Plasmid–Lipid Nanoparticles: A Potent Tool for Manipulating Neuronal Genes *In Vitro* and *In Vivo*

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Introduction

Purpose

Gene transfection in neuroscience has been limited due to the lack of an effective gene delivery tool. Recently, lipid nanoparticles (LNPs) have gained interest as safe and effective nucleic acid 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 plasmid-LNPs using the scalable microfluidics-based NanoAssemblr™ platform. The plasmid-LNPs achieve 95% transfection efficiency and high degrees of exogenous gene expression in difficult-to-transfect neurons *in vitro* and *in vivo*.

Results

GFP plasmid was successfully encapsulated into LNPs using the NanoAssemblr™ platform. The LNPs containing 3.5 kb and 8 kb plasmids were 90 nm and 100 nm in diameter respectively with identical PDI (~ 0.1). Plasmid encapsulation efficiencies > 95% were observed. Flow cytometry analysis after a 48 h treatment of 0.6 μg/mL GFP plasmid-LNP in rat primary neurons at DIV 7 showed > 95% uptake of nanoparticles (based on a fluorescent probe within the nanoparticle) leading to > 60% of cells expressing GFP. The high expression and uptake did not significantly impact cell viability even at doses of 2.5 μg/mL of GFP plasmid as measured using Presto Blue Cell Viability Assay. *In vivo*, GFP expression was observed 48 h following striatal injection of GFP plasmid LNPs in mice. Fluorescent markers for the LNPs and GFP fluorescence were found to colocalize in the striatum, and were found to spread to surrounding regions including parts of the ventricular system and corpus callosum.

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.
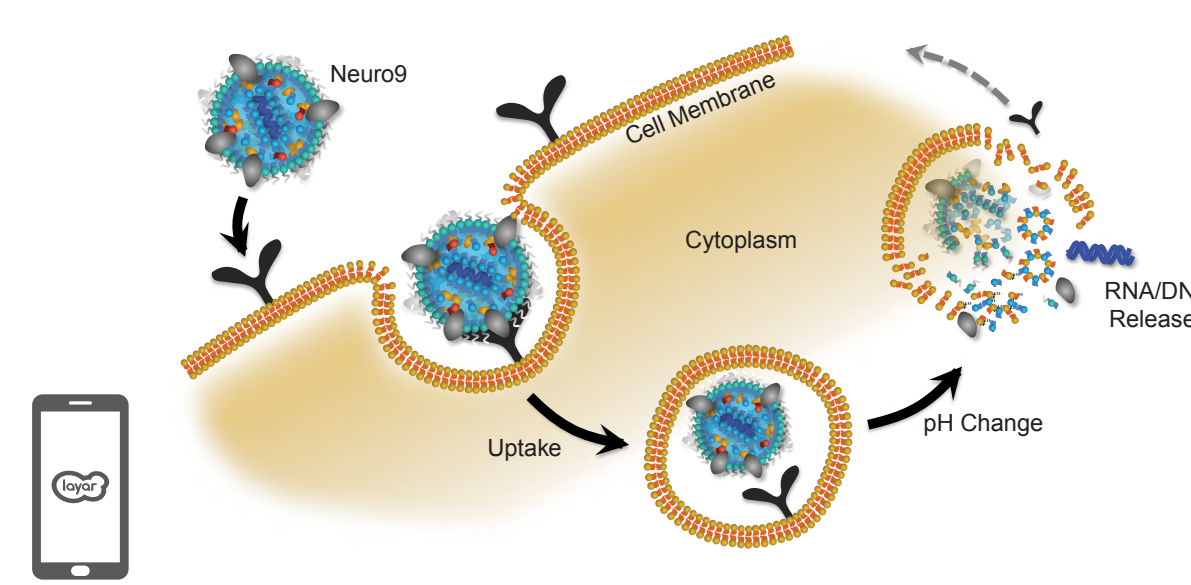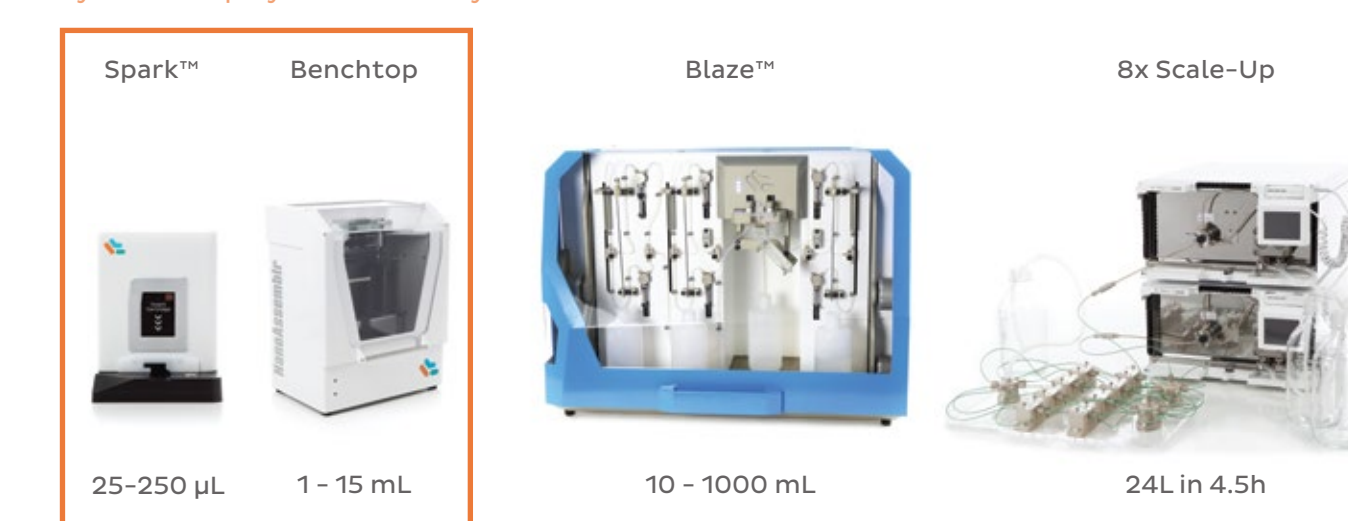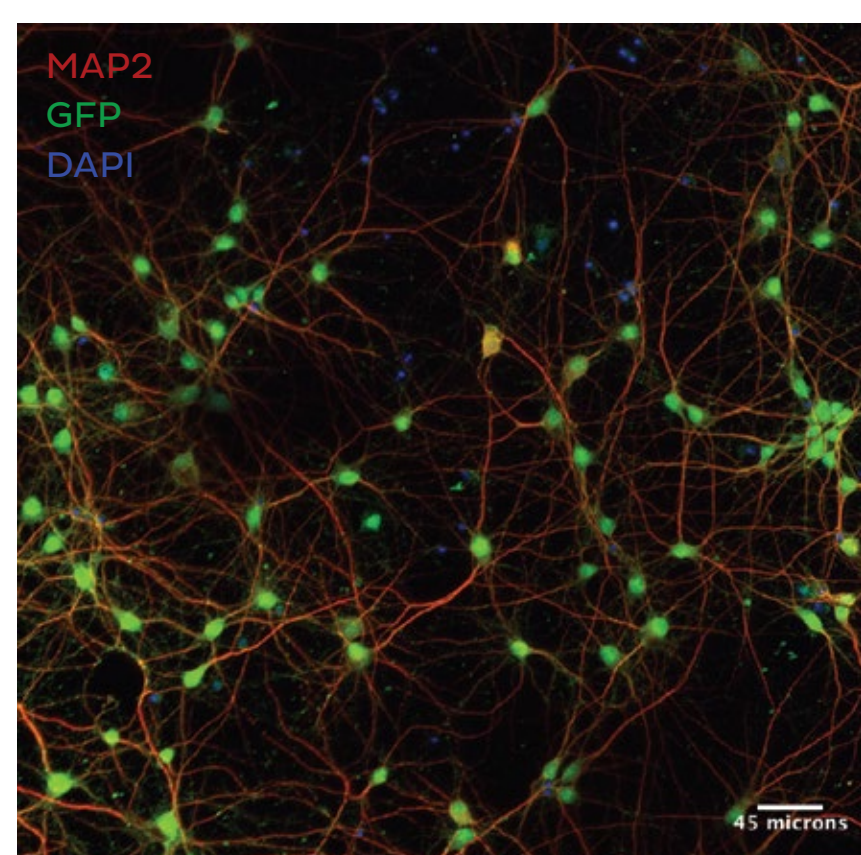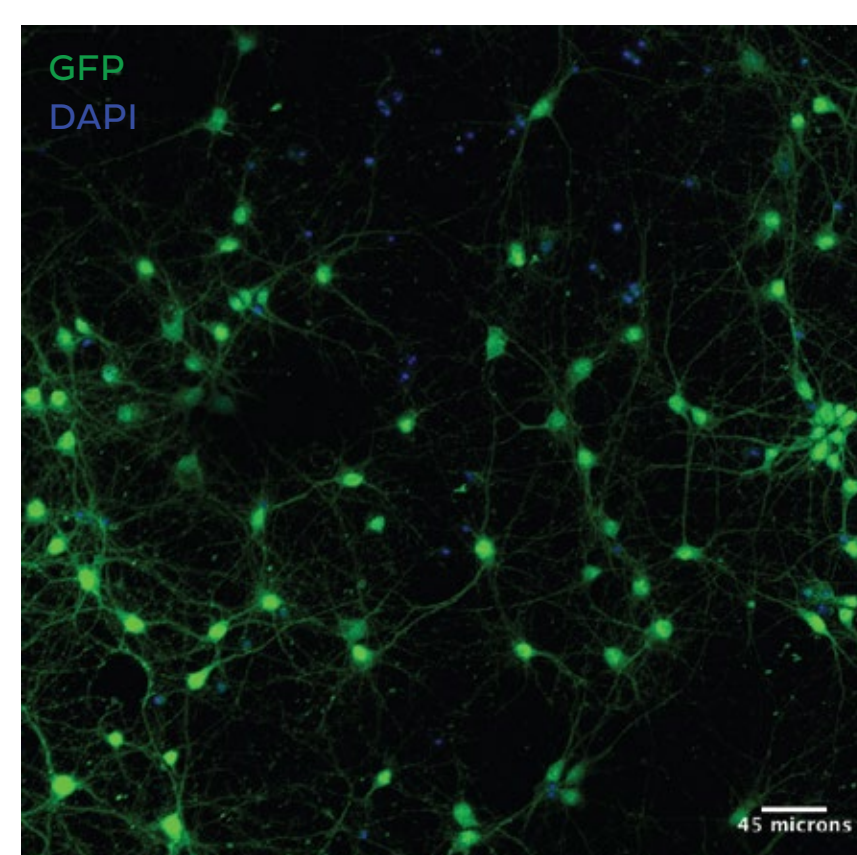
Video: LNP uptake and transfection

NanoAssemblr™ Systems

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

Systems employed in this study

| Spark™	Benchtop	Blaze™	8x Scale-Up
25-250 μL	1 - 15 mL	10 - 1000 mL	24L in 4.5h

In Vitro GFP Expression in Rat Primary Neurons

Supporting Information: Higher magnification images

Confocal fluorescence microscopy showing GFP expression (green) in MAP2 positive neurons (red) after treatment with LNPs encapsulating plasmid DNA encoding GFP 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).

## In Vivo GFP Expression in Rat Brain
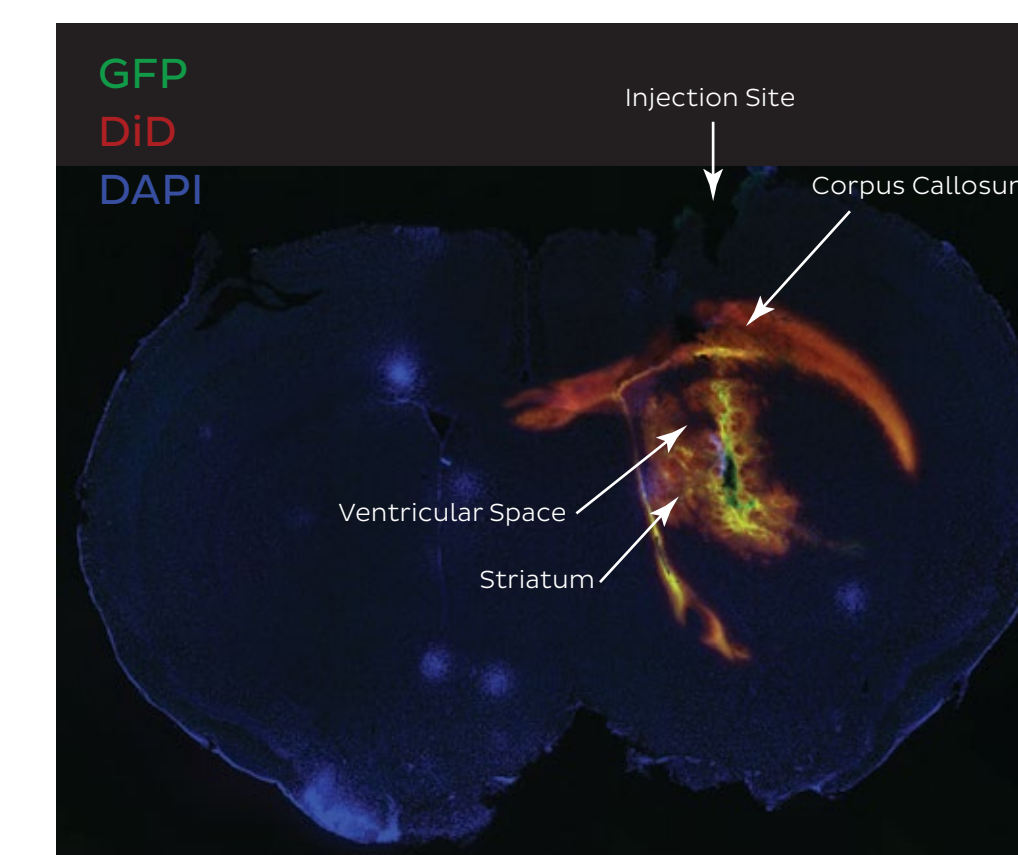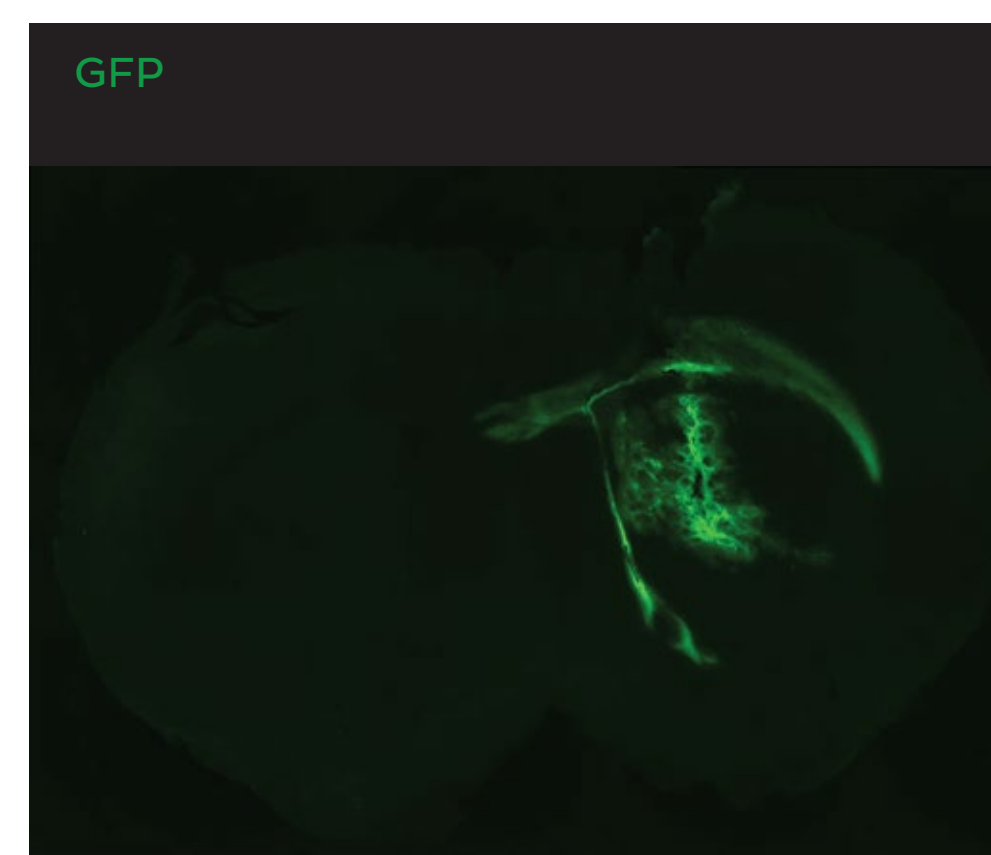
Fluorescence micrographs of brain slices showing GFP expression (green) 48H following striatal injection of LNPs encpasulating plasmid DNA encoding GFP in of 10 month old, FVB mice. Colocalization of nuclear staining with DAPI (blue), LNP uptake using DiD (red), and GFP expression (green) in the same image. Both LNP uptake and GFP expression co-localize within the area of injection, the striatium, and have spread to addtional surrounding regions including parts of of the ventricular system and the corpus callosum.

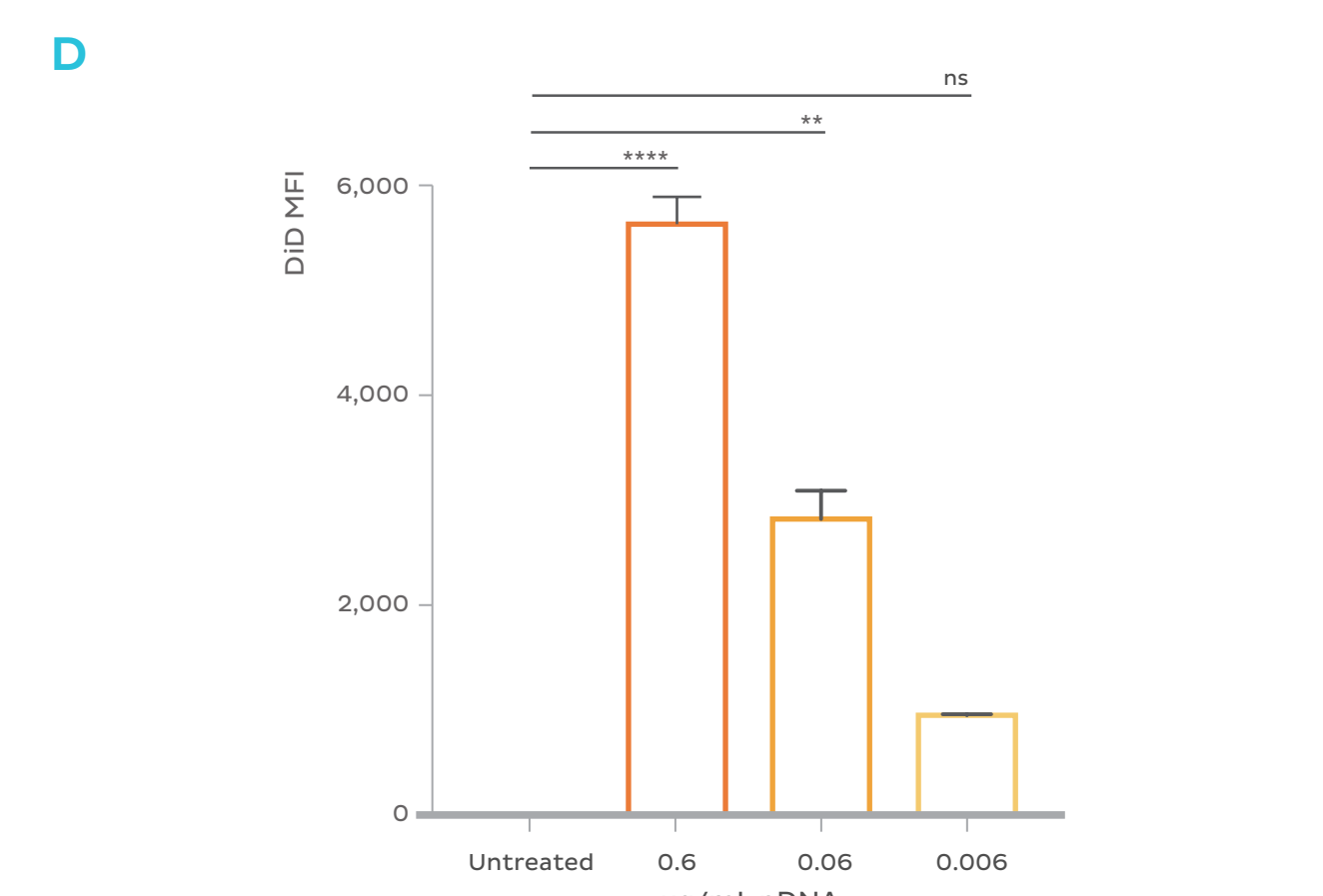## Flow Cytometry Shows GFP Expression and Particle Uptake

A

Dose μg/mL	% GFP +
Untreated	0.01
0.006	1.46
0.06	20.7
0.6	61.5
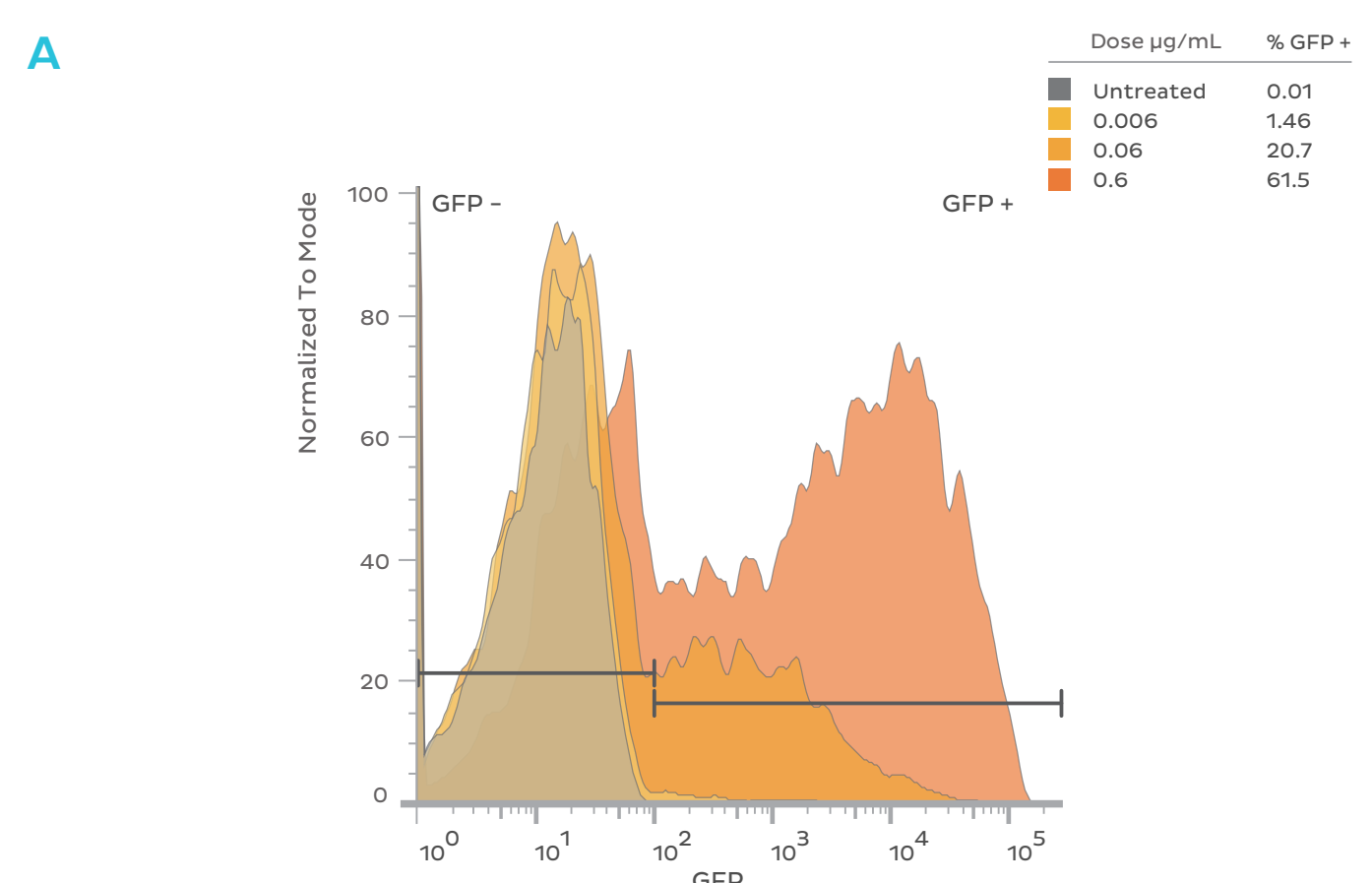
The percentage of GFP + neruons indicates expression of the plasmid and was dose-dependent, with 0.6ug/mL having the higest percentage.

B
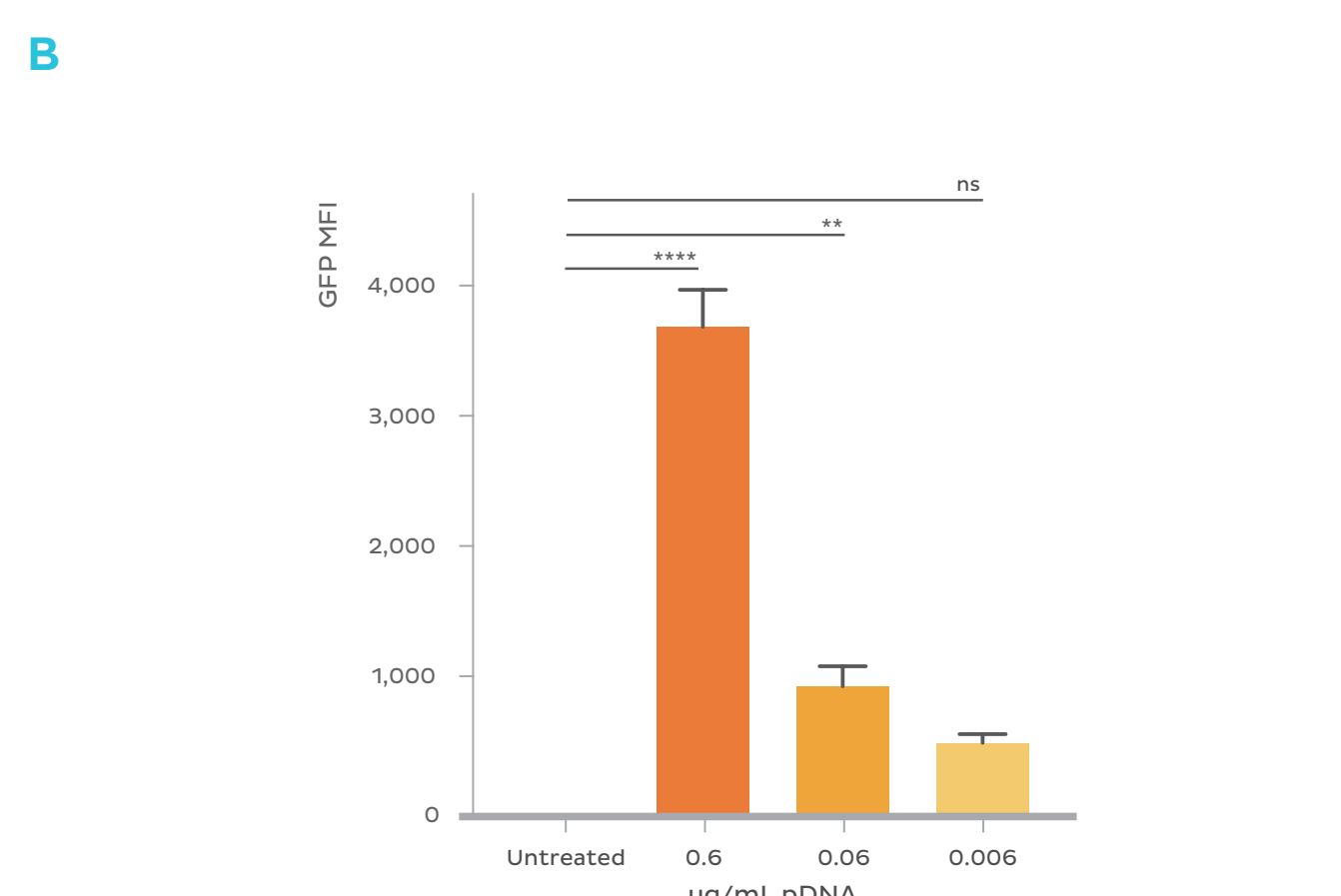
This dose reponse pattern is also observed in the GFP MFI, n=3 for each treatment one-way ANOVA with Dunnett's multiple comparisons test, **** p<0.0001, ** p<0.005

C

Dose μg/mL	% DiD +
Untreated	0.03
0.006	31.1
0.06	88.4
0.6	98.3
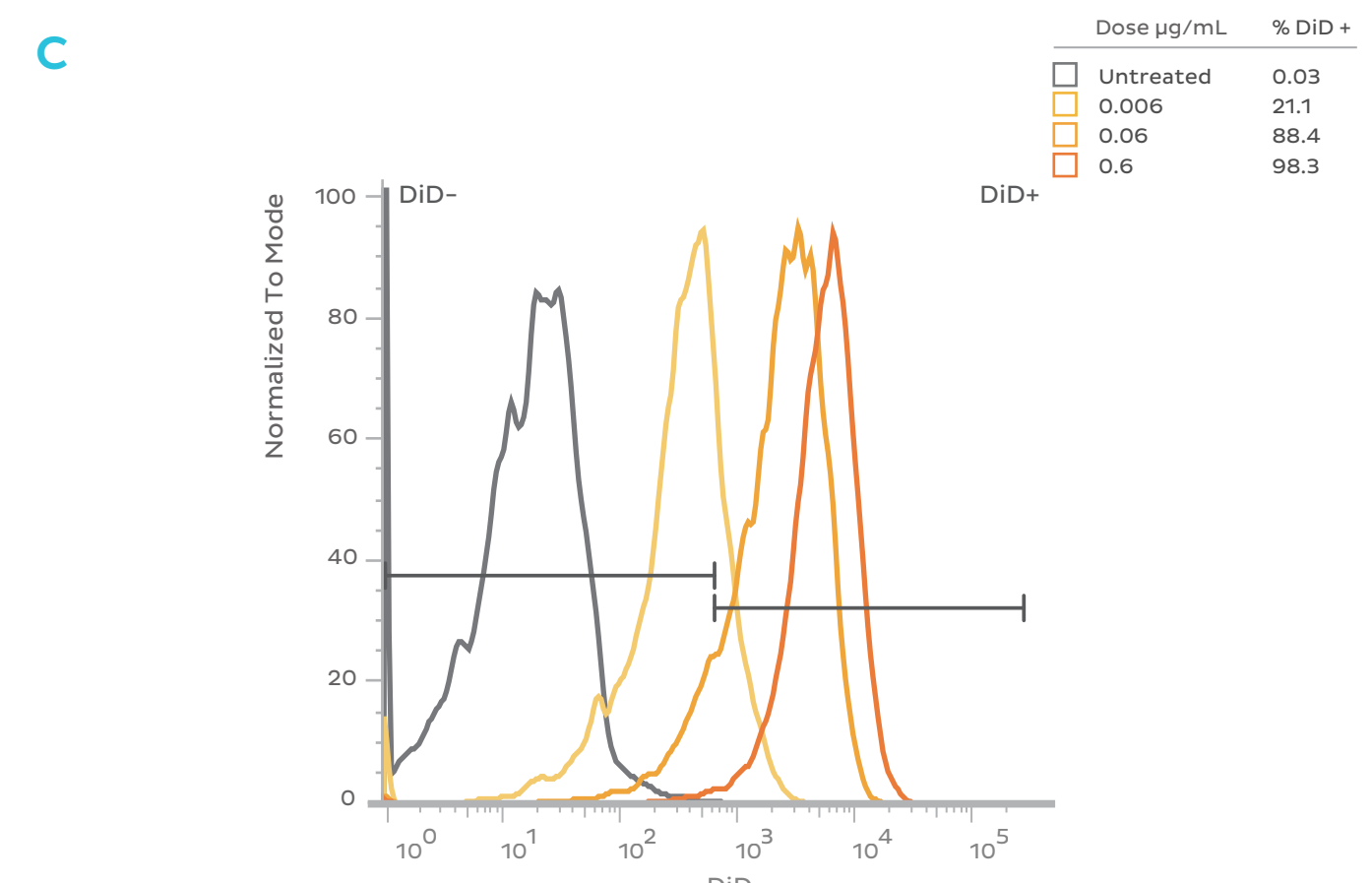
The percentage of DiD+ neruons indicates LNP uptake which was dose-dependent, with 0.6ug/mL having the higest percentage. `

D

This dose reponse pattern is also observed in the DiD MFI, n=3 for each treatment one-way ANOVA with Dunnett's multiple comparisons test, **** p<0.0001, ** p<0.005

Flow cytometry analysis of GFP expression in rat primary neurons treated at DIV 7 with LNPs encapsulating plasmid DNA encoding GFP. At the highest dosage (0.6ug/mL), > 60% of the neurons expressed GFP. Both percent GFP positive and intensity of GFP expression (Mean Fluoresce Intensity; MFI) are titratable by dosage of the GFP plasmid-LNP. Flow cytometry was performed after 48 h of treatment in the presence of 1 μg/mL of ApoE.

Flow cytometry analysis of plasmid LNP uptake in rat primary neurons. Cells were treated at DIV 7 with LNPs containing plasmid DNA and the fluorescent dye DiD. At a dose of 0.6 μg/mL plasmid > 95% of neurons showed uptake of LNPs and > 85% uptake at a one tenth that dose (0.06 μg/mL). Flow cytometry was performed after 48 h treatment with GFP plasmid-LNP in the presence of 1 μg/mL ApoE.
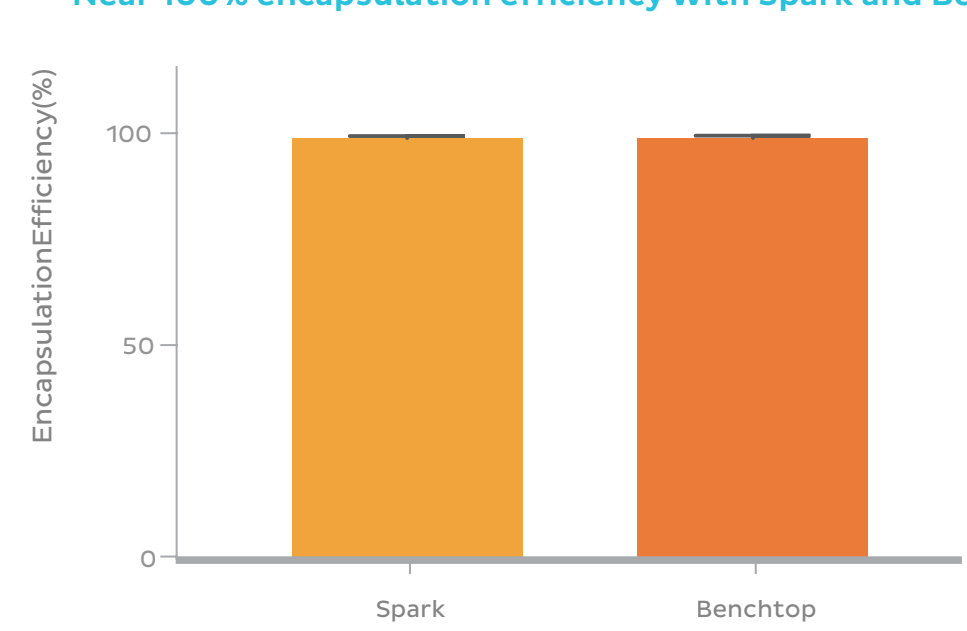
Additional Characterization


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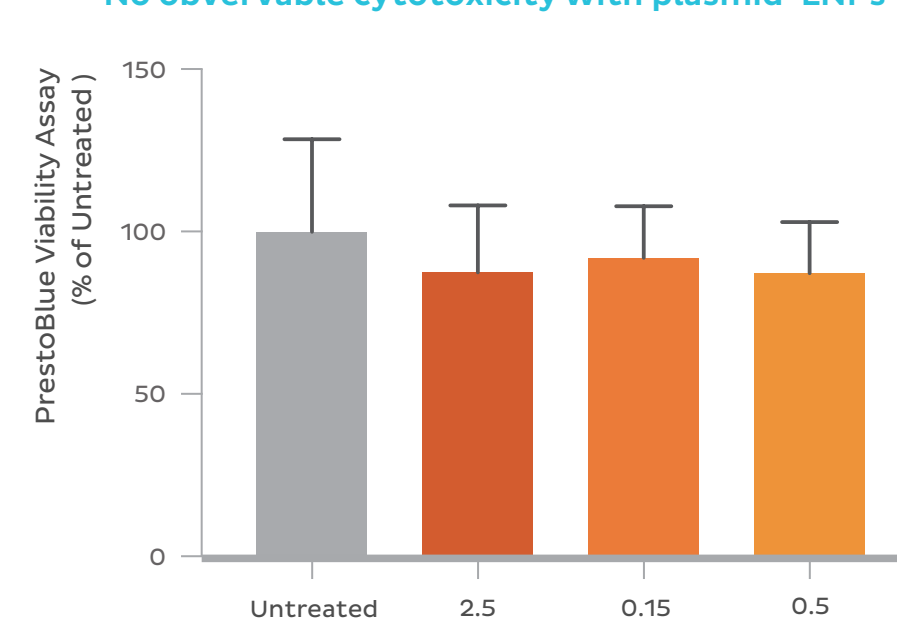
Cell viability was assayed by the PrestoBlue® Cell Viability Reagent. No toxicity was observed at any dose employed, n=6 for each treatment, one-way ANOVA with Dunnett's multiple comparison test.

**Near 100% encapsulation efficiency with Spark and Benchtop**

Encapsulation efficiency of plasmids in LNPs formulated with the Spark and Benchtop approach 100%. Encapsulation determined by PicoGreen Assay. n=3 per group represented as mean with error bars representing SD.

LNP size unaffected by plasmid length
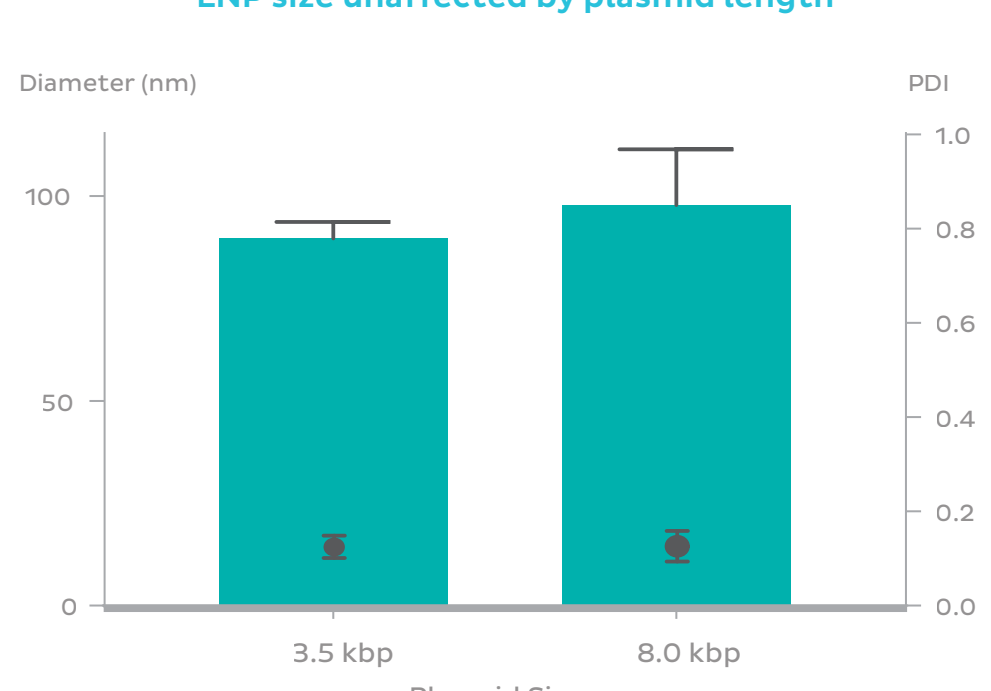
Hydrodynamic size of plasmid-LNP encapsulating different sized plasmids and formulated using the NanoAssemblr Benchtop. Particle size does not change significantly with pasmid size. n=3 per group represented as mean with error bars representing SD.

Plasmid LNPs made on Benchtop are smaller due to Sample Switching
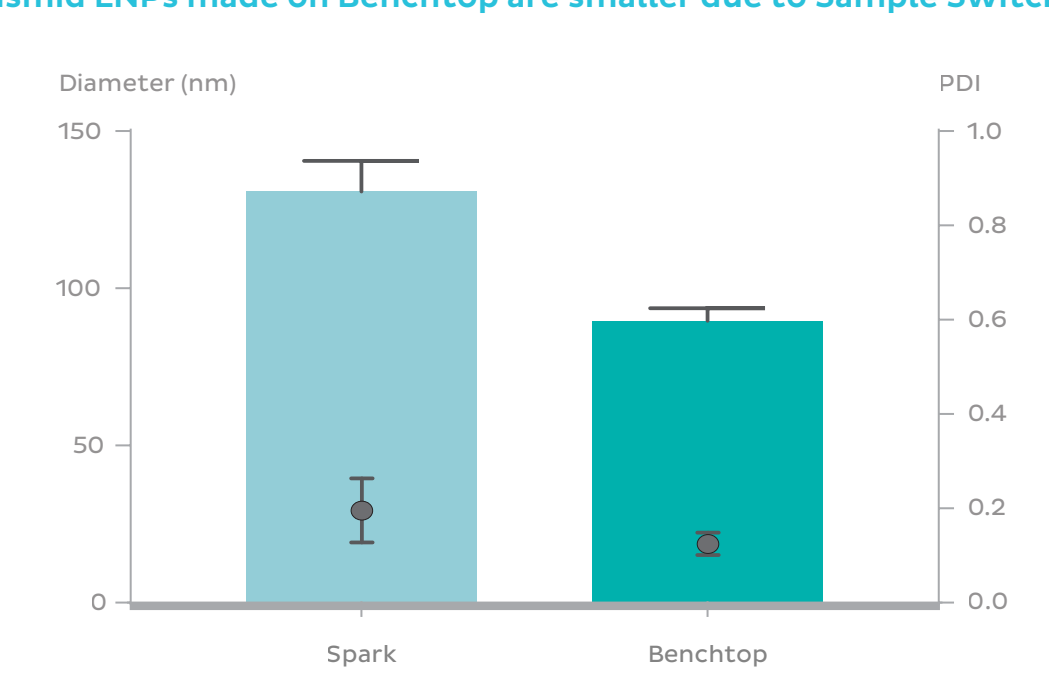
Hydrodynamic size of plasmid-LNP formulated on Spark and Benchtop. Spark particles tend to be larger. n=3 per group represented as mean with error bars representing SD.

Methods

Primary Rat Cortical Neuron Culture: E18 rat cortical tissue (BrainBits, LLC) were removed from shipping media and trypsinized with 0.25% trypsin-EDTA (ThermoFisher). Trypsin-EDTA was inactivated by washing with 10% FBS in DMEM (ThermoFisher), then DMEM alone. The tissue was triturated in Neuronal Media (NeuroCult™ Neuronal Basal Medium with NeuroCult™ SM1 Neuronal supplement, and L-Glutamine (StemCell) supplemented with L-Glutamic Acid (Sigma), and then passed through a 40μm cell strainer to form single cell suspension. Suspension was 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), 5μg/mL of ApoE was added followed by specified LNP at the specified dose. Neurons were incubated for 48h then harvested for endpoint assessment.

Flow Cytometry: Following treatment and incubation with specified LNP 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. 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. Suspended, stained neurons were passed through a 35μm cell strainer, 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: Neurons were plated on PDL coated coverslips, following cultivation, treatment and incubation, neurons were washed with PBS and fixed with 4% PFA. 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, 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. 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 was extracted using PureLink® RNA Mini 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 (IDT Technologies), and iTaq Universal Probes Master Mix (BioRad), run on a thermocycler (BioRad, CFX96) triplicate wells per biological replicate were used. Expression values of targeted mRNA were normalized to ActB values using the ΔΔCt method.

In Vivo Plasmid Transfection: YAC128xNSG mice at 5 months of age were intercranially injected with LNP-encapsulating plasmid encoding a protein with an eGFP reporter. Mice were anesthetized with 1% isofluorane. The heads were prepped and a single craniotomy was made +0.5mm anterior and -1.75mm lateral to bregma. A Hamilton syringe was loaded with 5 μl of plasmid-LNP and lowered -2.5mm from the dura. 5 μl of plasmid-LNP was injected at a rate of 0.5 μl/minute. Needle was left in place for 5 min before being retracted over course of 3 minutes. Skull was sealed with bone wax and sutured. Mice were observed for recovery until ambulatory. 48 hrs following injection mice were intercranially perfused with PBS and formalin. Brains were extracted and cryopreserved in 30% sucrose before flash frozen in isopropanol. Brains were sectioned using a cryostat at 30 μm thick serial sections with every 6th section being collected for analysis. Brains were mounted and cross-labeled with Hoescht (1:1000) for 15 min. Slices were imaged (Keyence BZ-9000) at 4x and stitched together using Keyence Viewing Software.

Plasmid-LNP Preparation on the NanoAssemblr Platform

NanoAssemblr Spark Up To 250 μL

1. Pipette RNA and Neuro9 solutions.
2. Insert into Spark and press "Run".
Pipette out and apply to cells.

NanoAssemblr™ Spark™ Reactor
1. Neuro9 Mix
2. Plasmid in Buffer
3. RNA-Neuro9 Output

NanoAssemblr Benchtop Up To 15 mL

1. Prepare solution of nucleic acids, thaw lipid mix, and load into separate syringes.
2. Load syringes into NanoAssemblr Benchtop and run formulation. (3 Min)
3. Purify with centrifugal filtration or dialysis.
Localized injection or systemic delivery.

Conclusion

The NanoAssemblr™ microfluidics-based technology reproducibly manufactured plasmid-LNPs that effectively mediated gene expression in primary neurons *in vitro* and *in vivo*, demonstrating plasmid-LNPs produced on the NanoAssemblr™ platform as effective delivery tools for neuroscience applications.

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