

Nota applicativa

SIMI: A Different Approach to Revealing Dangerous Subvisible Impurities

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Introduction

Foreign particulate matter, also known as extrinsic particles, has become a serious threat to patient safety in recent years, with cases linked to severe immunogenic reactions, deaths, and costly recalls.¹ In 2021 alone, Takeda recalled 1.6 million doses of the Moderna COVID-19 mRNA vaccine in Japan after several patient deaths were linked to steel particulates shed from a malfunctioning pump.² Over the span of seven years, from 2014–2017, there were more than 110 drug recalls as a result of glass delamination,^{3–4} and several more due to remnant crystals,⁵ owing to the difficulty of identifying the presence and source of foreign particulate matter. To prevent against such threats, manufacturers require high throughput technologies to better identify foreign particulate matter in real time.

Results and Discussion

Shining a Light on Extrinsic Particles with SIMI

Side illumination membrane imaging (SIMI) is a proprietary illumination, imaging, and analysis technique exclusively available to Aura instruments. Utilizing the principles of darkfield microscopy,⁶ SIMI is conducted on

an Aura membrane plate using an oblique illumination source which emits light at an angle parallel to the surface of the plate.

Compared to backgrounded membrane imaging (BMI), which utilizes brightfield microscopy (epi-illumination) where particles scatter the incident light isotropically (*i.e.*, at all angles), side illumination membrane imaging (SIMI) is a technique that is specifically designed to detect light scattering from high refractive index particles, as well as those that protrude out of the plane from the membrane surface. A particle's average SIMI intensity is measured in a relative scale where the inherent SIMI scattering of the membrane is defined as $SIMI = 0$. Positive SIMI values indicate particles protruding out of the membrane, as is the case with microplastics, fibers, and other common extrinsic particles, whose average SIMI intensities vary from 0–255. On some occasions, like with metallic particles and absorbing oils, particles will have reduced light refraction relative to the membrane and thus display negative SIMI values, varying from 0 to -122.

The majority of foreign, extrinsic particles exhibit a high refractive index and are mechanically rigid in structure, causing them to protrude from the membrane surface and scatter side illumination to a high degree, ideal for SIMI analysis. In contrast, biologic particles exhibit the opposite properties—low refractive index and malleable structures, causing them to lie flat with minimal side scatter profiles, which is best analyzed with BMI or fluorescence membrane microscopy (FMM). Utilizing all three microscopy approaches is most ideal to facilitate the determination of all extrinsic (non-biologic) and inherent (biologic) particles in biologic formulations.

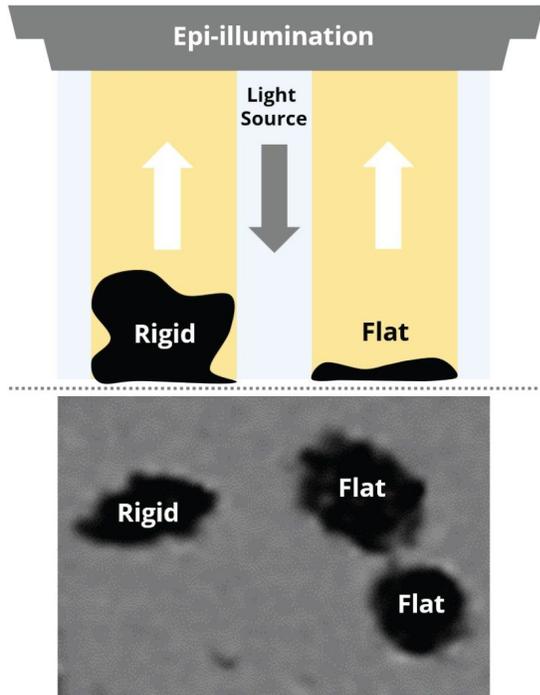
Using SIMI to Distinguish the Indistinguishable: Extrinsic Particles

Protein Aggregates vs. Ethylene Tetrafluoroethylene

The National Institute of Standards and Technology (NIST) created a protein aggregate mimetic reference material made from ethylene tetrafluoroethylene (ETFE). ETFE (NIST RM 8634) particles were designed to have similar morphological features, refractive index, and size distribution of common sub-visible protein aggregates.⁷ Using standard brightfield illumination, ETFE particles are indistinguishable from protein aggregates in a mixed population.⁸ The addition of the SIMI channel enables the rapid and accurate differentiation of plastic (rigid) from protein (flat, soft) particles.

Biological aggregates, such as protein and viral vectors, are often mechanically compliant and thus lay flat on the surface of the membrane after filtration (Figure 1). Coupled with low refractive index (proteins have similar optical properties as water), their SIMI signature is very weak and often indiscernible from the membrane, as evidenced in Figures 1b and 2b.

A. Backgrounded Membrane Imaging (BMI)



B. Side Illumination Membrane Imaging (SIMI)

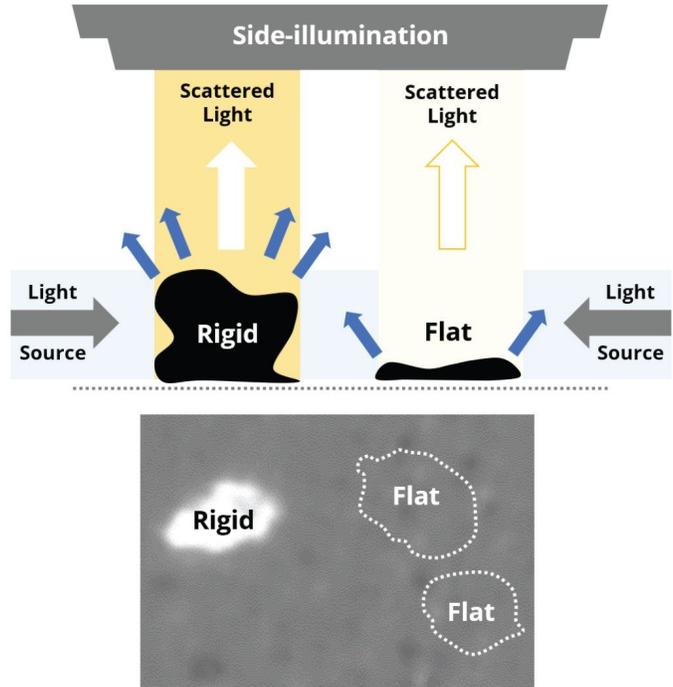


Figure 1. Schematic of the impact of light scattering on rigid and flat (soft) particles via (a) backgrounded membrane imaging (BMI) demonstrating isotropic scattering and (b) side illumination membrane imaging (SIMI) with enhanced side scattering.

In Figure 2, we show subvisible IgG protein aggregates imaged with both BMI and SIMI. BMI provides high contrast images of protein aggregates appearing as dark particles, while in the SIMI channel they are virtually invisible.

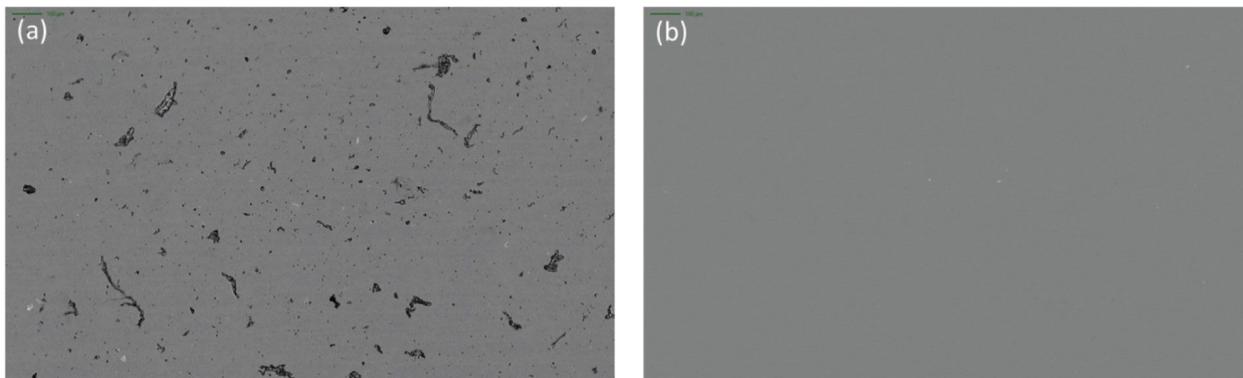


Figure 2. IgG protein aggregates imaged in (a) BMI and (b) SIMI.

While ETFE particles display similar high refractive index contrast to protein aggregates, they appear morphologically and optically similar. However, ETFE's rigid nature makes these particles exhibit bright scattering signatures in the SIMI channel (Figure 3). As extrinsic and inorganic particles sit high on the membrane after filtration, distinguishing extrinsic particles is both facile and rapid, avoiding the requirement for complex machine learning algorithms that are often required in flow-based techniques.

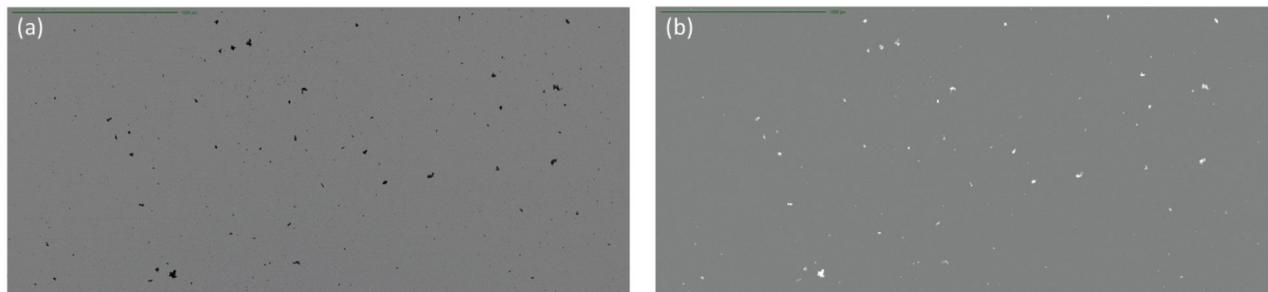


Figure 3. ETFE imaged in (a) Brightfield and (b) SIMI.

To highlight the increased side-scattering of rigid materials, a solution containing a mix of ETFE and IgG aggregates was used, which was fluorescently stained with thioflavin T (ThT), and detected using FMM. When used in conjunction with SIMI, it is possible to define subpopulations on Aura instruments using appropriate

characteristics for both extrinsic particles (ThT -ve; SIMI +ve) and protein aggregates (ThT +ve; SIMI -ve) as visualized in Figure 4.

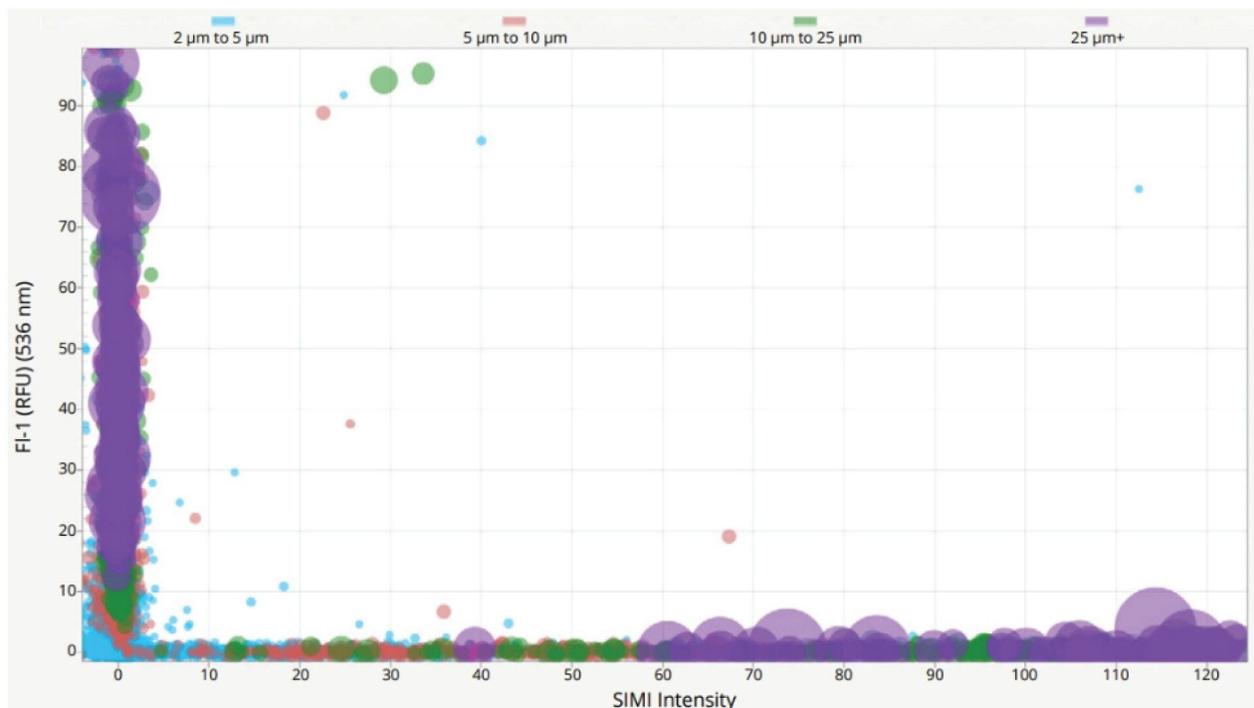


Figure 4. A 3D scatterplot of SIMI positive particles (ETFE, x-axis) and ThT positive aggregates (Protein, y-axis) of a mixed sample of ETFE and protein.

Particles Shedding from Common Biological Containers

Glass Particles

Glass particles, often generated from defective vials, are considered dangerous subvisible particles due to their ability to puncture capillaries and accumulate in tissues. In fact, this is one of the leading causes of product recalls.³ They present a significant analytical challenge as glass particles are optically transparent and have low refractive index similar to commonly used drug medias ($n = \sim 1.5$). Flow based technologies like flow imaging microscopy and light obscuration (LO) have inherently low refractive index contrast (water refractive index, $n = 1.33$ making detecting these particles nearly impossible. Aura System utilizes BMI and SIMI to effortlessly detect glass particles, thanks to the inherent high contrast imaging (Figure 5a) and strong side scattering due to the

inherent rigidity of glass (Figure 5b) provided by separating the particles of interest from the media it is in.

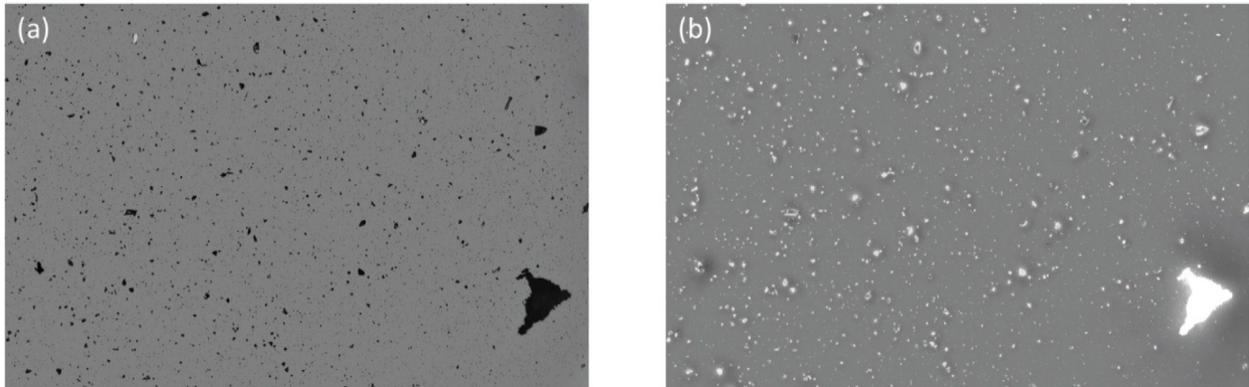


Figure 5. Glass particles imaged in (a) BMI and (b) SIMI.

Polypropylene Particles

Another common source of contaminating extrinsic particles is those generated from a drug vial, container or tubing. For example, threaded polypropylene vials tend to shed many subvisible particles, which are distinguishable by their SIMI signature. Particles greater than 5 μm equivalent circular diameter (ECD) represent significant side scatter and thus are easily detected (Figure 6). This is essential to properly identify the source of subvisible particles.

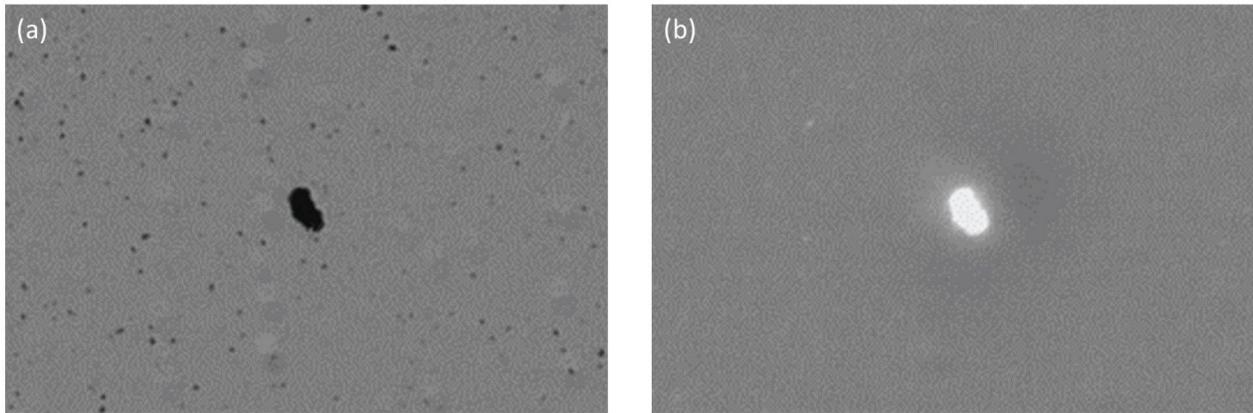


Figure 6. Polypropylene particles derived from a Corning Cryovial® containing WFI imaged using both (a) BMI and (b) SIMI.

Other Extrinsic Contaminants

Extraneous Fibers

The complexity of biologics manufacturing results in the increased potential for extraneous materials and fibers contaminating the final drug product. Fibrous subvisible particles, including those generated from clothing, filtration membranes, and syringe filters, all within the manufacturing environment, have two key identifying features, rod-like morphology defined by a low aspect ratio and strong SIMI intensity. Extraneous contaminants compare very favorably against inherent particles derived from the drug product when using SIMI as the distinguishing feature (Figure 7).

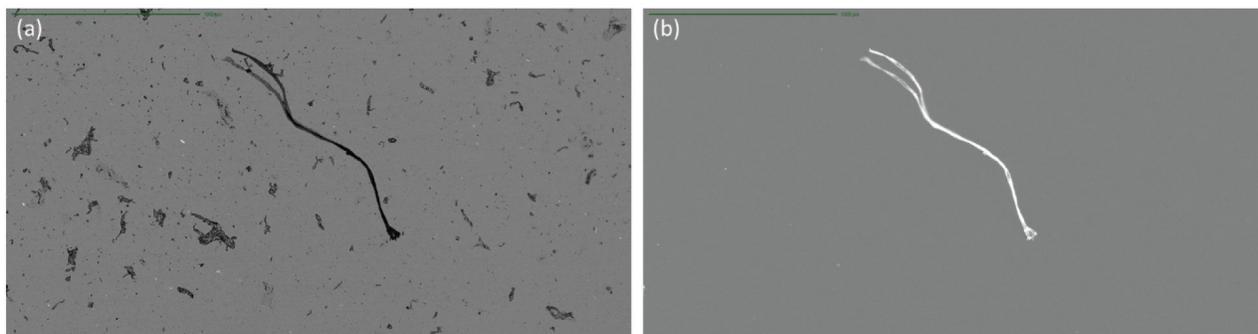


Figure 7. Brightfield image of mixed population of inherent particles of IgG and a single extraneous fiber using BMI and (b) when imaging in SIMI inherent particles disappear while the extraneous fiber exhibits a strong simi signature and an aspect ratio of 0.12.

Residual Dynabeads

In certain instances, as a key part of the biomanufacturing process, extrinsic particles may be intentionally added to drug product and removed prior to product release. One such instance is the addition of CD3+/CD28+ Dynabeads™ (Thermo Fisher Scientific®) to stimulate the activation of CAR-T cells to drive clonal expansion. These magnetic beads are subsequently removed from the product, however residual Dynabeads populations have the potential to present an immunogenic risk to the patient.

Current methods for quantifying residual Dynabeads rely on painstaking manual hemocytometry. Additionally, distinguishing Dynabeads from cells or cell debris is a significant challenge due to the high cell concentrations present in cell therapies, creating a longstanding challenge in CAR-T cell quality control. Aura systems can use morphology, sizing, and SIMI analysis to identify these "needle in a haystack" particles. The relative SIMI signature of Dynabeads, on a white plate, result in a negative SIMI profile which enables their distinction from the CAR-T cell therapy and other extraneous particles, including polystyrene beads (Figure 8). In addition, Aura instruments have the ability to perform particle analysis (counting and sizing particles) directly in SIMI mode to count Dynabeads directly without having to worry about the presence of high cell concentrations, as expanded in Tech Note 4.

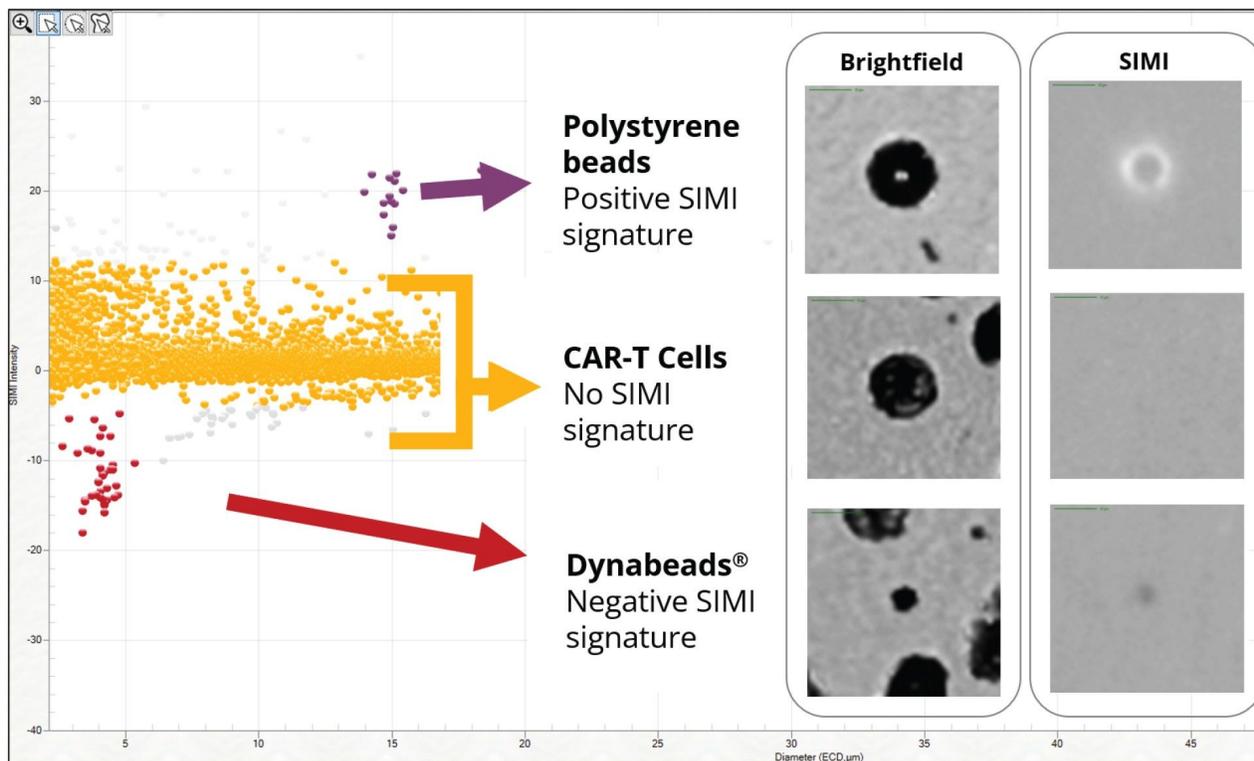


Figure 8. Dynabeads (red) can be distinguished from other subvisible particles, CAR-T Cells (yellow) and 15 μm Count-Cal polystyrene microspheres (purple) using ECD (x-axis) and SIMI intensity (y-axis). Inset are example brightfield and SIMI images for polystyrene microspheres (top), CAR-T cells (middle) and Dynabeads (bottom).

Using SIMI to Distinguish the Indistinguishable: Intrinsic/Inherent Particles

Small Molecule Crystals

The ability to define subpopulations of particles derived from the inherent drug product is an important distinction. We have previously demonstrated how this can be achieved in biologic drug formulations, however in small molecule therapies being able to determine crystal forms of the API is critical. It is possible to characterize the insoluble particles as either amorphous or crystalline by combining both BMI and SIMI. Small molecule crystals have very characteristic angular rod like morphology and exhibit strong SIMI signature, compared to amorphous particles (Figure 9).

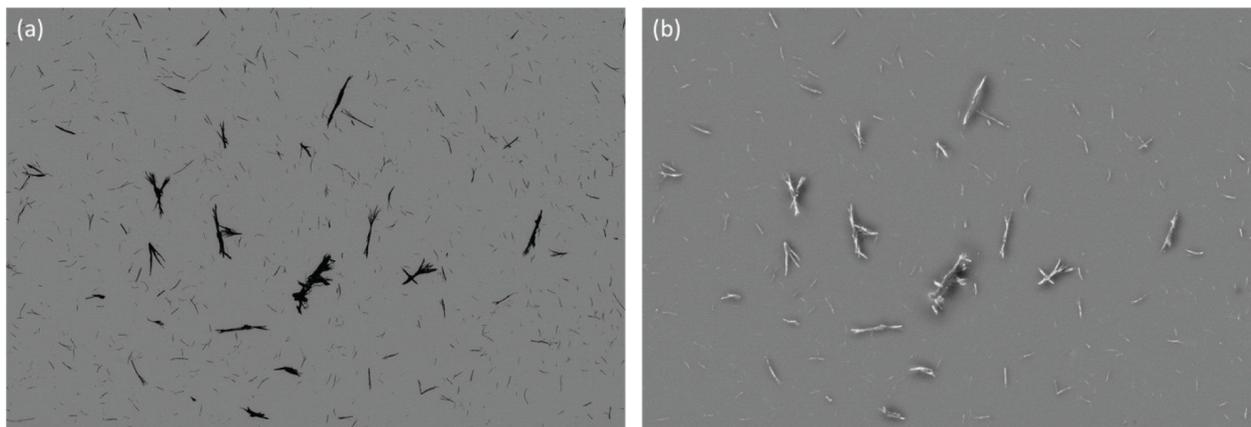


Figure 9. Insoluble dipyrnidamole crystals imaged in (a) BMI and (b) SIMI helping reveal the intricate three-dimensional morphology of these drug crystals.

Embedded Extrinsic and Intrinsic Particles

The complex relationship between drug product, excipients, and other particles can result in significant mixing of distinct types of particulate matter in a biopharmaceutical sample. Particles, be they extrinsic or intrinsic/inherent to the therapeutic, can provide nucleation sites for aggregation. The combination of BMI and SIMI can help elucidate occurrences of biological material interacting with non-biological particles (Figure 10). Composite particles, like these, are challenging to characterize using flow imaging systems or systems without multiple light sources and detection capabilities. The SIMI module in Aura instruments provides an additional dimension to analyzing extrinsic and intrinsic particle mixes.

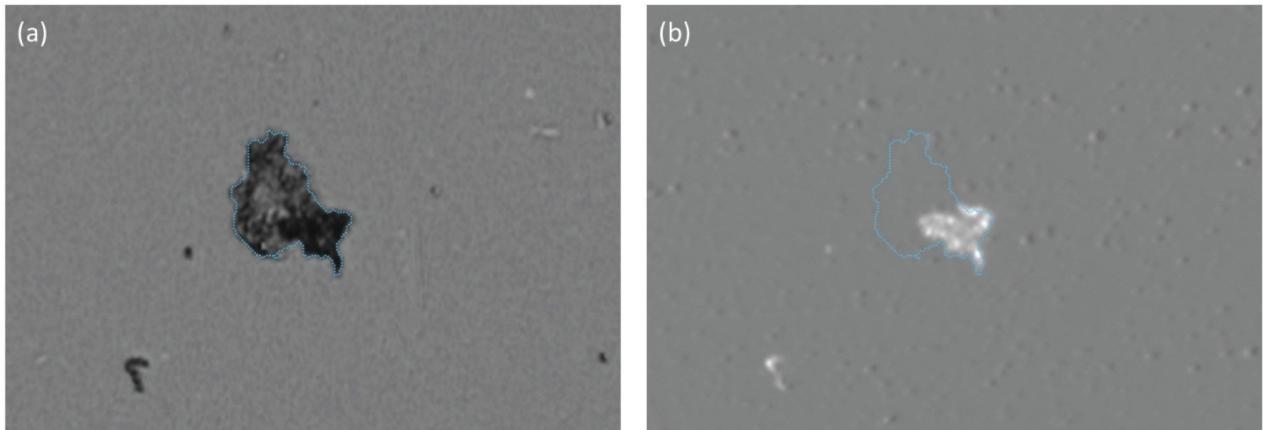


Figure 10. (a) In BMI mode a large (44 μm particle) from a mixture of ETFE and human IgG aggregates can be clearly identified, (b) in SIMI mode the particle can be defined as a composite of both plastic (ETFE, bright SIMI region) and biologic (IgG, invisible SIMI region).

Conclusion

SIMI is a unique approach to providing additional characterization of subvisible particles and is available in all Aura models to help rapidly identify foreign material in biological samples with a high degree of specificity. A key tertiary imaging mode in addition to BMI and FMM, SIMI provides selective, orthogonal morphological information to identify tall and rigid materials, a defining feature of most extrinsic particles (Figure 11). While particle sizing and counting is done via BMI, SIMI provides illumination, imaging, and analysis that can be useful across a variety of biological products to reveal foreign subvisible particles from the earliest to the latest of stages in the bioprocess.

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