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APPLICATION NOTE

Detection of Glass Shards in Protein Therapeutics with the FlowCam

OVERVIEW

One of the challenges facing formulators of protein therapeutics is the ability to properly characterize subvisible particulates. Some of the particles that may be found, such as inherent protein aggregates or silicone droplets, may not be as potentially harmful to a patient as other types of particles such as rubber or glass shards.

On March 25, 2011 the US FDA, in their Drug Safety and Availability section, issued an "Advisory to Drug Manufacturers: Formation of Glass Lamellae in Certain Injectable Drugs"[1]. This warning advised of "the potential formation of glass lamellae (glass fragments) in injectable drugs filled in small-volume glass vials". The FDA warned that the presence of these glass fragments (or shards) had caused several drug recalls[2].

While these shards may well be spotted during visual particle inspection of the finished vial, the literature shows that subvisible glass particulates occur long before they would be seen by visual inspection[3]. Due to their transparency, these glass shards are extremely difficult to detect, as can be seen in Figure 1.

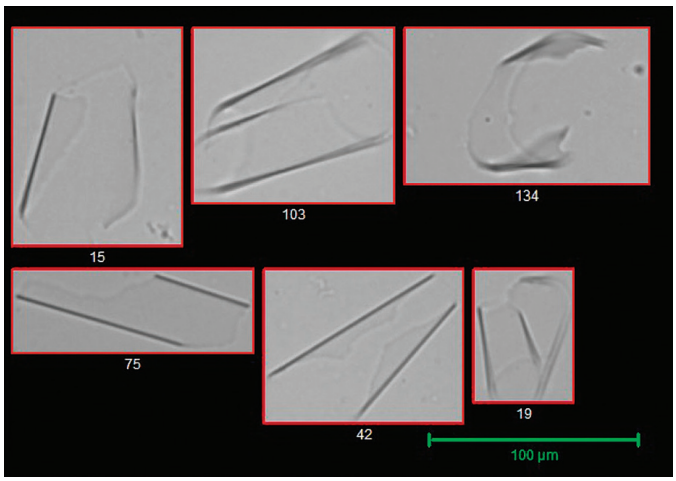

Figure 1: FlowCam images of glass shards

An investigation was made into the best methods for detecting glass shards using the FlowCam and VisualSpreadsheet® software.

METHOD

A test sample was created by combining a heat-stressed BSA sample with buffer spiked with subvisible glass shards (obtained from another vendor studying the same problem). The final sample had an overall particle load of around 250,000 particles/ml. Three runs of data were collected using the FlowCam for this sample, showing good repeatability (±3%). For each run, the software also collected the raw files (entire field of view captured by the camera for each frame) for subsequent reprocessing. One of these three runs was selected for detailed analysis, and then 20 raw files which contained glass shards were isolated and saved along with a calibration image for the final analysis.

A unique capability of the VisualSpreadsheet software is the ability to reprocess raw files for a specific run in order to vary acquisition parameters to see the effect of this variation. Prior research has shown this process to be an invaluable tool for optimizing Flow Imaging Microscopy (FIM) acquisition parameters to best capture protein aggregates without fractionating larger aggregates into smaller pieces[4]. The same technique used in that prior research was used here to determine which settings would be best to capture the glass shards while still maintaining the integrity of the other particulate data, especially the protein aggregates.

SUMMARY RESULTS

While all 20 of the raw files were carefully studied with each rerun of the data using different settings, a single raw file will be used here to demonstrate the results (see Figure 2). As discussed in the prior research[4], a combination of increasing

APP NOTE CONTINUED ON REVERSE

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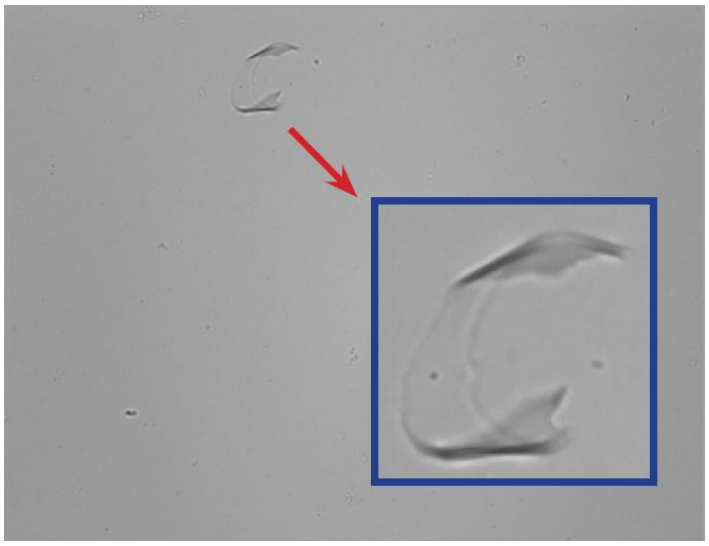

Figure 2: Raw file chosen for example, showing enlargement of the glass shard it contains in the inset

threshold sensitivity (for both darker and lighter pixels than the background) and "neighborhood analysis" is used to reduce fractionation of transparent particles, thus better characterizing the size of larger protein aggregates.

An algorithm has been introduced into VisualSpreadsheet which provides more accurate neighborhood analysis for grouping together fractionated particles. The intent was to use both sensitivity and the new neighborhood algorithm to optimize the settings.

Each time the raw file is reprocessed with different threshold settings, a collage image file is created showing the particles as detected from the raw file using the current threshold settings. The only variance between each run are the threshold settings, so those settings are isolated as the only potential cause of differences in the resultant collage file output.

Figure 3 show the results of processing the rawfile using a threshold of 25 levels darker than the background level (where levels refers to gray scale intensity of each pixel, from 0 to 255 with 0 being black and 255 being white). As can be seen, the glass shard in the rawfile has been fractionated into two large pieces which would not be recognizable as glass shards (by either an algorithm or by human eye.

Using the technique described in the prior research[4], it was found that the optimum thresholding settings for this raw file would be 12 levels darker and 12 levels lighter.

Figure 4 shows the resultant collage file using these threshold settings. You can see that although the shard is still cut into two pieces, each piece is now large enough to at least be recognizable as glass shard by the human eye.

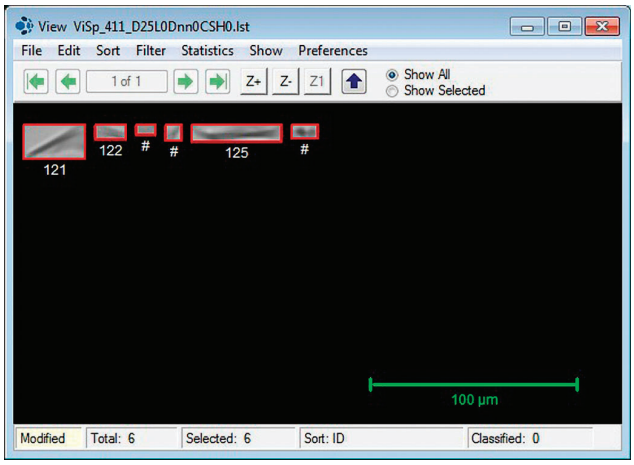

Figure 3: Raw file reprocessed with threshold 25 Dark only

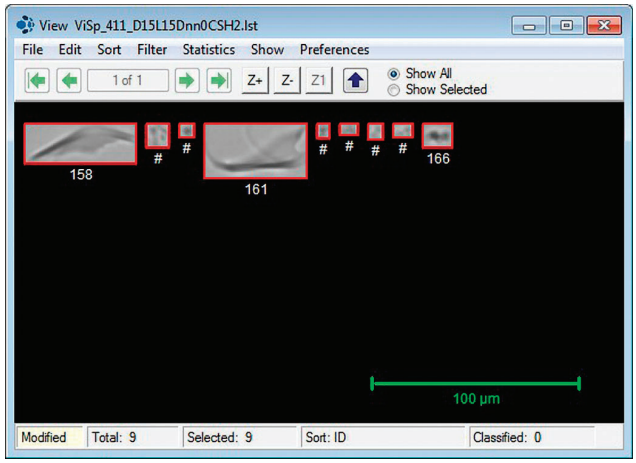

Figure 4: Raw file reprocessed with threshold 12 Dark and 12 Light

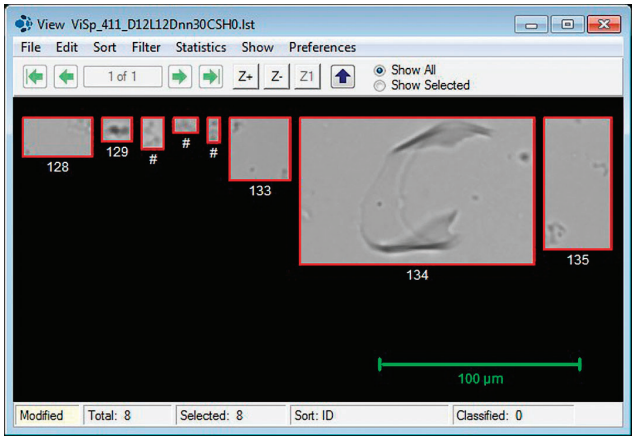

Figure 5: Raw file reprocessed with threshold 12 Dark and 12 Light, plus DNN= 30 µm

The final step of the process is to use the neighborhood processing algorithm of VisualSpreadsheet to pull the two pieces of the shard together. Figure 5 shows the results of adding in a Distance to Nearest Neighbor (DNN) of 30µm to the thresholding. The algorithm essentially looks for pixels that might be a part of the same particle within a distance of 30µm of any current particle pixel.

As can be seen in Figure 5, the glass shard has now been pulled together into a single, complete particle image. While increased sensitivity does pull the two parts of the shard together, it may also add artifacts by counting pixels that do not appear to be particles ("noise") as particles, while also potentially grouping them together into larger pseudo-particles. If the sensitivity and neighborhood size is increased too far, the results will become skewed to show higher concentrations than are really present.

After extensive experimentation, a "compromise" set of threshold/neighborhood parameters was found that best kept the shard intact and visibly identifiable without introducing artifacts.

CONCLUSIONS

FIM systems can be used to detect glass shards in protein therapeutics. FlowCam and VisualSpreadsheet enable the combination of dark and light thresholding, along with neighborhood analysis necessary to optimize the system in order to best capture these very transparent particles without introducing artifacts.

REFERENCES

1. US FDA (2011) Advisory to Drug Manufacturers: Formation of Glass Lamellae in Certain Injectable Drugs.
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3. R.G. Iacocca, N. Toltl, et al. (2010) *Factors Affecting the Chemical Durability of Glass Used in the Pharmaceutical Industry* AAPS PharmSciTech, 11(3): 1340-9. DOI: 10.1208/s12249-010-9506-9
4. L. Brown, W. Bernt (2014) *The Importance of Thresholding in Imaging Analysis of Protein Aggregates* AAPS National Biotechnology Conference 2014, San Dieg, CA

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