High-resolution Quantitative Analysis of Multiple AAV Capsids in Rodent and Primate Models Using Multiplexed Reporter Protein Tagging Platform

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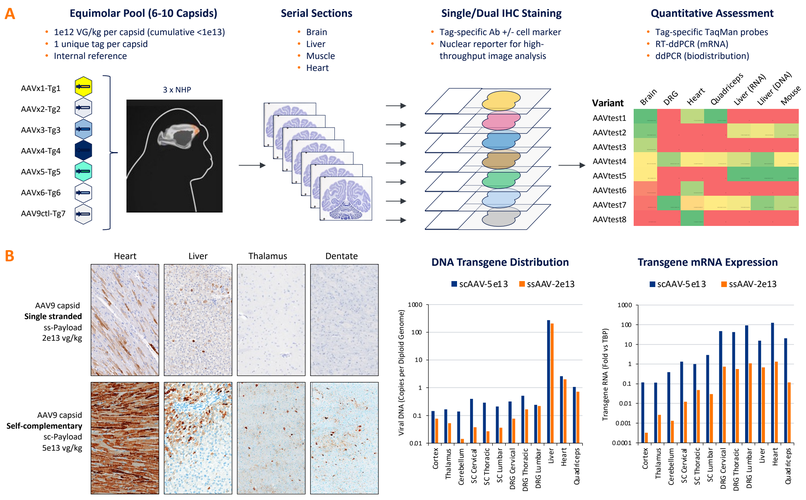
INTRODUCTION

Technologies for directed evolution of Adeno-Associated Virus (AAV) capsid libraries have dramatically improved in the last decade, and the generalization of high-throughput functional in vivo screens (iTransduce, M-CREATE, TRACER™, TRADE, BRAVE, DELIVER, etc.) is generating vast number of capsids with promising characteristics. This abundance of capsid variants can generate a bottleneck at the characterization stage, especially in non-human primate models. For each capsid variant to move forward as a clinical candidate or an experimental tool, it is of paramount importance to acquire high-quality quantitative and qualitative data, such as viral genome quantification, transgene mRNA expression, percentage of transduced cells, cell type specificity and regional distribution. Existing platforms combining RNA barcoding and single cell RNA-seq have been validated with up to 30 capsid variants in a single animal, but they are limited by low spatial resolution and the lack of protein expression data.

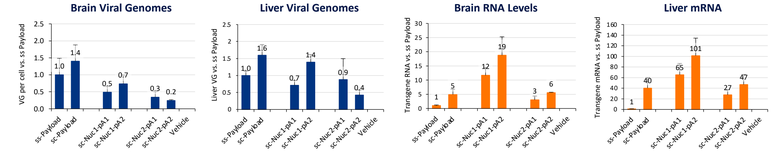
Here we designed a medium-throughput platform for simultaneous analysis of up to 10 capsid variants in a single animal while providing transgene DNA quantitation, transgene mRNA quantitation and transgene protein immunohistochemical detection. This was accomplished by 1) engineering a high-sensitivity reporter transgene scaffold to allow RNA and protein detection at low dose and 2) optimizing a series of C-terminally fused peptide tags that can be detected with distinct tag-specific antibodies. The Multi-Tag platform was first optimized in a mouse model using the neurotrophic 9P31 capsid and showed highly consistent detection in the brain at a well-tolerated dose. We then further demonstrated the validity of the platform in a 10-capsid pilot marmoset study and in cynomolgus macaque studies where we routinely tested 5 capsids per animal. Finally, we successfully demonstrated the compatibility of the multi-tag system with spatial transcriptomics in a mouse pilot experiment.

Figure 1. Transgene Design Allows for Efficient Multiplexed Transduction Assays



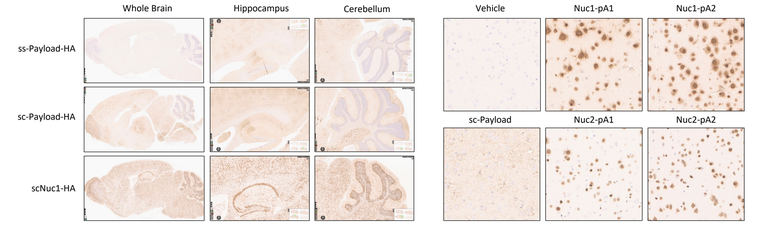
(**A**) Schema of multiplexed design, allowing up to 10 vectors to be injected simultaneously in a single animal with DNA, RNA and protein experimental readouts through IHC and tag-specific quantification of DNA and mRNA. (**B**) Comparative NHP transduction by AAV9 with a single-stranded (at 2E13 VG/kg) or self-complementary version (at 5E13 VG/kg) of the same cytoplasmic payload. Vectors were injected intravenously into adult NHPs and transduction was measured after 28 days by IHC, RT-ddPCR, and ddPCR. Self-complementary transgenes showed similar DNA biodistribution but markedly increased RNA and protein expression. (**C**) Schema of optimized transgene designs used in this study. HA-tagged cytoplasmic payload, Nuc1 or Nuc2 nuclear reporter followed by various polyA signals were used. Wild-type (ssAAV) and mutant (scAAV) ITRs are indicated.

Figure 2. scNuc1-HA Transgenes Show Superior Expression in Brain and Liver



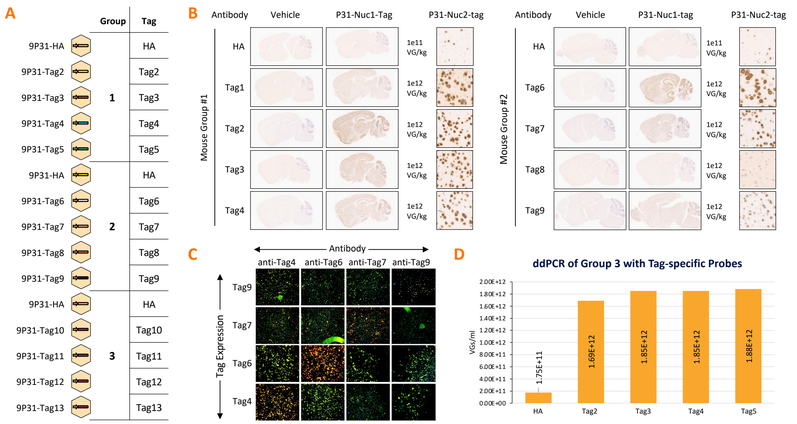
Mice were injected with 5e13 VG/kg 9p31 capsid carrying indicated transgenes (cytoplasmic payload, Nuc1 or Nuc2), each fused to an HA tag, with pA1 or pA2 polyadenylation sequence. After 28 days the transduction and expression was measured in the brain and liver.

Figure 3. scNuc1-HA Transgenes Show Superior Immunohistochemistry Detection



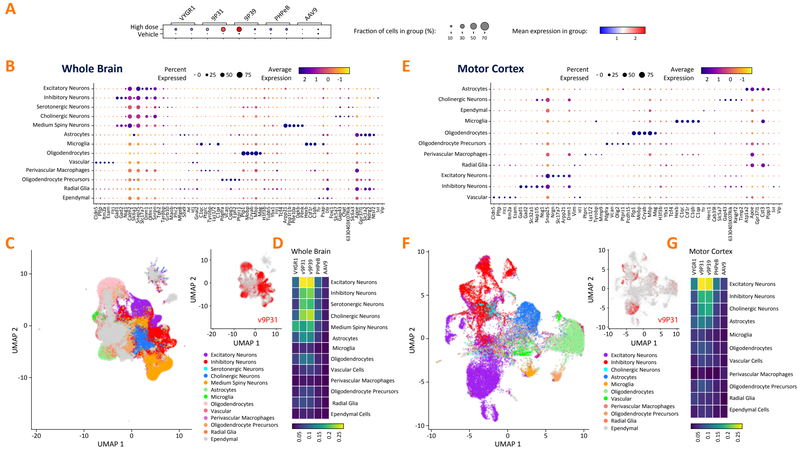
Mice were injected with scAAV9p31 carrying a cytoplasmic payload, Nuc1 or Nuc2, each fused to HA tag and followed by pA1 or pA2 polyadenylation signals. After 28 days sagittal sections are taken and stained with an anti-HA antibody.

Figure 4. Multiplexed *in vivo* Analysis with 5 Peptide Tags Per Animal



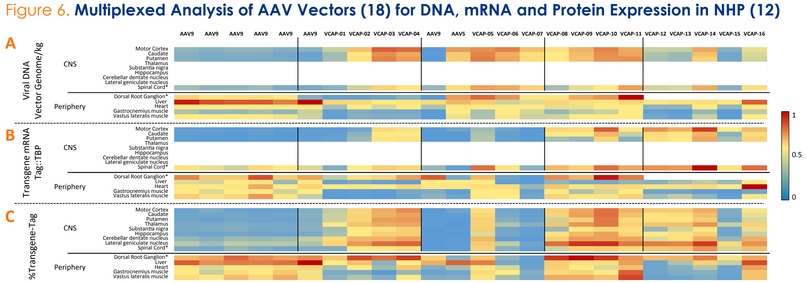
(**A**) Each mice group was injected with 1:10:10:10:10 cocktails of 9p31 capsids carrying Nuc1-HA and Nuc1 fused to 4 other peptide tags. (**B**) Consecutive brain sagittal sections were stained against each tag. (**C**) *In vitro* cross-reactivity assay. Representative example of HeLa cells transduced with 9p31 containing Nuc1 fused to the indicated tags, stained with antibodies against each tag. No cross-reactivity was observed between the 10 tag antibodies used in this study. (**D**) Multiplexed detection of transgene DNA was performed by ddPCR with tag-specific probes on brain tissue from mice dosed with 5-vector pools cocktail. Group 3 example is represented.

Figure 5. Multiplexed Analysis of Cell-specific Expression of Vector mRNA by Spatial Transcriptomics



A multiplexed evaluation of brain tissue of 5 capsids following an equimolar intravenous dosing at 1e12 vg/kg in C57Bl/6 mice (n=3), each capsid had 2 unique tags (**A**) in whole brain (**B**-**D**) and in motor cortex (**E**-**G**). Expression of cell-type specific marker genes (B, E), multi-dimensional plotting of cell-types based on differentially expressed genes and inset of representative capsid cell-type specific transduction (C, F), capsid enrichment across cell type clusters (D, G).

Figure 6. Multiplexed Analysis of AAV Vectors (18) for DNA, mRNA and Protein Expression in NHP (12)



Cynomolgus macaques (n=12, males, >2 years of age) received intravenous infusion of equimolar mix of novel AAV-derived capsids with multiple transgenes. After 28 days, vector genomes per kilogram (**A**), transgene mRNA (**B**), and the % positive transgene-tag expression (**C**) to examine central nervous system (CNS) and peripheral tissue tropisms in regions of interest. *Cervical spinal cord and dorsal root ganglia

CONCLUSIONS

- Improved vector design using nuclear-localized reporter allows highly sensitive detection and easy image quantitation in multiple tissues.
- Multiple peptide tags allow unbiased parallel quantification and do not produce cross-reactivity.
- Multi-tag capsid system is compatible with spatial transcriptomics allowing for simultaneous in-depth analysis of up to 10 capsids.
- The multi-tag capsid system enables the enhanced testing of multiple capsids in fewer non-human primates with efficient transduction in CNS and peripheral ROIs while also de-targeting the liver.