Evaluating Transduction Efficiencies of AAV Vectors into Human iPSC-derived Cell Types

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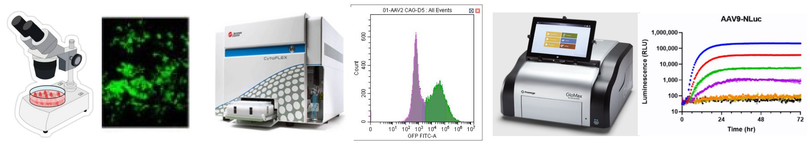
ABSTRACT

INTRODUCTION: The differentiation of induced pluripotent stem cells (iPSC) into specialized cell types of the human body represents a major advancement for the development of biologically relevant *in vitro* models. These cellular systems enable "disease-in-a-dish" studies and are compatible with various gene targeting approaches, including adeno-associated viral (AAV) vectors, to directly correct the genetic mutation. However, the permissibility of AAV transduction across different iPSC-derived cell types needs to be defined. This requires the implementation of a sensitive and versatile technology to monitor transduction efficiencies and quantify the impact of various factors, including viral vector serotype, multiplicity of infection (MOI), choice of promoter, transduction media, and/or timing of transduction.

Multiple Technologies for Characterizing AAV Transduction



Fluorescent microscopes can visualize GFP-expressing cells

CytoFLEX can quantify GFP by flow

Luminescence detection of AAV-NanoLuc

Figure 1. Transduction of efficiency of AAV in cells can be determined by various techniques. Generally, AAV constructs driving the expression of a fluorescent marker (e.g., GFP) are used and transduction efficiency is quantified by scoring the number of cells that fluorescent compared to the total number of cells observed. Example techniques include fluorescent microscopy to image the cells, flow cytometry to quantify fluorescent events, or image-based cytometry that can both obtain images of the cells as well as quantify GFP expression in situ. Alternatively, Promega offers an AAV construct that encodes a NanoLuc® luminescent reporter with a signal that can be measured on a standard plate reader. Some instruments are equipped with environmental control systems that enable kinetic cell-based assays to monitor AAV-driven transgene expression in real time.

MATERIALS and METHODS

CELLS: Commercially available cryopreserved human iPSC-derived cell types or "iCell products" from FUJIFILM Cellular Dynamics, Inc. (FCDI) and all culture media and supplements were used according to manufacturer's recommendations. Cell types (name, iPSC donor, abbreviation, and catalog #) that were used in this overview study included: iCell Cardiomyocytes[2], 01434 (CM2; C1016), iCell Hepatocytes 2.0, 01434 (Hep 2.0; C1026), iCell Retinal Pigment Epithelial Cells, 01279 (RPE; C1046), iCell Astrocytes, 01434 (C1037), iCell Astrocytes 2.0, 01279 (C1249), iCell Pericytes, 01279 (PERI; C1241), iCell Brain Microvascular Endothelial Cells, 01279 (BMEC; C1239), iCell Motor Neurons, 01279 (MNC; C1048), and iCell Sensory Neurons, 01279 (SNC; C1259).

PROMEGA: In collaboration with Promega (Madison, WI), we generated AAV vectors engineered with the NanoLuc®-HaloTag® Dual Reporter System and AAV viruses were manufactured by VectorBuilder (Chicago, IL). Serotypes examined here include AAV1, 2, 3, 4, 5, 6, 7, 8, 9, rh10, and PHP.eB, all built with a CMV or synapsin (SYN1) promoter to drive gene expression. This panel of AAV reagents was used to transduce several different iCell® products at MOI ranging from 10 to 10^6. If transduction was successful, the expressed NanoLuc® luciferase produced a luminescent signal in the presence of substrate, and the resulting signal intensity was proportional to the transduction efficiency of that AAV. Alternatively, AAV-positive cells could be quantified by imaging or flow cytometry when cells were labeled with Janelia Fluor® HaloTag® 646 Ligand post-transduction. Kinetic assays were performed over 48-72 hours on a BMG Labtech CLARIOstar equipped with the ACU (atmospheric control unit) to run environmentally-controlled, plate-based experiments.

PHENOVISTA: A parallel set of imaging-based testing was performed by PhenoVista Biosciences (San Diego, CA) with AAV viruses made by Sab Tech, Inc. (Philadelphia, PA) using chicken beta-actin (CBA) promoter driven expression of green fluorescent protein (eGFP). Six serotypes were tested here AAV 1, 2, 5, 6, 8, and 9. Cells were thawed and cultured in 384w format, transduced for 48-72 hours, and fixed for imaging on Day 7 or Day 14 with a 20X objective on a Yokogawa CQ1 or ThermoFisher Cell Insight CX7 LZR. Cell viability was assessed with Draq7 and Hoechst nuclei staining.

FUJIFILM DIOSYNTH BIOTECHNOLOGIES: This FUJIFILM group company based in College Station, TX is an industry-leading Contract Development and Manufacturing Organization (CDMO) capable of producing AAV viruses. Collaborative projects focused on evaluating transduction efficiency of select AAV serotypes (AAV1, 2, 4, 5, 6, 8, and 9) and comparing CAG and CMV promoters driving GFP. AAV viruses were manufactured at Vector BioLabs (Malvern, PA). Cells were cultured in 96-well plates and empty AAV vector viruses were used as negative controls. BacMam GFP was used as positive transduction control where appropriate. MOI of 100K was the maximum concentration of virus tested, instead of 10^6 as above. The Celigo S Image Cytometer from Nexcelom (Lawrence, MA) and a CytoFLEX flow cytometer from Beckman Coulter (Indianapolis, IN) were used to quantify GFP expression in the different cell types.

Evaluation of a NanoLuc®–HaloTag® Dual Reporter System for AAV Transduction

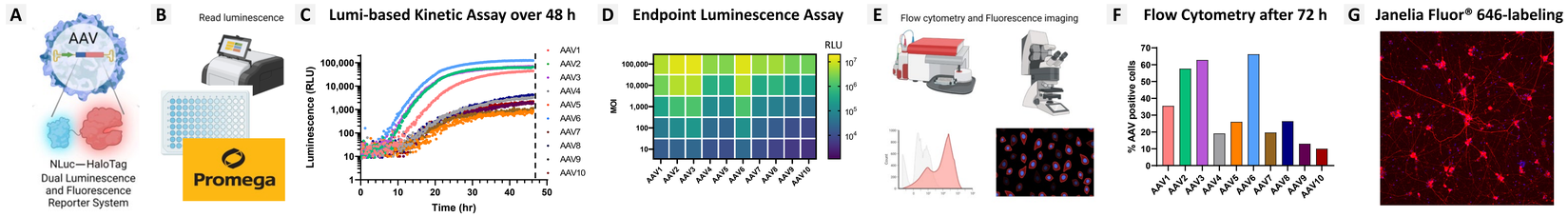


Figure 2. Results from Promega. (A) The NanoLuc®-HaloTag® dual luminescence and fluorescence AAV reporter system from Promega enables a high degree of assay flexibility and multiplexing. **(B)** Luminescent signals can be read in a plate-based assay and **(C)** are extremely sensitive with detection in iCell CM2 as early as 24 h post-transduction. Transduction with 10 different AAV serotypes in 96-well format was monitored on a CLARIOstar plate reader equipped with atmospheric control unit (ACU) for kinetic reads of luminescence over a 48-h period. AAV6, AAV3, and AAV2 showed robust transduction of iCell CM2 as compared to other serotypes. **(D)** Luminescence endpoint assays can be performed on parallel plates of cells instead. **(E)** This AAV construct also encodes HaloTag®, which is compatible with both flow cytometry and fluorescence imaging analysis. **(F)** The same plate used for CM2 endpoint luminescence was analyzed by flow cytometry (similar results). **(G)** iCell Motor Neurons transduced w/ AAV6-NanoLuc-HaloTag for 96 h and labeled w/ Janelia Fluor® 646 HaloTag® Ligand (red) and nuclei (blue).

Image-based Analysis of AAV-driven Expression of GFP in iCell products

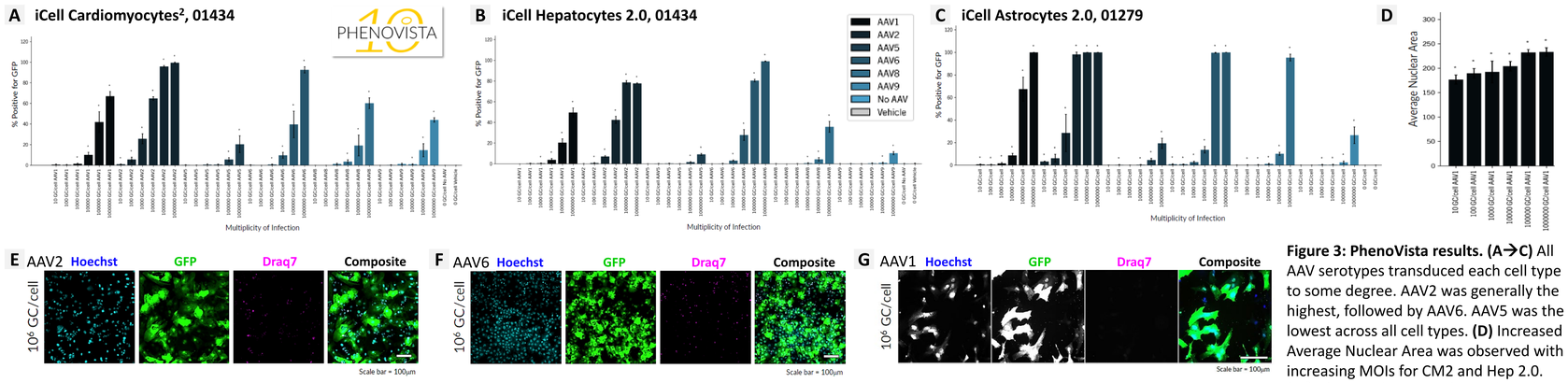


Figure 3: PhenoVista results. (A→C) All AAV serotypes transduced each cell type to some degree. AAV2 was generally the highest, followed by AAV6. AAV5 was the lowest across all cell types. **(D)** Increased Average Nuclear Area was observed with increasing MOIs for CM2 and Hep 2.0. **(E→G)** Example images for each analysis.

Summary Table of AAV Transduction Results with iCell Products

Serotype	Collaborator	Promoter	CM2	Hep 2.0	RPE	BMEC	PERI	ASC 1.0	ASC 2.0	MotorN	SNC
AAV1	Promega	CMV-NLuc	50	50	50	100	50	50	75	50	10
AAV1	Promega	SYN1-NLuc	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	50
AAV2	Promega	CMV-NLuc	90	75	90	90	60	75	75	50	10
AAV3	Promega	CMV-NLuc	100	75	100	30	30	75	75	75	10
AAV4	Promega	CMV-NLuc	20	50	75	10	10	20	30	N/A	N/A
AAV5	Promega	CMV-NLuc	30	10	75	10	50	50	30	25	N/A
AAV6	Promega	CMV-NLuc	100	100	100	75	60	100	100	75	20
AAV6	Promega	SYN1-NLuc	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	90
AAV7	Promega	CMV-NLuc	30	20	25	10	20	20	20	25	N/A
AAV8	Promega	CMV-NLuc	20	20	25	20	30	50	50	50	10
AAV9	Promega	CMV-NLuc	10	10	10	10	20	10	10	25	10
AAV9	Promega	SYN1-NLuc	N/A	N/A	N/A	N/A	N/A	N/A	40	10	90
AAVPHP.eB	Promega	SYN1-NLuc	N/A	N/A	N/A	N/A	N/A	N/A	40	25	90
AAV1	PhenoVista	CBA-eGFP	70	50	N/A	N/A	N/A	N/A	100	N/A	N/A
AAV2	PhenoVista	CBA-eGFP	100	80	N/A	N/A	N/A	N/A	100	N/A	N/A
AAV5	PhenoVista	CBA-eGFP	20	10	N/A	N/A	N/A	N/A	30	N/A	N/A
AAV6	PhenoVista	CBA-eGFP	90	100	N/A	N/A	N/A	N/A	100	N/A	N/A
AAV8	PhenoVista	CBA-eGFP	60	40	N/A	N/A	N/A	N/A	75	N/A	N/A
AAV9	PhenoVista	CBA-eGFP	50	10	N/A	N/A	N/A	N/A	20	N/A	N/A
AAV1	Diosynth	CMV-GFP	15	N/A	40	N/A	N/A	N/A	N/A	N/A	N/A
AAV1	Diosynth	CAG-GFP	10	N/A	50	N/A	N/A	N/A	N/A	50	N/A
AAV2	Diosynth	CMV-GFP	15	N/A	40	N/A	N/A	N/A	N/A	N/A	N/A
AAV2	Diosynth	CAG-GFP	50	N/A	50	N/A	N/A	N/A	N/A	5	N/A
AAV4	Diosynth	CMV-GFP	0	N/A	30	N/A	N/A	N/A	N/A	N/A	N/A
AAV4	Diosynth	CAG-GFP	0	N/A	75	N/A	N/A	N/A	N/A	N/A	N/A
AAV5	Diosynth	CMV-GFP	5	N/A	70	N/A	N/A	N/A	N/A	N/A	N/A
AAV5	Diosynth	CAG-GFP	5	N/A	90	N/A	N/A	N/A	N/A	30	N/A
AAV6	Diosynth	CMV-GFP	40	N/A	100	N/A	N/A	N/A	N/A	N/A	N/A
AAV6	Diosynth	CAG-GFP	60	N/A	100	N/A	N/A	N/A	N/A	60	N/A
AAV8	Diosynth	CMV-GFP	5	N/A	10	N/A	N/A	N/A	N/A	N/A	N/A
AAV8	Diosynth	CAG-GFP	2	N/A	10	N/A	N/A	N/A	N/A	30	N/A
AAV9	Diosynth	CAG-GFP	2	N/A	10	N/A	N/A	N/A	N/A	20	N/A

Table 1. Multiple iCell products were transduced with various AAV serotypes containing different promoters (CAG, CBA, CMV, or SYN1). Overall, AAV6 showed the highest transduction efficiency across iPSC-derived cell types. Moreover, SYN1 drove higher expression in neuronal cells, whereas CMV was better in other cells. CAG was also very effective in CM2. Neuronal cell types required longer transduction times (i.e., 96 h exposure to AAV) than did cardiomyocytes (24 h to peak signal, for example). In general, transductions were more efficient at earlier timepoints in culture (i.e., Day 7 vs. Day 10 for CM2 and Day 14 vs. Day 21 for RPE) with some exceptions.

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Comparison of AAV Promoters using Cytometry Methods

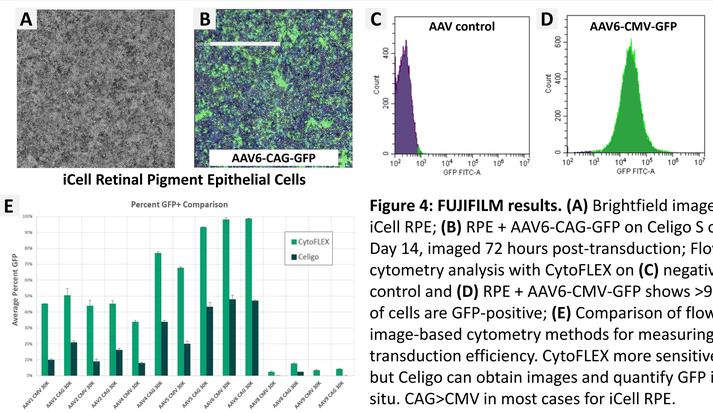

Figure 4: FUJIFILM results. (A) Brightfield image of iCell RPE; **(B)** RPE + AAV6-CAG-GFP on Celigo S on Day 14, imaged 72 hours post-transduction; Flow cytometry analysis with CytoFLEX on **(C)** negative control and **(D)** RPE + AAV6-CMV-GFP shows >98% of cells are GFP-positive; **(E)** Comparison of flow & image-based cytometry methods for measuring transduction efficiency. CytoFLEX more sensitive, but Celigo can obtain images and quantify GFP in situ. CAG>CMV in most cases for iCell RPE.

Conclusions

iCell products make a great model to evaluate transduction efficiency of different AAV serotypes preclinically. The systematic exploration of the numerous variables that impact the efficiency of AAV transduction for any given cell type is required. Cryopreserved cells from commercial sources that are manufactured reproducibly at scale are an ideal reagent for use in these studies because of their "thaw-and-go" consistency. Proper handling and storage of AAV material is critically important as it can affect transduction efficiency. Finally, the combination of iPSC-derived cells and AAV technology to modulate *in vitro* cellular disease models for pre-clinical studies and potentially even potency release assays is a powerful system that is now available as a drug manufacturing process for material release.