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🌐 Synergistic Enhancement of AAV Gene Delivery in 2D Cells and 3D Organoids Using Polybrene and Hydroxychloroquine V.1

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Hyeon-Jin Na^{1,2}, Yongbo Shin^{2,3}, Seung-Hyun Kim¹, Seung pil Jang¹, Myung Jin Son^{2,3}, Yong Min Choi^{1,2}, Hyeon Gyeol Jeon^{2,3}, Ok-Seon Kwon^{1,2}, Kyung-Sook Chung^{1,2}

¹Center for Gene and Cell Therapy, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Republic of Korea;

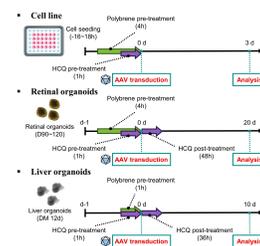
²Department of Advanced Bioconvergence, Korea University of Science & Technology (UST), Republic of Korea;

³Stem Cell Convergence Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea



Hyeon-Jin Na

Korea Research Institute of Bioscience and Biotechnology



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We use this protocol and it's working

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Abstract

Recent advances in three-dimensional (3D) culture platforms have enabled organoids to serve as physiologically relevant models for recapitulating human biology and assessing therapeutic efficacy and toxicity. Despite their promise, their complex architecture presents significant challenges for efficient gene delivery, thereby limiting their broader application in drug discovery and translational research. To overcome this challenge, we developed a sequential treatment strategy that combines polybrene (PB), which facilitates viral entry, and hydroxychloroquine (HCQ), which modulates endosomal processing. By applying PB as a pre-treatment and HCQ as a post-treatment, we achieved an approximate 1.3- to 2-fold increase in adeno-associated virus (AAV) transduction efficiency in both retinal and liver organoid models compared to single-agent treatments, and a 1.7- to 2.5-fold increases compared to treatment with virus alone. Importantly, this combinatorial treatment preserved cellular integrity, as confirmed by minimal TUNEL assay and high overall viability. Our findings demonstrate that sequential administration PB and HCQ significantly improves AAV transduction in 3D retinal and liver organoid systems, offering a robust method to improve gene delivery. This approach not only overcomes current limitations in organoid-based research but also supports the development of more predictive platforms for evaluating AAV vectors and advancing gene therapy application.

Guidelines

- The stock concentration may vary depending on experimental requirements.
- Sterilize 1.5 mL micro-centrifuge tubes prior to use.
- The cell culture process can be optimized based on cell line characteristics, such as trypsin-EDTA incubation time.
- The optimal maturation day for retinal organoids is between day 90 to day 120.

Note

Differentiation day may vary according to the experimental objectives.

- For chemical treatment, add the diluted chemical directly to the media, adjusting for the buffer volume.
- When conducting multiple reactions, it is advisable to prepare a master mix that includes a 10% excess to account for pipetting errors.

Materials

- Laboratory Equipment

1. CO2 incubator, clean bench, Vacuum pump
2. Pipette, pipette aid, table-top centrifuge, centrifuge, vortex
3. Refrigerator (4 °C), freezer(-20 °C) and deep-freezer (-80 °C)
4. Bright-field microscope, ZEISS confocal microscope (LSM800), Cytation 5
5. Real-time PCR, Cryostat microtome
6. GraphPad Prism, ZEISS blue software, BioTek Gen5
7. Personal Protective Equipment (PPE) at a minimum laboratory coat with fitted sleeves, latex or nitrile gloves and safety glasses

- Materials

A
50-mL, 25-mL, 10-mL, 5-mL, 2-mL sterile pipette
1000-μl, 200-μl, 10-μl pipette tips
50-mL, 15-mL conical tubes
1.5-mL sterile micro-centrifuge tubes
35-mm, 100-mm cell culture dishes
24-well, 48-well cell culture plates
96-well v-bottom ultra-low attachment plates

- Reagents for stock solution preparations

Reagent	Company	Cat #
DPBS, no calcium no magnesium	ThermoFisher	14190144
UltraPure DNase/RNase-Free Distilled Water	Invitrogen	10977015
Dimethyl Sulfoxide (DMSO), cell culture reagent	Santa Cruz	sc-358801

⊗ DPBS, no calcium, no magnesium **Thermo Fisher Catalog #14190144**

⊗ UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977015**

⊗ Dimethyl Sulfoxide (DMSO), cell culture reagent (CAS 67-68-5) **Santa Cruz Catalog #sc-358801**

- Reagent for cancer cell line culture

Reagent	Company	Cat #
DMEM	ThermoFisher	11995-065
Fetal Bovine Serum	ThermoFisher	14000044
Penicillin/Streptomycin	ThermoFisher	15140-122
Trypsin-EDTA(0.5%), no phenol red	ThermoFisher	15400054
PBS pH 7.4(10X)	ThermoFisher	70011-044

☒ DMEM Gibco - Thermo Fisher Scientific Catalog #11995065

☒ Penicillin-Streptomycin (10,000 U/mL) Thermo Fisher Scientific Catalog #15140122

☒ Trypsin-EDTA (0.5%) no phenol red Thermo Fisher Scientific Catalog #15400054

☒ 10X PBS pH 7.4 Thermo Fisher Scientific Catalog #70011044

■ Reagent for hPSCs and Organoid culture

Reagent	Company	Cat #
mTeSR1 complete kit	STEMCELL	ST85850
DMEM/F12	ThermoFisher	11320-033
IMDM	ThermoFisher	12440053
Ham F12	ThermoFisher	11765054
dPBS	ThermoFisher	14190144
Matrigel	Corning	354277
Knockout Serum replacement	ThermoFisher	10828028
Fetal Bovine Serum	ThermoFisher	14000044
ReLeSR	STEMCELL	ST05872
Gentle cell dissociation reagent	STEMCELL	ST07174
N2 supplement	ThermoFisher	17504044
GlutaMAX	ThermoFisher	35030061
Chemically Defined Lipid Concentrate	ThermoFisher	11905031
Penicillin/Streptomycin	ThermoFisher	15140-122
Cell Recovery solution	Corning	354253

Reagent	Company	Cat #
Trypsin-EDTA	Thermo	25200056
Y-27632	Tocris	4423
Advanced DMEM/F12	Thermo Fisher	12634028
HEPES	Thermo Fisher	15630080
B27 Supplement with Vitamin A	Thermo Fisher	17504044
N-Acetylcysteine	Sigma-Aldrich	A9165
A83-01	Tocris	2939
DAPT	Sigma-Aldrich	D5942
Dexamethasone	Sigma-Aldrich	D4920
[Leu]-Gastrin I human	Sigma-Aldrich	G9145
Human HGF	Peptidech	100-39
Human EGF	Peptidech	AF-100-15
Human BMP7	Peptidech	120-03
Human FGF19	Peptidech	100-32

⊗ mTeSR™1 **STEMCELL Technologies Inc. Catalog #85850**

⊗ DMEM/F-12 **Thermo Fisher Scientific Catalog #11320033**

⊗ IMDM **Thermo Fisher Catalog #12440053**

⊗ Hams F12 **Thermo Scientific Catalog #11765054**

⊗ DPBS **Thermo Fisher Scientific Catalog #14190144**

⊗ Corning® Matrigel® **Corning Catalog #354277**

⊗ KnockOut® Serum Replacement **Thermo Fisher Catalog #10828028**

⊗ ReLeSR **STEMCELL Technologies Inc. Catalog #05872**

⊗ Gentle Cell Dissociation Reagent **STEMCELL Technologies Inc. Catalog #07174**

⊗ Chemically Defined Lipid Concentrate **Thermo Fisher Catalog #11905031**

⊗ Penicillin/Streptomycin **Invitrogen - Thermo Fisher Catalog #15140-122**

⊗ Cell Recovery solution **Corning Catalog #354253**

⊗ Trypsin-EDTA (0.25%), phenol red **Thermo Fisher Catalog #25200056**

⊗ CHIR 99021 **Tocris Catalog #4423**

⊗ Advanced DMEM/F-12 **Thermo Fisher Catalog #12634028**

⊗ HEPES 1M **Thermo Fisher Scientific Catalog #15630080**

⊗ N-Acetyl-L-cysteine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A9165**

⊗ A 83-01 **Tocris Catalog #2939**

⊗ DAPT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D5942**

⊗ [Leu15]-Gastrin I human **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G9145**

⊗ Gibco™ Human HGF Recombinant Protein, PeproTech® **Fisher Scientific Catalog #100-39-10UG**

⊗ Epidermal growth factor **peprotech Catalog #AF-100-15**

⊗ Human BMP-7 Recombinant Protein, PeproTech® **Thermo Fisher Scientific Catalog #120-03P-10UG**

⊗ Human FGF-19, Animal-Free Recombinant Protein, PeproTech® **Thermo Fisher Scientific Catalog #AF-100-32-25UG**

■ Reagent for AAV transduction

Reagent	Company	Cat #
Hydroxychloroquine (HCQ)	Tocris	5648
Polybrene (Hexadimethrine bromide)	Sigma	107689

⊗ Hydroxychloroquine sulfate **Tocris Catalog #5648**

⊗ Hexadimethrine bromide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #107689**

■ Preparation of Medium

Medium	Composition
10DMEM medium	10% Fetal Bovine Serum, 1% Penicillin and Streptomycin supplied in DMEM media
mTeSR1 medium	100ml of mTeSR1 5X supplement supplied in mTeSR1 basal media
gfCDM Neural induction medium	10% Knockout Serum replacement, 1% Chemically Defined Lipid Concentrate, 450 μM monothioglycerol, 1% Penicillin and streptomycin supplied in basal media (IMDM:Ham F12 (1:1))
Neural retina maturation medium	10% Fetal Bovine Serum, 1% GlutaMAX, 1% N2, 0.5μM Retinoic acid, 100 μM Taurine supplied in DMEM/F12 media
Virus medium	10% Fetal Bovine Serum, 1% GlutaMAX, 1% N2, 1% Penicillin and Streptomycin supplied in DMEM/F12 media

Medium	Composition
Liver organoid differentiation medium(DM)	1% Penicillin/Streptomycin (P/S), 1% GlutaMAX, 1% HEPES, 1X N2 Supplement, 1X B27 Supplement with Vitamin A, N-Acetylcysteine 1 mM, A83-01 0.5 μ M, DAPT 10 μ M, Dexamethasone 3 μ M, [Leu]-Gastrin I human 10 μ M, Human HGF 25 ng/ml, Human EGF 50 ng/ml, Human BMP7 25 ng/ml, Human FGF19 100 ng/ml supplied in Advanced DMEM/F12 media
Basal medium	1% Penicillin/Streptomycin (P/S) supplied in Advanced DMEM/F12 media

■ Cell lines

Cell line	Description	Company, Cat #
HEK293F T	fast-growing, highly transfectable clonal isolate derived from human embryonal kidney cells	ThermoFisher, R70007
661W	immortalized cone photoreceptor cell line derived from the retinal tumor of a mouse	kindly provided by Prof. Muayyad Al-Ubaidi, University of Houston
H9	Human embryonic stem cell (hESC)	WI Cell, WA09

■ Material for analysis preparation

Reagent	Company	Cat #
4% Paraformaldehyde	Biosesang	PC2031-050-00
Tritone X-100	Sigma-Aldrich	X100
Tween-20	Sigma-Aldrich	P7949
Sucrose	LPS Solution	SUC05
Tissue-Tek O.C.T. Compound	SAKURA	4583
Aqueous Mounting Medium	Vector Laboratories, Inc	H-5501
ImmEdge Pen	Vector Laboratories, Inc	H-4000
In Situ Cell Death Detection Kit, Fluorescein	Roche	11684795910

Troubleshooting

Safety warnings

- ⚠ Proper Personal Protective Equipment (PPE) needed; a minimum laboratory coat with fitted sleeves, latex or nitrile gloves and safety glasses

Before start

Before you start, cell lines should be ready for sub-culturing and organoids should be differentiated and matured until they reach appropriate developmental stage of each organ for analysis.



Formulation of chemical

1 Polybrene

- 1.1 Prepare autoclaved tertiary distilled water.
- 1.2 Dissolve the polybrene powder in tertiary distilled water to make a final concentration of  5 mg/ml ~  10 mg/ml .
- 1.3 Filter polybrene solution with 0.22 μ m filter.
- 1.4 Aliquot polybrene stock into 1.5 mL sterile micro-centrifuge tubes.

2 Hydroxychloroquine

- 2.1 Prepare autoclaved tertiary distilled water.
- 2.2 Dissolve the HCQ tablet in tertiary distilled water to make a final concentration of  10 millimolar (mM) .
- 2.3 Filter HCQ solution with 0.22 μ m filter.
- 2.4 Aliquot HCQ stock into 1.5 mL sterile micro-centrifuge tubes.

Preparation of cell line

3 Sub-culture of cell line

- 3.1 Aspirate culture media and wash with 1X PBS.



3.2 Add  1 mL of 0.05% Trypsin-EDTA in the culture plate and incubate culture plate in 5% CO₂,  37 °C culture incubator for approximately  00:02:00 .

2m



3.3 Add  1 mL of 10DMEM medium and detach the cancer cell by tapping the plate with hands.



3.4 Transfer the detached cells to 15 mL conical tube and centrifuge it under  1.000 rpm, 00:03:00 .

3m



3.5 Aspirate supernatant and add  1 mL of 10DMEM medium to resuspend cells.



3.6 Seed 1/5 of the cells into a new 100-mm cell culture dish with 10DMEM medium.

4 Seeding of cell lines

4.1 Repeat steps in **Sub-culture of cell lines** [ [go to step #3.1](#)  [go to step #3.5](#)].

4.2 Dilute  100 µL to 1/10 by adding  900 µL of fresh media, making a final volume of  1 mL .



4.3 Mix  20 µL of 1/10 diluted cell resuspension with  20 µL of Trypan Blue in a 1.5 mL sterile micro-centrifuge tube.



4.4 Clean a hemacytometer and load  10 µL of the sample in each side.



4.5 Count the cells and seed appropriate number of cells into a new 48-well plate.

Preparation of organoids

5 Retinal organoids

5.1 Prepare retinal organoids and transfer retinal organoids to a new 96 well U-bottom or flat-bottom low attachment plate.

5.2 Change medium into fresh pre-warmed virus medium up to  200 μL

6 Liver organoids

6.1 Prepare liver organoid differentiation medium (DM) and basal medium, and store them at  4 $^{\circ}\text{C}$ until use. 

6.2 Dilute Matrigel in cold DPBS at a 1:100 ratio and coat a flat-bottom 24-well plate for  01:00:00 in a  37 $^{\circ}\text{C}$ incubator. (Keep the Matrigel  On ice at  4 $^{\circ}\text{C}$.) 

6.3 Harvest the organoid embedded in Matrigel dome using a 1000 μL pipette tip, transfer it into microcentrifuge tubes, and briefly spin down using a mini centrifuge to pellet the organoids. 

6.4 Remove the supernatant, resuspend the organoid pellet in basal medium, and briefly spin down again using a mini centrifuge. 

6.5 After removing the supernatant, add cold Cell Recovery Solution (60 μL per dome) and incubate the cultures in Cell Recovery solution at  4 $^{\circ}\text{C}$ for approximately  00:10:00 . 

6.6 Briefly centrifuge the cultures to separate the organoids from the Cell Recovery solution. Remove the supernatant and wash the cultures with cold DPBS twice.  

6.7 Remove the supernatant and resuspend the organoids in DM (100 μL per well of a flat-bottom 24-well plate).

6.8 Add  400 μL of fresh DM to each well of the Matrigel-coated 24-well plate ( [go to step #6.2](#)), and seed the 12-15 organoids per well by adding  100 μL of organoid suspension (as prepared in  [go to step #6.7](#)) (Additionally, prepare three wells of organoids for cell counting, to be used in  [go to step #10.1](#) below.) 

6.9 To anchor the organoids (but not fully attached) to the bottom of the Matrigel-coated plate, incubate the organoids for  04:00:00 -  06:00:00 in a  37 °C , 5% CO₂ incubator.

10h



Treatment of chemicals

7 Treatment of polybrene

7.1  16:00:00 to  18:00:00 after seeding an appropriate number of cells per well, treat with the polybrene at a concentration of  5 µg/ml ~  10 µg/ml .



7.2 In the case of organoids, on the day organoids are transferred, polybrene can be treated at a concentration of of  5 µg/ml ~  15 µg/ml .

7.3 About  01:00:00 to  04:00:00 after, AAV can be transduced in either cells or organoids.

8 Treatment of HCQ

8.1  16:00:00 to  18:00:00 after seeding an appropriate number of cells per well, treat with the HCQ at a concentration of  15 µM .

8.2 In the case of organoids, on the day organoids are transferred, HCQ at a concentration of  10 µM ,  15 µM ,  20 µM

8.3  01:00:00 after, AAV can be transduced in either cells or organoids.

8.4 In the sequential treatment protocol for organoids, HCQ is applied for  36:00:00 to  48:00:00 post-viral transduction.

Virus transduction

9 Transduction of AAV to cell line and retinal organoids

9.1 Observe the morphology and condition of cells.

9.2 Dilute AAV in 1X PBS at the desired MOI. (Ex. 2×10^4 vg/cell or 1×10^{10} vg/organoid)

Note

AAV stock needs to be diluted with the appropriate dilution factor. The Volume of transduced AAV should be over  1 μ L to avoid pipetting errors.

9.3 Transduce the diluted AAV into the cells.

9.4 Incubate in a  37 °C , 5% CO₂ incubator.

9.5 In the case of retinal organoids, Incubate for 15 days in a  37 °C , 5% CO₂ incubator. Change the medium every 2 days.

10 Transduction of AAV to liver organoids

10.1 To calculate the treatment concentration of AAVs, transfer organoids from three wells (n=3) as prepared in  [go to step #6.8](#) into microcentrifuge tubes, and briefly spin down using a mini centrifuge.

10.2 Remove the supernatant, resuspend the organoid pellet in basal medium, and spread the organoid suspension onto the cell culture dish.

10.3 To dissociate the organoids into single cells for counting, mechanically chop the organoids using a surgical blade.

10.4 Transfer the cells into microcentrifuge tube and incubate them with Trypsin-EDTA for  00:10:00 in a  37 °C incubator.



10m



Note

Every  00:05:00 , pipette up and down using a  200 μL pipette tip to fully dissociate the cells into a single-cell suspension.

- 10.5 Count the number of cells using a standard hemacytometer or automated cell counter. (10–12 organoids per well is approximately equivalent to 1×10^5 cells per well.)
- 10.6 Change the medium to  250 μL of DM supplemented with  10 μM Y-27632 and the appropriate concentration of AAVs. (Ex, Use 1×10^1 to 1×10^3 vg/cell for evaluating AAV cell entry, and 1×10^6 vg/cell for transgene expression analysis.)
- 10.7 Incubate the organoids on a bi-directional rocker in a  37 $^{\circ}\text{C}$, 5% CO_2 incubator. 1d  
- 10.8 For AAV entry analysis, transfer the AAV-transduced organoids ( [go to step #10.7](#)) from the matrigel-coated plate into microcentrifuge tubes and wash organoids with DPBS three times
- 10.9 The organoid pellet can be used for next generation sequencing (NGS) analysis or stored at  -80 $^{\circ}\text{C}$ for later analysis.
- 10.10 For further incubation of the AAV-transduced organoids ( [go to step #10.8](#)), Resuspend organoids with Matrigel ( 30 μL per dome) and add the Matrigel droplet to the center of each well in a new flat-bottom 24-well plate.
- 10.11 Incubate the cells for 5–10 minutes in a  37 $^{\circ}\text{C}$ incubator until the Matrigel is solidified.
- 10.12 Overlay the droplet with DM ( 500 μL per well for a 24-well plate) and incubate for 3–10 days in a  37 $^{\circ}\text{C}$, 5% CO_2 incubator. Change the medium every 2 days.

Analysis for protein expression with Flow cytometry

11 Sample preparation for Flow cytometry

11.1 Observe the morphology and condition of 2D cells.

11.2 Aspirate culture media and wash with 1X PBS.

11.3 Add  100 μL of 0.05% Trypsin-EDTA in the culture plate and incubate culture plate in  37 $^{\circ}\text{C}$, 5% CO_2 incubator for approximately  00:02:00 .



2m



11.4 Add  100 μL of 2% FBS in 1X PBS and detach the cells by tapping the plate with hands.

11.5 Pipette and transfer the detached cells to a 1.5 mL sterile micro-centrifuge tube.



11.6 Add  300 μL of 2% FBS in 1X PBS and strain using a 5 mL polystyrene round-bottom tube with a cell strainer cap.



12 **Flow cytometry**

12.1 Run the flow cytometry software.



12.2 Load the samples one by one.

12.3 Analyze the data.

Analysis for protein expression with immunofluorescence chemistry

4h

13 **Sample preparation for immunofluorescence staining**

13.1 Collect retinal organoid samples using a 2 mL pipette aid and transfer them to 1.5 mL sterile micro-centrifuge tube.



Note

In the case of liver organoids, follow the steps ( [go to step #6.3](#)  [go to step #6.6](#))

13.2 Wash the samples twice with 1X PBS. 

13.3 Remove the 1X PBS and add  1 mL of 4% paraformaldehyde (PFA), incubating for  01:00:00 at  Room temperature .    

13.4 Remove the 4% PFA and wash the samples 3 times with 1X PBS. 

13.5 Incubate the samples in 15% sucrose in 1X PBS for  01:00:00 at  Room temperature .   

13.6 Remove the 15% sucrose and add 30% sucrose in 1X PBS, incubating  Overnight at  4 °C .    

13.7 Embed the samples in OCT compound and freeze them in a deep-freezer  Overnight at  -80 °C .   

13.8 Section the samples at a thickness of  10 μm using a cryotome and mount three sections per silane-coated slide.

Note

The slides should be stored  -20 °C unless staining is performed immediately.

14 **Immunofluorescence staining of slides**

14.1 Wash the slide with tertiary distilled water to remove remaining OCT compound around the samples. 

14.2 Gently remove excess moisture around the samples using Kimtech wipes. 

Note

The samples must not be dried.

14.3 Create a barrier around the samples using a barrier pen.

14.4 Add blocking and permeabilization buffer consisting of 5% FBS with 0.1% Triton X-100 in 1X PBS and incubate for at least  00:30:00 at  Room temperature in a humid chamber.   30m

14.5 Dilute the primary antibody with the appropriate dilution factor in 1% FBS in 1X PBS.

14.6 Aspirate the blocking and permeabilization buffer and apply the diluted primary antibody solution, incubating the slides in the humid chamber  Overnight at  4 °C .    30m

14.7 Wash the slides in 0.1% PBST containing 1% FBS five times, for  00:10:00 each.  10m

14.8 Dilute the secondary antibody and Hoechst with the appropriate dilution factor in the same solution used for the primary antibody. 

14.9 Gently remove excess moisture around samples and apply the diluted secondary antibody, incubating the slides in the humid chamber for  02:00:00 at  Room temperature .   2h

14.10 Wash the slides in 0.1% PBST containing 1% FBS five times, for  00:10:00 each.  10m

14.11 Gently remove excess moisture around the samples. 

14.12 Apply 1~2 drops of mounting medium on each sample and cover with a cover glass.

14.13 Let the slides dry in a dark place  Overnight at  Room temperature .

10m



15 **TUNEL assay of slides**

15.1 Follow the manual of In Situ Cell Death Detection Kit, Fluorescein from Roche.

16 **Imaging slides**

16.1 Run the imaging software.



16.2 Load the slides upside-down, ensuring cover glass faces microscope lenses.



16.3 Capture images at 10X to 40X magnification



Acknowledgements

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