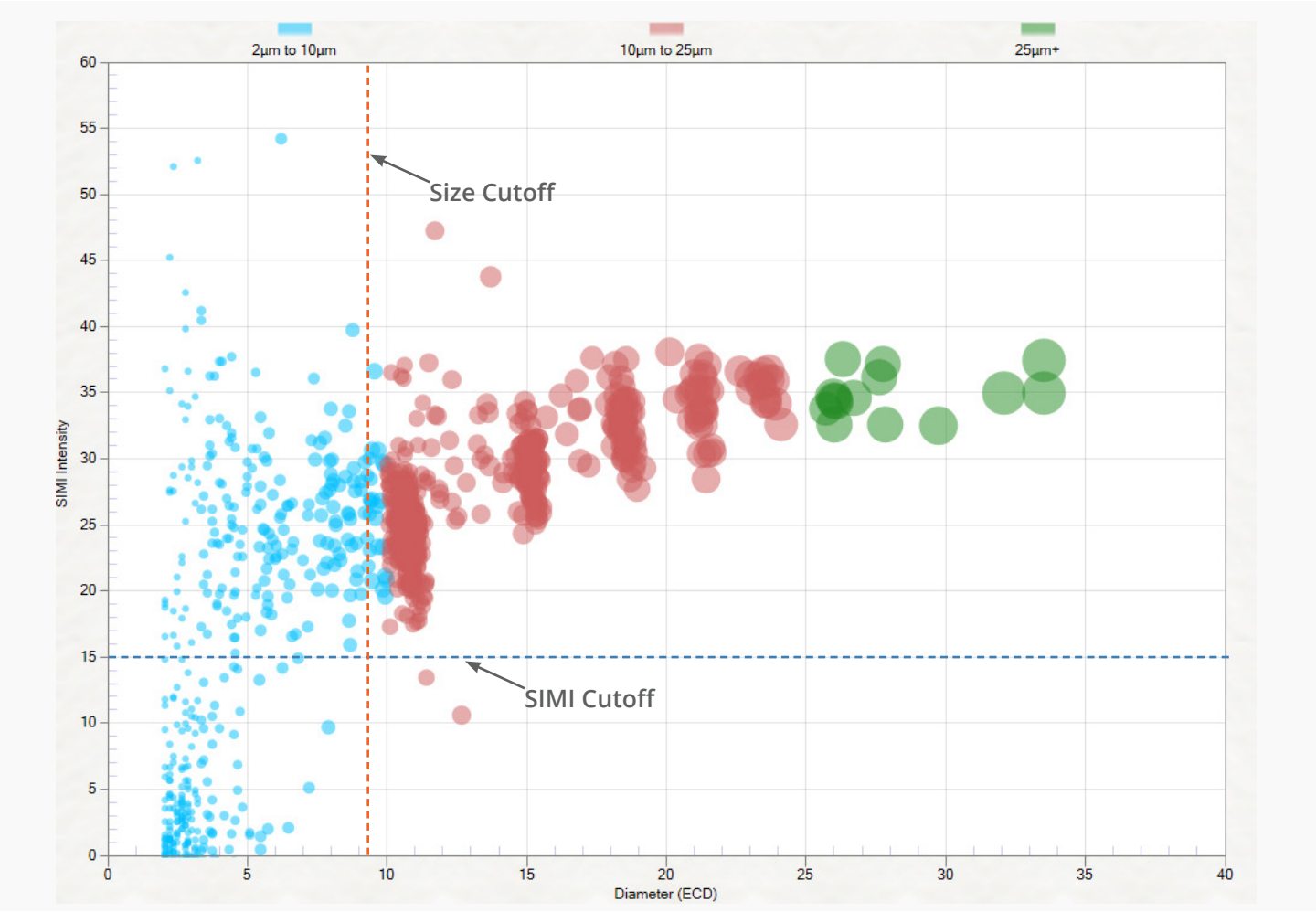


FIGURE 4: Introducing SIMI cutoffs to remove dust particles improves the area calculation.

SIMI intensity <15. On very rare occasions, the software may experience clipping issues due to beads rolling on the membrane during analysis, and as a result will report lower than expected counts. To address this, simply lower the diameter threshold to include clipped singlets. We recommend lowering the threshold by another 10% of the total diameter to 8 µm for the 10.01 µm beads.

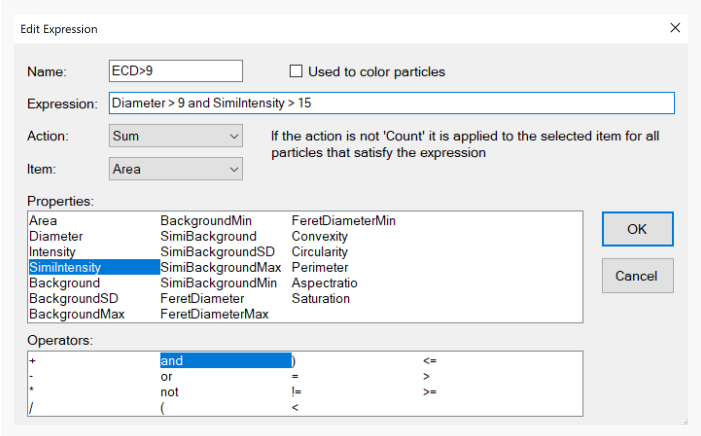


FIGURE 5: Cumulative Area Calculation using the expression engine with a SIMI filter applied.