Messenger RNA-Lipid Nanoparticles: A Potent Tool for Manipulating Neuronal Genes

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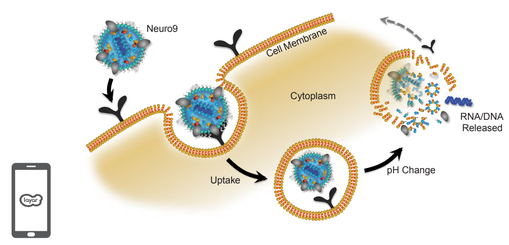
Introduction

Purpose
The use of RNA to manipulate gene expression in neuroscience has been limited due to the lack of an effective delivery tool. Recently, lipid nanoparticles (LNPs) have gained interest as safe and effective RNA delivery vehicles both *in vitro* and *in vivo*. However, traditional methods for the manufacturing of LNPs pose numerous challenges such as operator variability, and poor scalability. Here, we describe the robust and reproducible manufacture of mRNA-LNPs using the scalable microfluidics-based NanoAssemblr™ platform. The mRNA-LNPs exhibit exceptional transfection and exogenous gene expression efficiency in difficult-to-transfect primary neurons.

Results
mRNA was successfully encapsulated into LNPs using the NanoAssemblr™ platform. The LNPs encapsulating the two different mRNA lengths both exhibited the same size of ~120 nm, low PDI (< 0.2), and high mRNA encapsulation efficiency (> 95%). Flow cytometry analysis after a 48 h treatment of 1 µg/mL GFP mRNA-LNP in rat primary neurons at DIV 7 showed > 95% uptake of nanoparticles (based on a fluorescent probe within the nanoparticle) leading to > 95% of cells expressing GFP. Similar results for GFP expression were observed using ELISA. The high expression and uptake did not significantly impact cell viability even at doses of 5 µg/mL of GFP mRNA as measured using Presto Blue Cell Viability Assay.

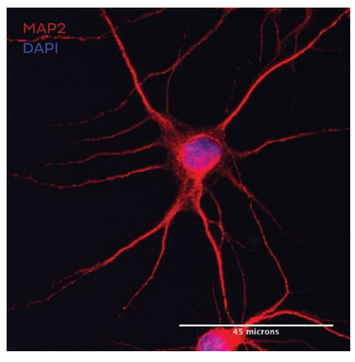
Gene Delivery via Lipid Nanoparticles
Lipid Nanoparticles (LNPs) are a platform that can be used to deliver nucleic acids to cells. LNPs mimic low density lipoproteins (LDLs), which are taken up by an endogenous pathway. LNPs are pH sensitive, and designed to release their payload into the cytoplasm.

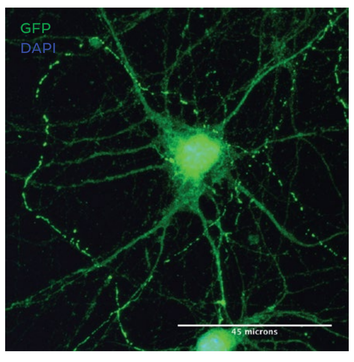


Video: LNP uptake and transfection

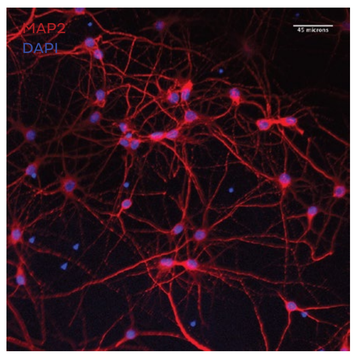
NanoAssemblr™ Systems
The NanoAssemblr™ platform uses microfluidics to precisely control the manufacturing of nanoparticles from µL to mL scales.

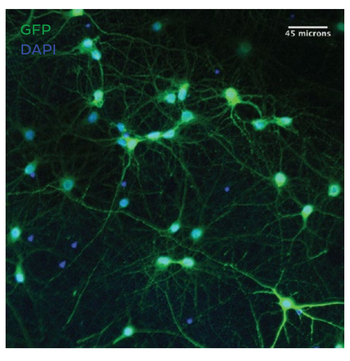
mRNA Delivery in Rat Primary Neurons





Confocal microscopy 63x: MAP2 positive neurons (red) show expression of GFP (green) after treatment with Neuro9 GFP mRNA-LNP in the presence of 5 µg/mL ApoE. Cells were stained with anti-MAP2 primary antibody/alexa fluor-594 conjugated secondary and anti-GFP primary antibody/alexa fluor-488 conjugated secondary antibody. Nuclei are stained with DAPI (blue)

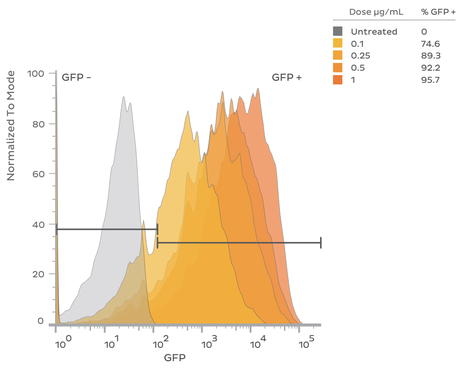




Confocal microscopy 40x: MAP2 positive neurons (red) show expression of GFP (green) after treatment with Neuro9 GFP mRNA-LNP in the presence of 5 µg/mL ApoE. Cells were stained with anti-MAP2 primary antibody/alexa fluor-594 conjugated secondary and anti-GFP primary antibody/alexa fluor-488 conjugated secondary antibody. Nuclei are stained with DAPI (blue)

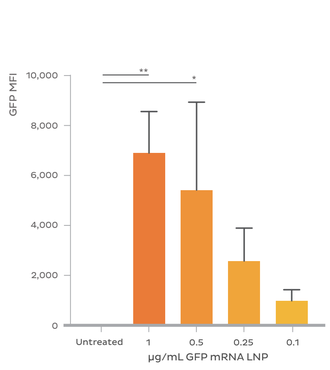
Flow Cytometry Shows GFP Expression and Particle Uptake

A



The percentage of GFP+ neurons was dose dependent, with 1 ug/mL having the highest percentage.

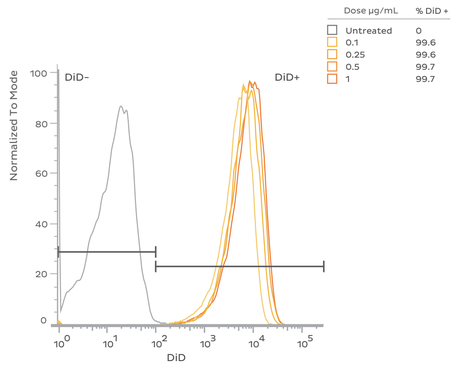
B



The mean fluorescent intensity (MFI) was highest for the GFP mRNA dose of 1 µg/mL, n=3 for each treatment, one-way ANOVA with Dunnett's multiple comparisons test, ** p<0.005, * p<0.05

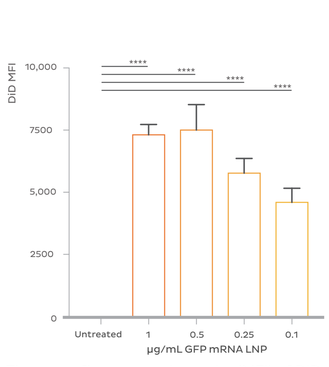
Flow cytometry analysis after 48 h treatment with Neuro9 GFP mRNA-LNP in the presence of 5 µg/mL ApoE in rat primary neurons, DIV 7, show >95% of neurons express GFP at a dose of 1 µg/mL and > 90% at 0.5 mg/mL.

C



The percentage of DiD+ neurons indicate nearly all cells take up mRNA LNPs at all tested doses.

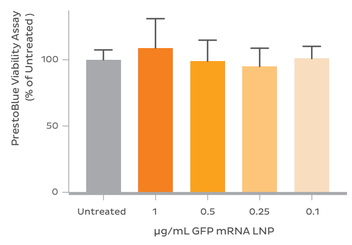
D



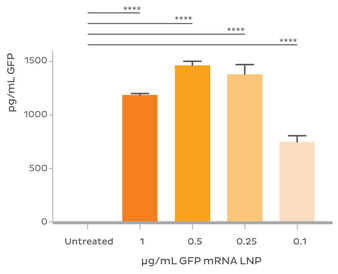
The mean fluorescent intensity (MFI) of DiD indicates Nanoparticle uptake, n=3 for each treatment, one-way ANOVA with Dunnett's multiple comparisons test, **** p < 0.0005

Flow cytometry analysis using the fluorescent probe DiD incorporated within the nanoparticle. >95% of neurons showed uptake of LNPs at all tested doses of mRNA. Flow cytometry performed after 48 h treatment with Neuro9 GFP mRNA-LNP in the presence of 5 µg/mL ApoE in rat primary neurons, DIV 7.

Cell Viability and ELISA

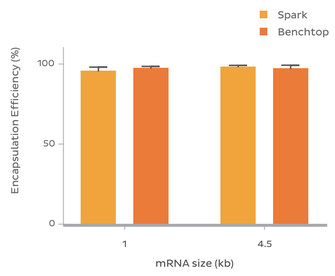


Cell viability was assayed by the PrestoBlue® Cell Viability Reagent. No toxicity was observed at any dose employed, n=6 for each treatment One-way ANOVA with Dunnett's multiple comparison test.

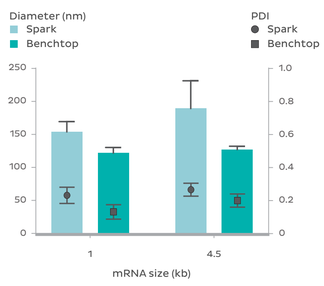


ELISA assay for GFP expression of DIV7, 48H mRNA LNP treated primary neurons, n=3 per treatment. Oneway ANOVA with Dunnett's multiple comparison test. **** p < 0.0005

Particle Characterization



mRNA encapsulation efficiency in LNPs formulated on the Spark and Benchtop instruments using two different sized mRNAs. n=3 per group represented as mean with error bars representing SD.



LNP hydrodynamic diameter and polydispersity index (PDI) for two mRNAs of different size formulated on the Spark and Benchtop instruments. n=3 per group represented as mean with error bars representing SD.

Conclusion

The NanoAssemblr™ microfluidics-based technology reproducibly manufactured mRNA-LNP that effectively mediated gene expression in primary neurons, demonstrating mRNA-LNPs as an effective delivery tool for neuroscience applications.

Methods

Primary Rat Cortical Neuron Culture
E18 rat cortical tissue was purchased from BrainBits, LLC. The cortices were removed from the shipping media and trypsinzed with 0.25% trypsin-EDTA (ThermoFisher). The trypsin-EDA was then inactivated by washing the tissue with 10% FBS in DMEM (ThermoFisher), followed by DMEM alone. The tissue was triturated in Neuronal Media (NeuroCult™ Neuronal Basal Medium (StemCell) with NeuroCult™ SM1 Neuronal supplement (StemCell) and L-Glutamine (StemCell)) supplemented with L-Glutamic Acid (Sigma), and then passed through a 40µm cell strainer to form a single cell suspension. This suspension was then counted and plated on PDL coated culture plates or glass cover slips at a density of 4.8 x 104 cells/cm2. Half of the media was changed with Neuronal media without L-Glutamic Acid every 3-4 days.

Treatment of Primary Rat Cortical Neurons with LNPs
After 7 days in culture (DIV7) the 5µg/mL of ApoE was added followed by the specified LNP at the specified dose. The neurons were then incubated for 48h after which they were harvested for endpoint assessment.

Flow Cytometry
Following treatment and incubation with the specified LNP as noted above the neurons were washed with PBS and then trypsinized with 0.25% trypsin-EDTA to detach them from the culture plate. Trypsinization was inactivated by 3% FBS in PBS and the neurons were triturated to form a single cell suspension. The neurons were then pelleted and washed with PBS before being re-suspended in suspension buffer (BD Biosciences) and Propidium Iodine (BD Biosciences) was added to stain for apoptotic cells. The suspended, stained neurons were then passed through a 35µm cell strainer and then assessed using a BD Celesta Flow Cytometer.

Viability Assay
The standard protocol included with the PrestoBlue® Cell Viability Reagent (ThermoFisher), following the manufacture's instructions. Values were corrected to wells containing media without neurons.

Immunocytochemistry
For this assessment the neurons were plated on PDL coated coverslips, following cultivation, treatment and incubation, the neurons were washed with PBS and fixed with 4% PFA. The neurons were permeabilized using 0.1% triton-X and then blocked with NDS followed by incubation with a MAP2 antibody (Sigma M4403) for 1 hour. The neurons were then blocked in NGS followed by incubation with a GFP antibody (AbCam ab13970) in NGS overnight. Secondary antibodies were then added, AlexaFluor-594 for MAP2 and AlexaFluor-488 for GFP. The coverslips were mounted on glass slides using ProLong® Diamond Antifade Mountant (Thermofisher) and imaged on a confocal microscope. Confirmation of the selective binding of the primary and secondary antibodies was provided through no primary controls (data not shown).

RNA Extraction and RT-qPCR
Following treatment and incubation of the neurons, RNA extraction was preformed using the PureLink® RNA Mini Kit RNA extraction kit (ThermoFisher) following the manufacture's instructions. RNA concentrations were assessed using a NanoDrop (ThermoFisher) and then equal amounts of RNA were used for cDNA conversion using the SuperScript IV VILO Master Mix (ThermoFisher) and following the manufacture's instructions. RT-qPCR was performed using TaqMan primers and probes against the targeted mRNA and ActB, obtained from IDT, and iTaq Universal Probes Supermix master mix (BioRad), run on a BioRad CFX96 thermocycler (BioRad) triplicate wells per biological replicate were used. Expression values of the targeted mRNA were normalized to ActB values using the ΔΔCt method.

Enzyme-linked Immunosorbent Assay
Following treatment and incubation with the specified LNP as noted above the neurons were washed with PBS and lysed as specified in the GFP ELISA kit (Ab Cam Cat. # ab171581). Following lysis the concentration of total protein was assessed using a BCA assay (Ab Cam Cat.# ab102536). Protein concentration was normalized such that 100ng/well of total protein was used in the GFP ELISA kit.

mRNA-LNP Preparation on the NanoAssemblr Platform