Cryo and negative staining EM – a comparison study with AAV capsids -

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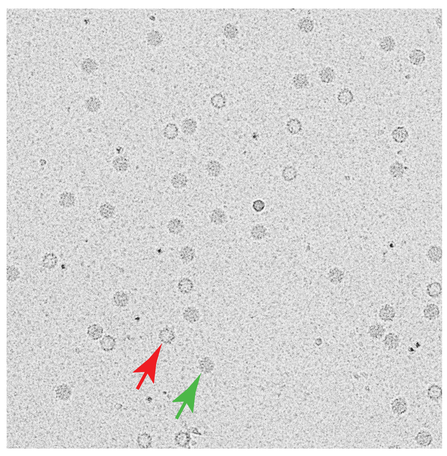
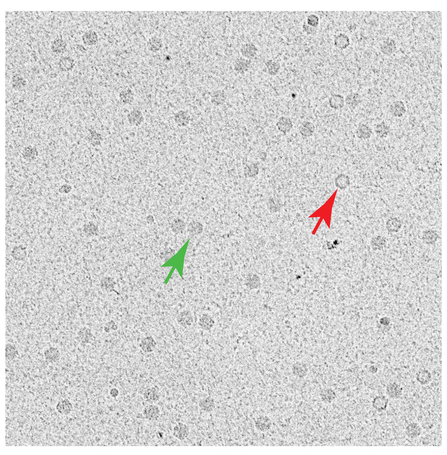
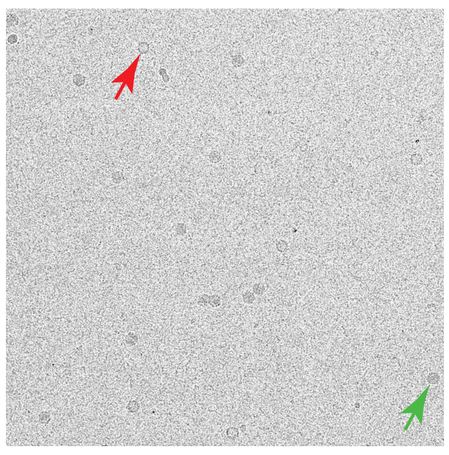
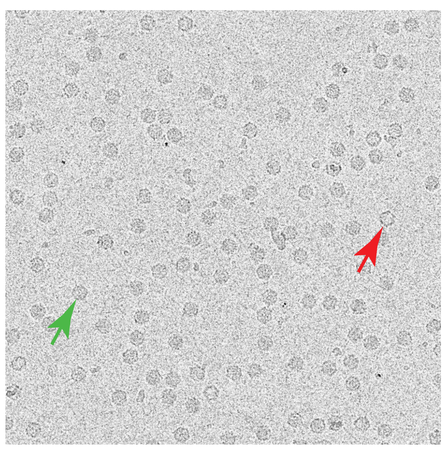
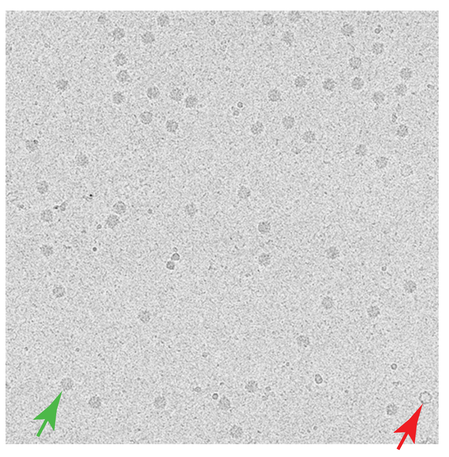
Abstract
Academic and industrial labs frequently use AAV vectors for the development of gene therapies. To ensure safety and efficacy a reliable quantification of rAAV titers is indispensable. There have been concerns about the reliability of negative staining electron microscopy (EM) for the determination of the full and empty capsid ratio. Cryo-EM has been considered as an alternative method, which is suggested to be less prone to artifacts, thus leading to more reproducible results.
In this study PROGEN compared negative staining EM and cryo-EM with regard to discrimination of full and empty capsids and accuracy. Therefore, the AAV serotypes 3, 5, 6, 8 & 9 were analyzed using both EM methods. The results support the validity of negative staining EM, using well-established protocols and confirm the quality & reliability of PROGEN´s calibration process and strict quality control of PROGEN´s AAV Titration ELISAs.

Introduction
Accurate titer determination is a critical step in the production process of AAV vectors since a high proportion of the virus capsids can be empty and thus lead to a significantly higher total capsid titer compared to the genomic titer. In order to accurately determine the ratio of full and empty capsids, PROGEN compares cryo-EM and negative staining EM using capsid material of different AAV preparations which have been used as internal gold standard material for the calibration of PROGEN's AAV Titration ELISAs.

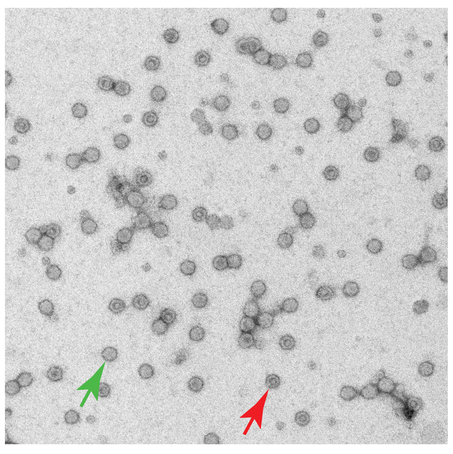
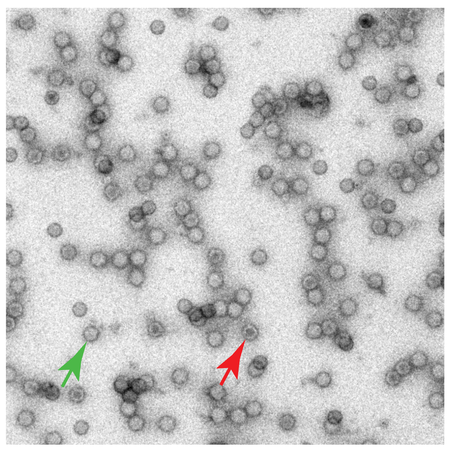
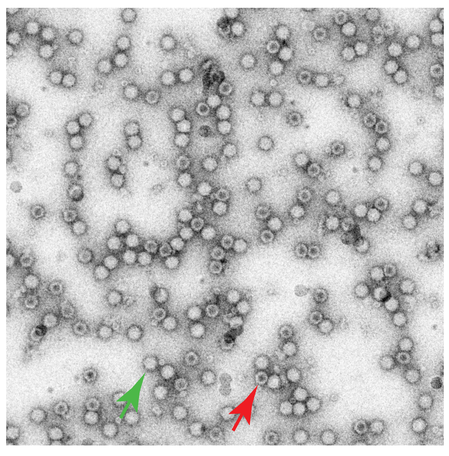
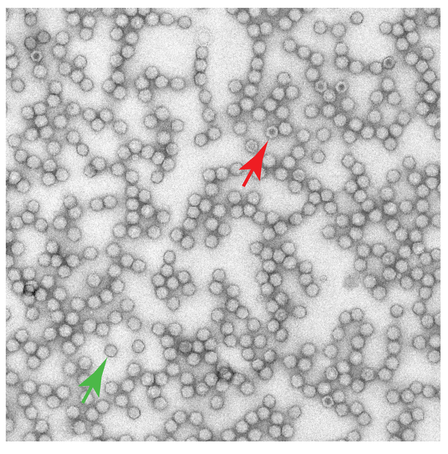
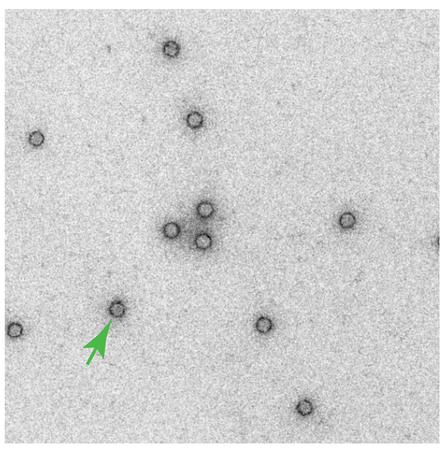
Results

Cryo-EM vs negative staining EM of different AAV serotypes

Cryo-EM

AAV 3	AAV 5	AAV 6	AAV 8	AAV 9
				
~ 71% full particles (1 experiment)	~ 89% full particles (1 experiment)	~ 77% full particles (1 experiment)	> 95% full particles (1 experiment)	> 95% full particles (1 experiment)

Negative staining EM

				
65-74% full particles (average: 68%) (2 experiments)	83-92% full particles (average: 85%) (2 experiments)	74-83% full particles (average: 78%) (2 experiments)	> 95% full particles (1 experiment)	> 95% full particles (1 experiment)

full AAV particles
empty AAV particles

All particle counts obtained by cryo-EM deviate less than 5% from the negative staining EM values, thus confirming the results of the widely used negative staining EM.

Methods
Aliquots of the same lot of each serotype were analyzed with both EM methods. The particle titers of the samples were between 10^{12} and 10^{13} p/ml. Counting of full and empty capsids was done manually using multiple images.

Sample preparation for Cryo-EM
2.5 µL were applied to glow discharged, holey carbon grids (Quantifoil 200 mesh R2/1 coated with 2 nm carbon). Grids were then blotted and flash-frozen in liquid ethane using a Vitrobot mark IV* (2 s at 8 °C / 100% humidity). Data acquisition was performed on a Talos Arctica microscope* (200 kV). Data sets were recorded on a Falcon 3 direct electron detector in linear mode (magnification: ×45,000, calibrated pixel size: 6.5 Å on the object scale, defocus: -3 µm). The images were recorded in a single frame (1 sec. exposure), accumulating a total dose of 33 e−/Å2 at the sample level. Data collection was performed fully automatically on a single grid using SerialEM.
*(ThermoFisher Scientific)

Sample preparation for negative staining EM
After adsorption onto glow-discharged, carbon-coated grids, samples were washed twice with deionized water followed by an incubation with 1% aqueous uranyl acetate. Micrographs were taken at 0.7 nm pixel size using a Zeiss EM912 TEM equipped with Proscan CCD Camera (Carl Zeiss Oberkochen, TRS Moorenweiss).

Conclusion
Our comparative study shows that the ratios of full end empty particles optained by negative staining EM and cryo-EM resulted in almost identical values. When performed by well-trained staff and with a well-established protocol, negative staining EM is as reliable as cryo-EM. It is important to note that the sample preparation for negative staining can be more challenging than for the cryo-EM and needs to be established by the EM user. On the other hand, cryo-EM requires a much more expensive setup and access to these instruments can be difficult in times of high demand by structural biologists. The comparable results of negative staining EM and cryo-EM confirm the reliability of the calibration procedure of the internal gold standard material for PROGEN's AAV Titration ELISAs.

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