TrueCourse Biosciences

WHITEPAPER — CRYO-TEM

Quality control of AAV production with cryo-TEM

Evaluation of viral vector purity is critical for AAV-delivered therapies

Cryo-TEM can be used to study particles in their native state, discriminating between partially full, empty and full capsids, to support the analysis of viral vector purity in the development and scale-up of vector production processes. Cryo-TEM images also provide information on morphology (size, structure) and the presence of aggregation.

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The Challenge

Adeno-associated virus (AAV) vectors are one of the leading platforms for gene delivery for the treatment of a variety of human diseases. The success of AAVs in preclinical and clinical testing in recent decades has led to a rapid growth in approvals of AAV-based therapeutics by regulators across Europe and the United States. Bringing ongoing and future AAV-based gene therapy projects to the clinic depends on overcoming several challenges and limitations in the AAV development process.

A central challenge is in the large-scale vector production of highly potent products. In particular, current methods of transgene packaging often result in incomplete packaging of genetic material into virions, which can reduce the quality of vector production and affect the safety and efficacy of the AAV gene therapy.

Accurate and rapid detection of these empty capsids is critical when scaling up production, and failure to detect these particles can lead to a high fraction of empty capsids remaining in vector batches. Empty capsids are problematic as they can compete for host attachment and promote immune responses without the benefit of delivering the gene therapy. Robust methods for quantifying DNA encapsidation by AAV particles have been fairly limited until recently. Current methods to assess the proportion of empty particles include optical density measurements, qPCR, and negative stain TEM.

However, these methods tend to be imprecise and can also damage the capsid, resulting in a need for more streamlined and comprehensive methods to analyze gene vector packaging in AAV production pipelines.

The Solution

In recent years, cryogenic transmission electron microscopy (cryo-TEM) has become a preferred method for monitoring AAV encapsidation. Cryo-TEM is an electron microscopy technique in which biological samples are rapidly vitrified, effectively trapping them in a native hydrated state for analysis. This method allows for the examination of a wide range of biological samples, including AAV particles, in native or near-native states. Additional benefits include the ability to work with small sample volumes and rapid turnaround times for image analysis, offering fast and accurate analysis at all stages of the AAV production pipeline.

Cryo-TEM offers an accurate method for analyzing and quantifying AAV particle morphology, including capsid integrity, purity, size, population fractions (DNA-filled versus empty capsid populations), aggregation and virus titer analysis. With these analyses, cryo-TEM can be used to perform routine quality control and determine sample composition by detecting the presence of aggregates, broken capsids or host cell debris, as well as any changes in capsid morphology or interior density (encapsidation state).

With the aid of computational analysis software, AAV particles can be identified and assessed by two-dimensional (2D) classification and can also be fully reconstructed into three-dimensional (3D) structural maps.

WHAT CRYO-TEM MEASURES

- Empty, partially full and full capsid population fractions
- Particle size, symmetry and virion surface features
- Capsid integrity — intact versus broken particles and free DNA
- Aggregation, host cell debris and virus titer

The Results

CRYO-TEM IMAGING AND ANALYSIS OF AAV SAMPLES

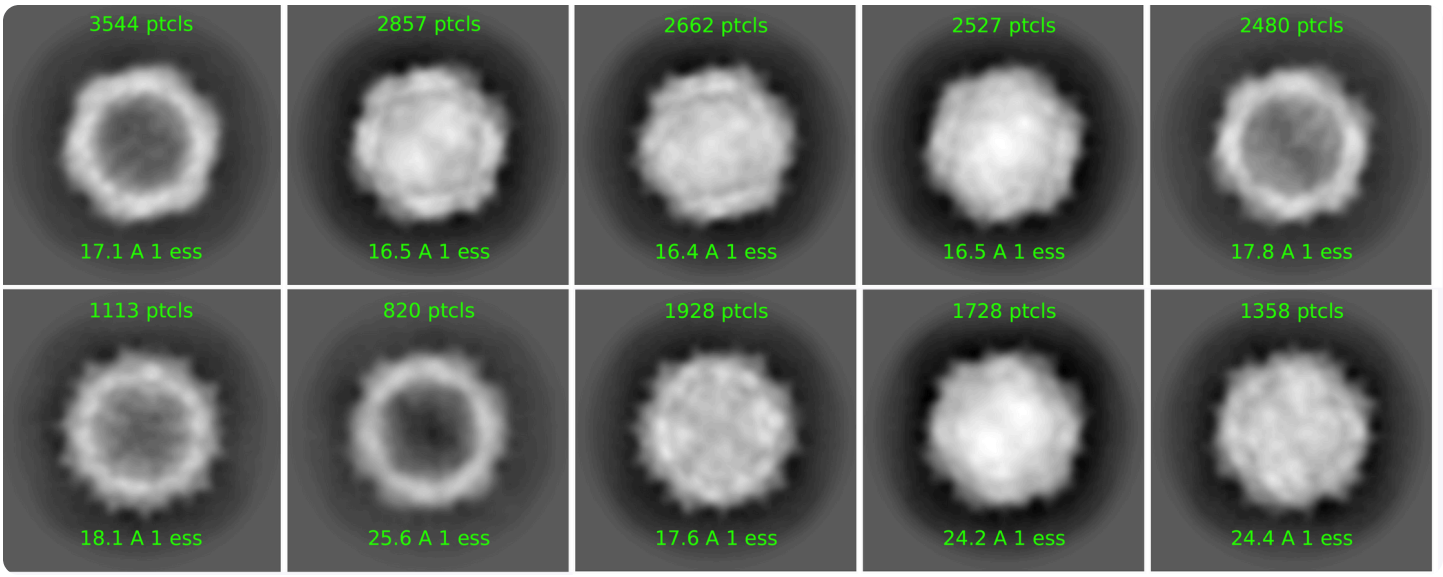
This case study highlights how TrueCourse utilizes cryo-TEM to image and analyze AAV particles. Structural analysis of AAV particles by cryo-TEM is typically straightforward because the capsid structure is well defined, has high symmetry, and the sample behaves well in ice. This allows for the collection of high-quality images of individual AAV particles by cryo-TEM imaging (Figure 1B).

Grid substrate and dilution conditions are routinely optimized as required and benefit from a highly developed AAV workflow created by experts at TrueCourse. In this process, samples are vitrified using a semi-automatic plunger, and a specialized grid is loaded into a TEM microscope. Images are acquired by highly sensitive cameras controlled by automated data collection software.

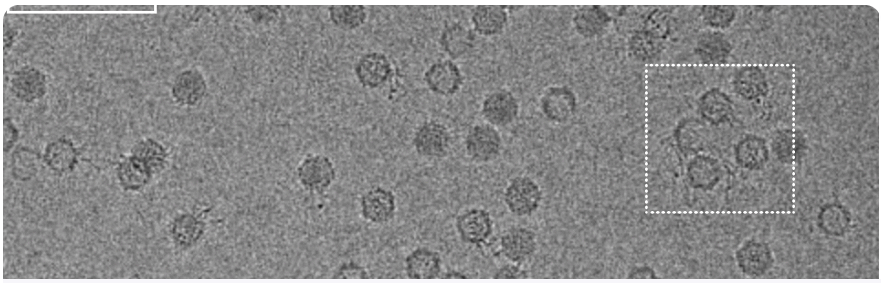
After collection, images can be viewed in a secure, web-based image viewer and then processed by advanced image analysis software and further analyzed by in-house particle sizing and classification software. Using these methods, AAV particles in their native state can be directly visualized at the resolution required to establish encapsidation status (Figure 1).

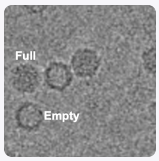
Figure 1B shows a cryo-TEM image with a wealth of information about the size, morphology and integrity of the particles. Measurements can be performed to quantify the physical properties of the AAV particles, such as the ratio of empty to full particles, and the ratio of intact to non-intact particles, providing a comprehensive analysis of the sample.

A 2D CLASS AVERAGES — EMPTY AND FULL PARTICLES



B CRYO-TEM IMAGE OF AAV CAPSIDS





DNA-filled (**full**) and **empty** capsids are distinguished by interior density.

Damaged capsids and **free DNA** are resolved in the same image.

FIGURE 1 (A) Automated particle classification by 2D analysis provides statistics on empty and full particles that are automatically sorted into different class averages. Additional high-resolution features can be enhanced by this analysis providing further structural details. (B) Cryo-TEM image and enlarged insets of AAV capsids showing examples of DNA-filled and empty capsids as well as damaged capsids and free DNA.

HIGH-RESOLUTION STRUCTURE DETERMINATION OF AAV

These images can also be used to assess the level and nature of particle aggregation and detect the presence of other material in the sample. Particle classification can also be performed automatically using 2D analysis (Figure 1A). These images provide detailed information including particle dimensions, symmetry, interior density (related to encapsidation state), and virion surface features.

These same cryo-TEM images are further processed to produce high-resolution 3D structures of AAV samples. 3D reference models are created to better sort AAV particles within a sample according to their genome density. Electron density maps are analyzed and protein side chains are easily identified, allowing for the visualization of surface-exposed residues to confirm potency and targeting capacity. 3D reconstruction also allows visual confirmation of alterations to the capsid, and investigation of amino acid variations, as well as antigenic hot spots to check for potential immune reactions.

Creating 3D reconstructions of AAVs is a multi-stage process that includes particle picking, 2D and 3D classifications, and refinement. In this process, each image contains multiple viral particles that are initially classified into 2D classes, where each class provides a different projection view of the virus particle; 2D classes can be mathematically combined to generate a 3D map, which provides information about high resolution features for the capsid, as well as general low-resolution morphology of internal density.

In Figure 2, a 3D reconstruction of an AAV particle carrying genomic material was determined to 1.8 Å resolution by TrueCourse (Figure 2A). High-quality density maps were obtained, with extremely well-defined side chain density (Figure 2B and C).

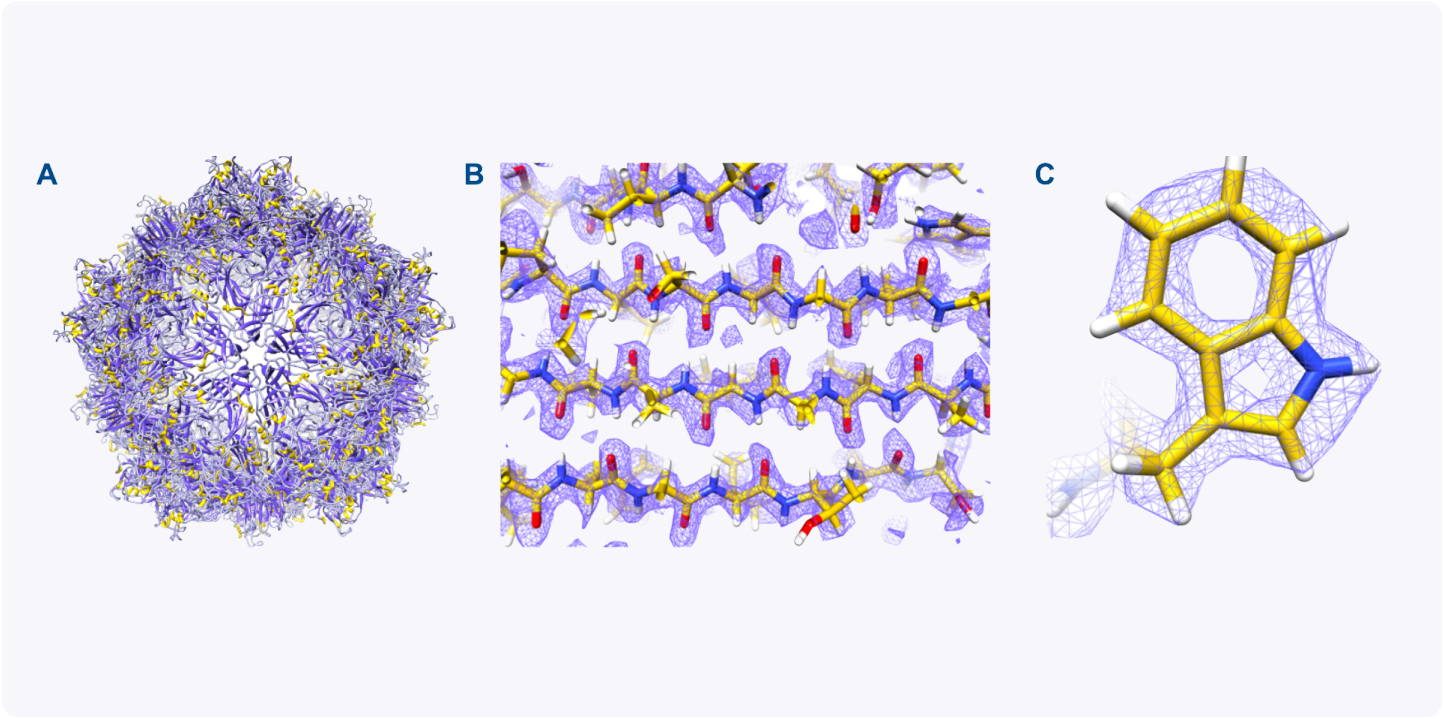


FIGURE 2 (A) 3D reconstruction of an AAV particle resolved to 1.8 Å resolution. A portion of the density map is shown in (B) fitted with the atomic model. (C) The density map for one of the tryptophans highlights the quality of the reconstruction.

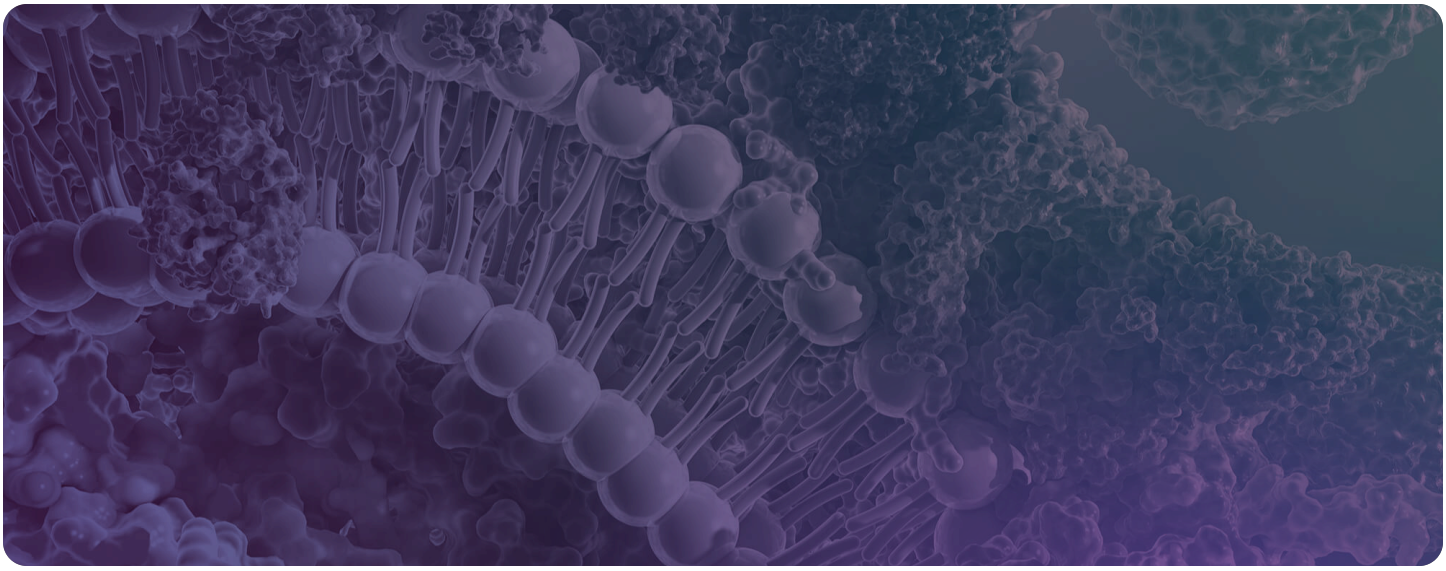
The density map from the 3D reconstruction provides information for the external volume of the particle, both surface features and internal morphology, and can be used as a benchmark structure for comparison to future potential alterations and structural changes.

Cryo-TEM with single particle analysis allows for high-resolution structure determination of the viral capsid to also support structure based vector design (SBVD), providing improved targeting of the vector to the appropriate tissue/cell-type and better avoidance of host immune responses through understanding vector recognition by neutralizing antibodies.

The Conclusion

Monitoring AAV production quality and consistency is critical for developing safe and efficacious drug delivery therapies, information that is required by the FDA for regulatory approval of gene therapies. Here, the TrueCourse cryo-TEM workflow presented shows the rapid and high-quality direct visualization and analysis of AAV particles that provides information about both quality and purity of AAV particles.

Cryo-TEM allows the detection of non-functional AAV virus particles, changes in the capsid structure, and quantification of genomic load; all of which are crucial to understanding, characterizing, and improving gene vector delivery systems.



For more information, or to speak to one of our cryo-EM experts, please email us at info@truecoursebio.com.

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