NEUROSCIENCES APPLICATIONS

Primary neuronal cultures from various territories or animals origins as well as primary microglial cells or neuronal cell line cultures are commonly used to understand mechanisms of neural function, dysfunction, and degeneration. In particular, primary neuronal and microglial cells are suitable to carry out in-depth studies of molecular events, inflammatory pathways, biochemical pathways or neuronal excitability, which are difficult to perform with in vivo models¹. The different types of experiments that are readily available when using primary culturing (high-resolution microscopy analyses, genetic manipulations, electrophysiological recordings, genomics and proteomic analyses, optogentics, genome editing...) are important for many fields of neuroscience research such as to study neurotoxic effects of drugs, neuronal plasticity, or deciphering pathway to understand the molecular basis of neuronal diseases.

Guidelines





This Application Note will focus on many aspects on the use of magnetic nanoparticles for neurosciences applications and will be illustrated with the most recent or insightful scientific publications.

A highlight will be done in vitro on our best sellers **Neuro-Mag™** & **Glial-Mag™** products and In vivo, a spotlight will be placed on our **BrainFectIN™** product. However for many reasons that are to be depicted in a following paragraph, all the results obtained from neuronal cultures cannot easily be extended to the whole organism physiology and thus there is still a need once the basis are settled using in vitro model, to work directly in vivo or to a lesser extent, ex vivo. As a matter of fact, among all the organs, tissues and territories that make up an organism, the nervous system is one of the most complex and the cells that make it up are among the most difficult to modify genetically using classic gene delivery methods.

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1 Millet, L. J. & Gillette, M. U. Over a century of neuron culture: from the hanging drop to microfluidic devices. Yale J Biol Med 85, 501–521 (2012).

OZ Biosciences has designed several optimized Magnetofection[™] Transfection Reagents according to defined applications:

NON VIRAL APPLICATIONS

- PolyMag[™] | PolyMag Neo[™]
- CombiMag[™]
- Magnetofectamine O2 Kit
- NeuroMag[™]
- Glial-Mag[™]
- SilenceMag[™]
- FluoMag[™]
- SelfMag[™]

VIRAL APPLICATIONS

- ViroMag[™]
- ViroMag R/L
- AdenoMag[™]
- Mag4C-LV / Mag4C-AD

IN VIVO APPLICATIONS

- In vivo PolyMag[™] & DogtorMag[™]
- In vivo ViroMag[™]

The gene delivery systems are classically organized into two main categories: viral and non-viral. Viral vectors (Lentiviral–LV, adenoviral–Ad, adeno-associated virus–AAV) are commonly applied to transduce primary neurons or Neural Stem Cells (NSC) due to their high efficiency but numerous drawbacks limit their use especially when addressing in vivo transduction.

With a reduced immunological response, the **non-viral approaches** have gained interest over the last decades even if they suffer from a low efficacy compared to viral methods.

Non-viral gene transfer methods can be classified in two groups:

(1) chemical delivery systems such as cationic polymers, cationic lipids or calcium phosphate

(2) **physical delivery systems** such as electroporation, ballistic gene transfer, microinjection and Magnetofection[™].

The existing gene delivery systems usually dedicated to primary microglia and post-mitotic neurons such as hippocampal, cortical, motor neurons or NSC have several limitations due to intrinsic characteristics of these cells. The post-mitotic neurons, indeed, are very sensitive to micro-environmental as well as pH changes, osmotic shocks.... In addition, a foreign nucleic acid cannot enter in the nucleus during the nuclear envelope breakdown, occurring only in mitotic cells. Moreover, the main barrier comes from the fact that the transfection procedures are often toxic to these cells.

Regarding NSC, the transfection techniques can also easily interfere with proliferation and differentiation and these two latter points are of huge importance when considering genetic modification of these cells. Actually a comparative study reviewed methods of gene delivery for neural primary cells and the main drawbacks of one or the other often pinpoint the high toxicity index, lack of reproducibility, the narrow age windows for transfecting neurons or the price of equipment.

Among the gene delivery systems tested a physical one, **the nucleofection**, **gave interesting results but only address the transfection of young neurons** (4 days in vitro -DIV) and cells in suspension at the time of plating². On the other hand, lipid-based methods routinely used for classic transfection in numerous cell models also show their limitation when dealing with neuronal cultures: effective up to a certain limit, large doses of cationic lipids and nucleic acids need to be used to ensure a correct transfection rate that in turn, lead to a massive neuronal death as these cells are very sensitive to any cellular shock as afore mentioned. OZ Biosciences supplies several solutions for Neuroscience Applications:

- NeuroMag[™]
- Glial-Mag[™]
- BrainFectIN™
- ViroMag[™] R/L
- In vivo ViroMag™
- AdenoMag[™]

As soon as 2007, Buerli et al. while stating that "efficient and long-lasting transfection of primary neurons is an essential tool for addressing many questions in current neuroscience using functional gene analysis", confirmed that neurons are sensitive to cytotoxicity and difficult to transfect with most existing methods.

The authors used a then recent technique called Magnetofection[™] to transfect and co-transfect DNA and short hairpin RNA (shRNA) into rat hippocampal neurons cultured from several hours to 21 days in vitro and achieved high efficiency with a long lasting expression of the transgene while inducing low toxicity. This leading original paper published in Nature protocol set up the basis of the genetic modification of neuronal cells with the use of Magnetofection[™], a magnetic nanoparticles (MNP)-based method that would become one of the most efficient and non-toxic gene delivery system.

Since more than a decade now, MNPs have received increasing attention for their use in promoting gene delivery under magnetic attraction, using viral or non-viral gene carriers. This very promising approach of combining magnetic vector targeting and gene delivery was called Magnetofection[™] by Plank and colleagues³ and was demonstrated over the years to be easy-to-use yet dramatically efficient for the delivery of genetic material to cells using magnetic nanoparticles controlled by an external magnetic field. Compared to conventional transfection based on polymers or lipids, magnetofection benefits from a number of obvious advantages, such as a higher efficiency and, consequently, a lower required dose of nucleic acid or viral vectors, shorter delivery time, and the ability to transfect locally and in a limited area⁴. In vitro, for instance MNPs can be applied to adherent cells in cell culture and directed with a magnet placed at the bottom of the plate or flask. Magnetic nanoparticles are then assumed to be internalized through endocytic pathway with high transfection efficiency (Figure 1). In vivo on the other hand, therapeutic gene/MNP complex are either injected intravenously in the systemic circulation, or directly into the vicinity or the close proximity of the organ or tissue to genetically modify. Then, a specific magnetic field delivered by a strong external magnet positioned near the site of interest directs the MNPs to a focal target, in order to increase the transfection efficiency. The gene of interest is thus retained abundantly only at specific target site.

3 Scherer, F. et al. Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo. Gene Ther 9, 102–109 (2002). 4 Plank, C., Zelphati, O. & Mykhaylyk, O. Magnetically enhanced nucleic acid delivery. Ten years of magnetofection-progress and prospects. Adv Drug Deliv Rev 63, 1300–1331 (2011). **Figure 1.** Endocytosis and intracellular trafic. Magnetic nanoparticles associated to nucleic acids or lipoplexes are concentrated onto the cell surface by a magnetic field - B - (1). This accumulation induced endocytosis (2). Then after, nanoparticles are captured into endosomes (3). Several mechanisms induce breakin of the endosomal membrane (4) and complexes are released into e cytoplasm (5). Complexes are dissociated (6), and the nucleic acid are transported to the nucleus (7a) (DNA) or to the DICER/RISK system (7b) (RNA). Transfection efficiency may be reduced by several barriers at each step (represented in red). Illustration adapted from Sapet C et al⁵.



Beside their use for improving gene transfection of non-viral and viral vectors, recent research based on MNPs has shown increasing use for biomimetic cell membrane nano-gene carriers, exosome-based gene delivery, cell-based drug delivery systems or CRISPR/Cas9 gene editing. Moreover, magnetic nanoparticles are routinely applied in magnetic resonance imaging or magnetic hyperthermia therapy, providing proof of concept of their total safety in vivo when injected locally or into the systemic circulation.

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5 Sapet, C., Laurent, N., Le Gourrierec, L., Augier, S. & Zelphati, O. [In vitro and in vivo Magnetofection : a move towards gene therapy]. Ann Biol Clin (Paris) 68, 133–142 (2010).

Specifically developed for Neuronal Applications or Microglial Cell Transfection by OZ Biosciences NeuroMag[™] (ref. #NL51000) and Glial-Mag[™] (ref. #GL00500) magnetic nanoparticles respectively were formulated to achieve high transfection of different types of nucleic acids, such as DNA, RNA, or oligonucleotides in serum compatible condition, for both transient and stable transfection. This versatility of applications is due to the very formulation of MNPs: based on organic and inorganic materials, their **chemical structure allows adaptable functionalization that offers a high potential for multi-tasking**. The inorganic core (iron oxide, silica, PLGA...) protected from corrosion by a lipid or polymer coating can be decorated with functional organic molecules/bioactive reagents. Generally the accuracy for application comes from the specificity of the coating that can serve also as a linkage layer for bioactive reagents such as genetic material, drugs or antibodies⁶.

In terms of neural cell therapy, magnetic nanoparticles have found a concrete application so far in medical imaging (magnetic resonance imaging -MRI), to label transplant populations for graft tracking in host neural tissue⁷. Nowadays, MRI is widely available in clinical setting and imaging which definitively confirms the total harmlessness of magnetic nanoparticles.

Opposed to electroporation, any kind of neurons can be transfected during their adherent state at any stage of differentiation or DIV and multiple transfections can also be performed in the same culture at different time points (Figure 2). As for any other magnetic nanoparticles developed by OZ Biosciences (such as PolyMag Neo[™], CombiMag[™], SilenceMag[™], ViroMag RL[™], ViroMag[™] Stem...) the complexation between cargo and magnetic core are based on electrostatic and hydrophobic interactions mediated by the specific coating as previously mentioned. This confers a fully biodegradable material and do not interfere with cellular homeostasis.



6 De Crozals, G., Bonnet, R., Farre, C. & Chaix, C. Nanoparticles with multiple properties for biomedical applications: A strategic guide. Nano Today 11, 435–463 (2016).

7 Zheng, B. et al. Magnetic Particle Imaging tracks the long-term fate of in vivo neural cell implants with high image contrast. Sci Rep 5, 14055 (2015).

Figure 2. Principles of Magnetofection[™] for in vitro applications in NeuroSciences. Nucleic acids (DNA, siRNA, mRNA...) or lipoplexes are incubated for 20 min with magnetic nanoparticles. Complexes are then added to primary neurons, neuronal cell lines, or glial cells and incubated for 20-30 min onto a magnetic plate delivering a specific magnetic field. The magnetic force pulls the magnetic complexes towards the cells and therefore promotes endocytosis. In order to focus on **Neurosciences Applications** a highlight will be done in vitro on:

(1) NeuroMag[™] that, over the years has demonstrating a phenomenal capacity for transfecting different primary neuronal populations, from dopaminergic, hippocampal, cortical to motor neurons with any kind of nucleic acid vector

(2) Glial-Mag[™], a magnetic nanoparticles formulation dedicated to microglia transfection.

This non-exhaustive note will focus on many aspects on the use of magnetic nanoparticles for neurosciences applications and will be illustrated with the most recent or insightful scientific publications.

In vivo, a spotlight will be placed on BrainFectIN[™], a polymer based transfection reagent dedicated to Brain transfection for gene expression or gene silencing and some example will use NeuroMag[™] to set an example on the capacity to target in vivo gene transfection.

.FORMATION OF COMPLEXES



PRIMARY NEURONS NEUR

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MAGNETIC PLATE : 20-30 min.

Summary

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More than 2000 publications show the efficiency of Magnetofection[™] and a large variety of cells was successfully transfected with NeuroMag[™] reagent.

Application for Primary Neurons, Cortical & Hippocampal

Originally developed for hippocampal and cortical neurons, it was thus logically obvious to begin this application note with the impact that Magnetofection[™] and more specifically NeuroMag[™] had on Neuroscience Research since many years while transfecting these cell types.

As previously mentioned, one of the most important advantages of Magnetofection[™] is that this method can be used to transfect neurons at any DIV from day 0 to more than 20 days after seeding; in the following, some examples will illustrate this unique capacity. Thereby Asselin L. and colleagues used **NeuroMag[™]** to transfect cortical neurons dissected from E15.5 CD1 mouse embryos in culture at DIV0 and DIV2 using the recommended instruction to follow the axonal growth and measure the neurite growth. Immunostaining 2 and 3 days later of transfected neurons confirmed that high gene expression has occurred allowing to dig deeper into pathogenic mechanisms⁸. Interestingly whereas researchers generally aim at genetically modifying the highest number of cells, depending on the conditions used, **NeuroMag[™]** efficiency can be modulated to transfect only a small fraction of cells to achieve the researched objective. Actually, as opposed to lentiviral methods that do not allow to efficiently controlling neither the transfer nor the quantity of vector copy that is delivered into cells, Magnetofection[™] has the capacity to induce transfection even with low doses of DNA allowing to finely tune the dose or the effect of the genetic modification. So, working on mice cortical neurons at DIV 5, Tagliatti E et al used this transfection reagent to deliver DNA only in a small (~3 to 5%) subpopulation of neurons "which was essential for imaging of vesicular release in individual synaptic boutons"⁹. Doing so, the authors demonstrated that the disruption of oligomerisation of Synaptotagmin1 (a neurotransmitter synchronizer) increased neurotransmitter release in response to single action potentials and abolishes clamping of Synaptotagmin7-mediated asynchronous release. These data allowed them to conclude to the importance of the Syt1 oligomerization for neurotransmitter release.

9 Tagliatti, E. et al. Synaptotagmin 1 oligomers clamp and regulate different modes of neurotransmitter release. Proc Natl Acad Sci U S A 117,

⁸ Asselin, L. et al. Mutations in the KIF21B kinesin gene cause neurodevelopmental disorders through imbalanced canonical motor activity. Nat Commun 11, 2441 (2020).

Application for Primary Neurons, Cortical & Hippocampal

The majority of the experiments use Magnetofection[™] to transfect mid-term cultured cortical or hippocampal neurons (from DIV 6 to 14) as illustrated by the works performed by Ramos-Fernandez et al. These authors published two papers mentioning the use of NeuroMag[™] to transfect primary cultures of hippocampal neurons at DIV8 and DIV 10 respectively. Cultivated in 6-well plates, 50x104 hippocampal neurons were transfected at DIV8 with 4µg DNA per well and immunostaining at DIV10 helped the researchers to conclude that Wnt signaling through Wnt5a was involved in synaptic formation and remodeling.

The same authors then transfected and co-transfected DIV10 primary hippocampal neurons to quantify dendritic spines 4 days later by immunofluorescence¹⁰. Not only had the transfection succeeded in genetically modify cells but also the total harmlessness of the method enabled to have access to fully viable cells allowing the measure of dendrite length. Data gained within the two sets of experiments allowed the authors to conclude that **Wnt signaling is essential to hippocampal synaptic plasticity**. DIV10 neurons were also transfected with a variety of constructs using **Magnetofection™** to study their regeneration capacities 3 to 7 days later by laser axotomy confirming the fact that **NeuroMag™** allows long lasting of transgene expression and thus low (or no) toxicity and does not interfere with any following experiments whatever the readout is¹¹.

As a corollary to transfect immature young neurons at early DIV, the difficulty to genetically modify neurons rises with aging and long termed cultured (>10 days in vitro). Once again, pioneering magnetofection for neuron gene expression, Buerli et al established a protocol to optimize cell viability while favoring the best transfection efficiency. Not only the culture conditions should be improved with the supplementation in B27 additive or with the replacement of half of the medium every 3 days after 10 days in vitro¹², but also the very conditions for transfection should be modified depending on the cell type, the territory and the age.

¹⁰ Ramos-Fernández, E., Tapia-Rojas, C., Ramírez, V. T. & Inestrosa, N. C. Wnt-7a Stimulates Dendritic Spine Morphogenesis and PSD-95 Expression Through Canonical Signaling. Mol Neurobiol 56, 1870–1882 (2019).

¹¹ Petrova, V. et al. Protrudin functions from the endoplasmic reticulum to support axon regeneration in the adult CNS. Nat Commun 11, 5614 (2020).

Application for Primary Neurons, Cortical & Hippocampal

The difficulty of transfection and the sensitivity of the cells rise with the aging of neurons in culture. This is why, it becomes more and more challenging **genetically modify primary neuronal culture** with DNA for gene expression or silencing and thus, only the Magnetofection[™] methods **provide a valuable tool to this end**. Cultivated in presence of AraC during 24H to avoid glial cell proliferation, rat and mouse cortical cultures were co-tranfected at 15 days in vitro using NeuroMag[™] with plasmids encoding for shRNA and control genes. This allowed the authors to study the contribution of the c-Abl kinase to learning and memory by favouring synaptic remodeling¹³. Latter in neuron aging, on their way to decipher the pathways that led to the evolution of the human neocortex and that play an important role during human brain development, Charrier et al. in 2014 expressed and silenced gene expression using shRNA vectors in mice cortical neurons from DIV 10 to DIV 20¹⁴.

At this latter stage, cotransfection were performed at a 1:1 ratio meaning that 1 µL NeuroMag[™] was used to complex and transfect 1 µg nucleic acid instead of the 3.5:1 ratio generally used and recommended, proving once again that Magnetofection[™] can adapt to any kind of cell culture. Since then, this study paved the way for many other works and long-termed mature neurons cultures now appear accessible to genetic modification using Magnetofection[™] whereas it was almost impossible before. And so, DIV 18 or 19 hippocampal neurons are routinely modified with DNA (simple or co-transfection) using NeuroMag[™] respectively for instance to monitor subcellular distribution of protein in synapse or dendrites¹⁵ or to decipher the role of miRNA into the maturation of neurons¹⁶.

Most of the published scientific articles focus their work on rodent (rat or mouse) or human neuronal cells. The versatility of **Magnetofection™** to transfect hippocampal or cortical neurons of any animal origins was recently demonstrated for the first time in bird brain cells by Biegler et al. The authors used magnetic nanoparticles to transfect zebra finch cortical neurons with plasmid DNA and transfected primary neurons showed differential localization on a cell-by-cell basis providing strong and consistent expression in the transfected post-mitotic cells¹⁷.

16 Guajardo, L. et al. Downregulation of the Polycomb-Associated Methyltransferase Ezh2 during Maturation of Hippocampal Neurons Is Mediated by MicroRNAs Let-7 and miR-124. Int J Mol Sci 21, E8472 (2020).

¹³ González-Martín, A. et al. c-Abl regulates a synaptic plasticity-related transcriptional program involved in memory and learning. Prog Neurobiol 102122 (2021) doi:10.1016/j.pneurobio.2021.102122.

¹⁴ Charrier, C. et al. Inhibition of SRGAP2 function by its human-specific paralogs induces neoteny during spine maturation. Cell 149, 923–935 (2012).

¹⁵ Gou, G. et al. SynGAP splice variants display heterogeneous spatio-temporal expression and subcellular distribution in the developing mammalian brain. J Neurochem 154, 618–634 (2020).

Application for Primary Neurons, Cortical & Hippocampal

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It is admitted that an efficient transfection is defined by the percentage of transfected cells or the overall protein amount produced that is generally measured after 48 or 72H. However, when addressing neurosciences applications other important parameters must be taken into account such as the viability index or the lasting of the transient expression of gene of interest. This latter criterion is of major interest since it not only includes a notion of efficiency but also and above all, it defines by itself the notion of viability. During NeuroMag[™] development and formulation, the experiments showed that the transgene was expressed over 15 days assessing the safety of the transfection reagent. Since then several articles confirmed these data as illustrated by the work of Tischbein M and colleagues that transfected primary rat cortical neurons at DIV 7 with NeuroMag[™] at a ratio of 1.75:1 and further collected the genetically modified neurons 7 to 9 days after assessing both the lasting of the gene expression and the intrinsic total lack of toxicity¹⁸.

18 .Tischbein, M. et al. The RNA-binding protein FUS/TLS undergoes calcium-mediated nuclear egress during excitotoxic stress and is required for GRIA2 mRNA processing. J Biol Chem 294, 10194–10210 (2019).

¹⁷ Biegler, M. T. et al. Induction of an immortalized songbird cell line allows for gene characterization and knockout by CRISPR-Cas9. (2021) doi:10.1101/2021.05.27.445896.

siRNA, ODN, miRNA

Main applications for gene expression using <u>NeuroMag[™]</u> in primary hippocampal and cortical neurons: Gene expression, Electrophysiological recording, immunostaining, gene rescue, gene KO with shRNA, genome edition (see below), subcellular localization...

Originally developed for primary hippocampal and cortical neurons, <u>NeuroMag™</u> has gained more interest over the years to transfect other types of neurons; among them, motor neurons are the second type the most employed for transfection. Beside the transfection of plasmid DNA for gene expression or inhibition (with DNA encoding for shRNA), NeuroMag[™] can be used to deliver any kind of nucleic acid such as oligonucleotides (ODN), micro RNA (miRNA), siRNA, mRNA...

Thanks to this versatility, Magnetofection[™] is also a very powerful tool to silence gene expression with siRNA in primary neurons as demonstrated by Kute PM et al., that directed knockdown in DIV12 primary cultures prepared from rat cerebral cortices with NeuroMag[™] to investigate the distinct and convergent roles of two proteins in the translation of mRNA¹⁹. Considering oligonucleotides, Lafourcade et al. succeeded in transfecting primary hippocampal neurons 3 days or 14 days after plating with oligonucleotides carrying the sequences of miRNAs. *"The efficiency of transfection was ~90%"*, the authors stated, as already observed in their previous work and lasted at least 7 Days since whole patch clamp recordings of the transfected neurons were done at DIV10²⁰. This high efficiency allowed them to consider a connection between their miRNA of interest and generalized stress response and moreover on glutamate neurotransmission.

In a quite similar approach **almost 90-95% of neurons were also transfected with siRNA and miRNA** in a work reported by Wang Y et al. on their way to relate miRNA-132 downregulation and Alzheimer's disease through tau phosphorylation²¹. The originality of this publication comes from the fact that NeuroMag[™] was used to transfect human neurons cultured for six months; far beyond the maximum 21 DIV time window in which neurons are generally employed.

21 Wang, Y. et al. Downregulation of miR-132/212 impairs S-nitrosylation balance and induces tau phosphorylation in Alzheimer's disease. Neurobiol Aging 51, 156–166 (2017).

¹⁹ Kute, P. M., Ramakrishna, S., Neelagandan, N., Chattarji, S. & Muddashetty, R. S. NMDAR mediated translation at the synapse is regulated by MOV10 and FMRP. Mol Brain 12, 65 (2019).

²⁰ Lafourcade, C. A. et al. A Role for mir-26a in Stress: A Potential sEV Biomarker and Modulator of Excitatory Neurotransmission. Cells 9, E1364 (2020).

Application for Primary Motor Neurons

Motor neurons (MNs) are neuronal cells located in the central nervous system (CNS) controlling a variety of are neuronal cells located in the central nervous system (CNS) controlling a variety of downstream targets and can be classified in upper MN (originate from the cerebral cortex) and lower MN located in the brain spinal cord ; spinal MN are responsible for the contraction of effector muscles in the periphery. They are the longest known cell type with axons extending through several meters in mammals and are extensively studied due to their implication in progressive or spinal muscular atrophy, primary lateral sclerosis or amyotrophic lateral sclerosis. Considering this latter pathology Magnetofection[™] was ap**plied in numerous publications**; Sama R. et al. demonstrated the implication of the p38 MAPK kinase activation in a novel gain-of-toxic function in ALS with the help of magnetic nanoparticles that were used to genetically modify DIV 2 motor neurons with plasmid DNA. In this study, the authors followed axon outgrowth by transfecting DNA with NeuroMagTM at a ratio 2:1 (2μ L of per μ g DNA)²². MN served as model in ALS pathology and multiple works found an efficient mean of transfection in the Magnetofection[™] methods with the help of NeuroMag[™]. Another example comes with Birsa N. and colleagues that unraveled new pathomechanisms of a multifunctional RNA binding protein (Fused in sarcoma) whose mutations affects crucial functions and could be implicated in this disease. In this study, motor neurons were transfected at DIV 2 with NeuroMag[™] with high efficiency²³. Interestingly, the authors followed the instructions settled by Fallini et al in 2010 in their paper named "High-efficiency transfection of cultured primary motor neurons to study protein localization, trafficking, and function".

The authors used NeuroMag[™] as a tool for studying the localization and transport of axonal proteins and stated an efficient transfection rate of > 45%. Moreover, they confirmed the minimized toxic effect this method has on survival and morphology. Not only they demonstrated an efficient DNA transfection but also they confirmed the suitability of Magnetofection[™] for gene knockdown with shRNA based constructs. And to conclude their work by this what-will-become true postulate: "a transfection method for primary motor neurons that is simple, efficient and non-toxic. [the authors] anticipate that this novel approach will have a broad application in the study of motor neurons development, axonal trafficking, and molecular mechanisms of motor neuron diseases"²⁴. Transfecting MN with Magnetofection[™] allowed the researchers over the years to address a range of pathologies, biological pathways or gene studies. As opposed to hippocampal or cortical neurons all the MN employed were transfected between DIV 2 and DIV 5.

22 Sama, R. R. K. et al. ALS-linked FUS exerts a gain of toxic function involving aberrant p38 MAPK activation. Sci Rep 7, 115 (2017).
23 Birsa, N. et al. FUS-ALS mutants alter FMRP phase separation equilibrium and impair protein translation. Sci Adv 7, eabf8660 (2021).
24 Fallini, C., Bassell, G. J. & Rossoll, W. High-efficiency transfection of cultured primary motor neurons to study protein localization, trafficking, and function. Mol Neurodegener 5, 17 (2010).

Motor Neurons Derived From Embryonic Stem Cells or IPSC

Thanks to its <u>versatility</u> and <u>low toxicity index</u>, not only <u>Magnetofection™ technology</u> can be applied to primary motor neurons as previously cited but also to motor neurons derived from embryonic stem cells (ES) or from induced pluripotent stem cells (IPSC). This is of particular interest when knowing the difficult and hazardous steps from animal brain to culturing cells; moreover when addressing human research, primary neurons are not easily accessible and iPSC derived motor neurons become an almost inexhaustible source of brain cells.

To illustrate this, Nishimoto et al. used Magnetofection[™] procedure for large vector (35 kDa) transfection into human ES cell-derived motor neurons. Following the standard protocol, they prepared a mix of plasmid encoding for both gene of interest and control GFP and NeuroMag[™] and incubated the cells post transfection seven days before performing experiments and monitoring viability.

Their results demonstrated that "transfection of [a] full length 35 fragment showed no significant toxicity against motor neurons" and allowed them to study mechanisms associated with motor neurons degeneration, a leading way for the identification of novel pathogenic factors and neuroprotective agents for the treatment of ALS²⁵. Recently, Tripathi P et al., employed the same methods with human IPSc-derived motor neurons generated from fibroblasts. Cells were first derived into neural precursor cells before being induced and differentiated into MN that were further genetically modified with plasmid DNA using NeuroMag^{™26}.

25 Terenzio, M. et al. Bicaudal-D1 regulates the intracellular sorting and signalling of neurotrophin receptors. EMBO J 33, 1582–1598 (2014). 26 Tripathi, P. et al. Pathomechanisms of ALS8: altered autophagy and defective RNA binding protein (RBP) homeostasis due to the VAPB P56S mutation. Cell Death Dis 12, 466 (2021).

Application for Other Types of Primary Neurons

Over the years **NeuroMag[™]** has built its reputation mainly on 3 pillars:

(1) high efficiency,

(2) lack of toxicity and

(3) versatility for any nucleic acid types (DNA, siRNA, ODN, LNA, miRNA, mRNA...).

This was principally achieved on hippocampal, cortical and motor neurons that cover the most majority of scientific works. However, NeuroMag[™] can also address any kind of brain cells and other types of primary neurons from various origins and territories have been genetically modified thanks to these

Gene Expression

To begin with, primary amygdal neurons are used to study pathologies linked with emotional learning and behaviors such as major depressive disorder. This in vitro model is issued from Amygdala that is a part of the limbic system, a neural network that mediates many aspects of emotion and memory, and has gained more and more interest in the last decade since depressive disorders represent one of the major causes of morbidity in the Western Worlds.

Exploring the hypothesis that this disease is associated to a single-nucleotide polymorphisms that affect the activity of an RNA binding protein, Davidson S. et al., co-transfected amygdal neurons prepared from P1-3 rat neonates with DNA using **NeuroMag™** that required only 250 ng nucleic acid for 150000 cells and further performed dual luciferase assay to monitor differences in allelic variants²⁷. Stress and anxiety find also their source in amygdala and these complex disorders are in the same way extensively studied as illustrated by Hay CW and colleagues that applied a combination of bioinformatics, electrophoretic mobility shift assay and reporter plasmid **Magnetofection™** into rat primary amygdala neurons to identify a highly conserved glucocorticoid receptor sequence. In their work, firefly luciferase plasmids were co-transfected with Renilla luciferase plasmid to normalize signals between transfections using **NeuroMag^{™28}**. Intimately linked to the limbic system with which it exerts control on the endocrine system and the autonomic nervous system to maintain homeostasis, the hypothalamus has gained since many years some growing interest in the field of neurosciences applications.

²⁷ Davidson, S. et al. Analysis of the effects of depression associated polymorphisms on the activity of the BICC1 promoter in amygdala neurones. Pharmacogenomics J 16, 366–374 (2016).

²⁸ Hay, C. W. et al. Functional effects of polymorphisms on glucocorticoid receptor modulation of human anxiogenic substance-P gene promoter activity in primary amygdala neurones. Psychoneuroendocrinology 47, 43–55 (2014).

Gene Expression

Several morbid disorders find their source in this specific area of the brain and in order to try understanding the mechanisms governing susceptibility to obesity and anxiety, McEwan AR. et al. co-transfected reporter plasmids into rat hypothalamic neurons by Magnetofection[™] with the application of Neuro-Mag[™] and demonstrated the implication of differential alleles in obesity²⁹.

As connectors that project from midbrain to the basal ganglia, the limbic system and the prefrontal cortex to mediate the role of dopamine modulatory transmitter in motor functions, reward mechanisms and learning process, the dopaminergic neurons have remarkable axonal arbors with numerous release sites that make them a model of choice to study synapse dynamism. In this way Lycas MD et al., recently studied the nanoscopic reorganizations with major impact on transmitter homeostasis that take place in dopaminergic varicosities. By transfecting primary cultures of Dopaminergic neurons at 14 DIV with NeuroMag[™] at a ratio of 2:1 and 4µg DNA encoding of dopaminergic transporter linked to a photoconvertible fluorescent protein, the authors revealed a clustered distribution into nanodomains which implies to have important involvement for understanding modulatory neurotransmitter physiology³⁰.

As for motor neurons, **IPSC** provide also a reliable source for dopaminergic neurons moreover when working with human cells. **Magnetofection™** appears again a **method of choice to genetically modify this specific cell type** and this becomes of interesting importance when addressing physiological pathway of pathologies such as Parkinson's Disease. To this end Guhathakurta S et al., carried out **transient transfection of IPSC** derived from Parkinson's Disease patients differentiated into dopaminergic neurons using **NeuroMag™** reagent. Grown in 12-well plate, the cells were transfected on day 25 of differentiation using 3µg of DNA and 9µL of magnetic nanoparticles and as marked by the authors "this process ensured a **very high transfection rate without any visible cell mortality**" and "to **increase transfection efficiency**, *cells were transfe*cted again the same way after 3 days of the first transfection and harvested 6 days after first transfection" demonstrating once again the total harmlessness of the Magnetofection™ method³¹.

29 McEwan, A. R. et al. An ancient polymorphic regulatory region within the BDNF gene associated with obesity modulates anxiety-like behaviour in mice and humans. 2021.07.20.452916 https://www.biorxiv.org/content/10.1101/2021.07.20.452916v1 (2021) doi:10.1101/2021.07.20.452916. 30 Lycas, M. D. et al. Nanoscopic dopamine transporter distribution and conformation are inversely regulated by excitatory drive and D2-autore-ceptor activity. 2021.03.09.434538 https://www.biorxiv.org/content/10.1101/2021.03.09.434538v1 (2021) doi:10.1101/2021.03.09.434538. 31 Guhathakurta, S. et al. Targeted attenuation of elevated histone marks at SNCA alleviates α-synuclein in Parkinson's disease. EMBO Mol Med 13, e12188 (2021).

A complete list of publications mentioning the use of Magnetofection[™] to transfect primary neurons can be found at <u>https://www.</u> <u>ozbiosciences.com/module/citationfinder/</u> <u>default</u>

Gene Expression

Also implicated in Parkinson's Disease, the striatum, a subcortical paired nerve structure involved in voluntary movements, food or sexual motivation, pain managements is also implicated in other pathologies such as Tourette's or Huntington's. Regarding the latter, Cherubini et al. used NeuroMag[™] to transfect primary striatal neurons at DIV10 with plasmids encoding a fluorescent protein and a mitochondrial targeting sequence. Applied to the genetic modification of striatal neurons, the Magnetofection[™] allowed to demonstrate excessive mitochondrial fragmentation that would lead to disturbance in Ca2+ efflux and Reactive Oxygen Species homeostasis a possible cause for the pathology³².

This application note cannot be exhaustive and cannot relate to all the different types of neurons that were efficiently transfected with Magnetofection[™], of whom, cerebellar granules, dorsal root ganglion, striatal are some other primary neurons that were genetically modified by NeuroMag[™].



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32 Cherubini, M., Lopez-Molina, L. & Gines, S. Mitochondrial fission in Huntington's disease mouse striatum disrupts ER-mitochondria contacts leading to disturbances in Ca2+ efflux and Reactive Oxygen Species (ROS) homeostasis. Neurobiology of Disease 136, 104741 (2020).

Application for Neural Stem Cells

With the incidence over the years of neurodegenerative diseases causing injuries in the Central Nervous System, numerous preclinical studies have demonstrated **improved functional and histological outco-mes following transplantation of genetically engineered Neural Stem Cells (NSCs)**. The options consist in replacing lost cells either by mobilization of endogenous NSC or by grafting exogenous stem cells pre-viously modified.

The NSCs are the only cells in the brain that bear the potential to promote endogenous/transplant mediated regeneration and functional recovery; offering benefits of self-renewal and multipotentiality for cell replacement. Moreover with their capacity to migrate long distances, especially towards foci of pathology, coupled to their amenability to genetic modification, the NSC appears to be the ideal vehicles for delivery of therapeutic molecules to injury sites³³.

Numerous works have demonstrated that viral vectors are very efficient to genetically modify NSC but dramatically affect their viability as well as their cell physiology and differentiation processes; non-viral vectors are thus again seen as the best alternative but also impact viability while above all altering differentiation. In vitro, these cells are cultivated using two formats either monolayers or neurospheres and as soon as 2011, **our team has demonstrated for the first time** that Magnetofection[™] method with NeuroMag[™] was efficient to transfect DNA into NSC in their adherent state with high viability index compared to other classic lipid-based methods and that it could be applied to study the involvement of genes during their neuronal differentiation³⁴. Since then, multiple articles have related the capacity of NeuroMag[™] to genetically modify NSC on various supports, such as in a semi-adherent model of culture on pre-formed collagen gels in a 3 dimension gel where NSC were propagated as 3D spheroids. In this works, Adams C. et al., confirmed that Magnetofection[™] was a "safe methodology to genetically engineer NSC"</sup> grown in a culture system which more closely represent the physiological tissue³⁵.

Recently, NeuroMag[™] was applied to transfect DNA minicircles (small DNA vectors encoding essential gene expression components but devoid of bacterial backbone, thereby reducing size) into neural stem cells cultivated as neurospheres. As opposed to previous experiments, NSCs were allowed to form neurospheres prior to transfection with NeuroMag[™] (ratio 3:1) and cells were analyzed several days after.

33 Phillips, M. I. & Tang, Y. L. Genetic Modification of Stem Cells for Transplantation. Adv Drug Deliv Rev 60, 160–172 (2008).
34 Sapet, C. et al. High transfection efficiency of neural stem cells with magnetofection. Biotechniques 50, 187–189 (2011).
35 Adams, C. F., Dickson, A. W., Kuiper, J.-H. & Chari, D. M. Nanoengineering neural stem cells on biomimetic substrates using magnetofection technology. Nanoscale 8, 17869–17880 (2016).

Application for Neural Stem Cells

The authors thus stated that after 24H grown in gel, "transfected NSC population demonstrated high viability [>92%]" and harbored "no statistical difference in the proliferation rate"³⁶. In addition, this promising approach using minicircles deployed with Magnetofection™ "achieved highest, safe non-viral DNA transfection level (up to 54%) reported so far for primary NSCs". Not only NeuroMag™ allow to reach high efficiency, but also "Minicircles-functionalized magnetic nanoparticles mediated gene delivery also resulted in sustained gene expression for up to four weeks". Most importantly, the authors to conclude that "all daughter cell types of engineered NSC (neurons, astrocytes, oligondendrocytes) were transfected in contrast to conventional plasmids which usually yield transfected astrocytes only, offering advantage for targeted cell engineering".

Primary cortical neurons DV 7 DV 7 DV 7 DV 20 (Charrier et al. ¹⁶) DV 20 (Charrier et al.

Figure 3. Example of cells successfully transfected with Magnetofection[™] in vitro. Primary cortical and hippocampal neurons at DIV7 and hippocampal neurons at DIV20 were transfected using **NeuroMag[™]** at ratio 3.5:1 or 1:1 respectively (upper line). Neural Stem cells and mouse hypothalamic cell line CLU-101 were transfected with **NeuroMag[™]** at the recommended conditions. BV2 cell line was genetically modified with the dedicated **Glial-Mag[™] reagent** following instruction manual.

36 Safe nanoengineering and incorporation of transplant populations in a neurosurgical grade biomaterial, DuraGen Plus TM, for protected cell therapy applications - PubMed. https://pubmed.ncbi.nlm.nih.gov/32087299/.

Application for Neural Stem Cells

As for other neural cells, NeuroMag[™] is also able to deliver other types of nucleic acids, even short ones, such as siRNA in NSC and this capacity allowed Mukherjee S. et al., to explore the role of the prohibitin (PHB- known to be a receptor protein mediating Chikungunya virus internalization) in Japanese Encephalitis Virus (JEV) infection of NSC.

Data demonstrated that JEV induced the expression of several proteins of whom PHB to promote NSC death by caspase activation ; paving the way for the discovery of therapeutic intervention to protect neural stem cells from neurotropic virus attack³⁷. Altogether these publications demonstrated **the ca-pacity of Magnetic nanoparticles** (aka, NeuroMag[™]) to transfect any nucleic acid in Neural Stem cells without endangering their pluripotentiality capacities, cultivated under any form, adherent, in 3D or in neurospheres.

37 Mukherjee, S. et al. Japanese encephalitis virus induces human neural stem/progenitor cell death by elevating GRP78, PHB and hnRNPC through ER stress. Cell Death Dis 8, e2556 (2017).

Application for Glial Cells

Glial cells are the most abundant cells in the human brain and have long been considered to just provide a passive structural support for neurons – the word glia literally meaning neural "glue". Consisting of astrocytes, oligodendrocyte lineage cells and microglia, as their major component, these cells that were originally considered as purely non-functional cement for neurons, have been found to have many other roles in regards to the function of the adult brain.

Considering their genetic manipulation in order to express or silence gene expression, the Magnetofection[™] technology appears to be the most efficient and less harmful for the cells with a limited impact on cell viability and no impact on the cell behavior or becoming. So far the best solution for Macroglial cells can be found with NeuroMag[™] transfection reagent; for microglia transfection, we have developed a dedicated magnetic nanoparticle formulation, Glial-Mag[™].

Macroglia: Astrocytes, Oligodendrocytes...

Astrocytes are the most common glial cells with irregular star-shaped cell bodies and broad endfeet on their processes. They interact extensively with neurons and provide them with important structural and metabolic support and have major role in the normal homeostatic functions of the central nervous system supporting neuronal survival. Astrocytes have **been found to have** also effective cellular secretory processes meaning that they can be engineered to release neurotrophic proteins such as NGF or BDNF; this characteristic rendering them a target of choice for genetic modification. Since more than ten years now, MagnetofectionTM has been used to transfect these glial cells with either DNA or siRNA. Considering the latter, co-cultures of neurons and astrocytes were knocked-down for β 2-microglobulin subunit from MHC-I complex, by using siRNA magnetically complexed to NeuroMagTM. This allowed the researcher to evaluate the astrocyte activation and reactivity related to MHC-I expression for generation of astrogliosis³⁸.

38 Bombeiro, A. L., Hell, R. C. R., Simões, G. F., Castro, M. V. de & Oliveira, A. L. R. de. Importance of major histocompatibility complex of class I (MHC-I) expression for astroglial reactivity and stability of neural circuits in vitro. Neurosci Lett 647, 97–103 (2017).

Macroglia: Astrocytes, Oligodendrocytes...

Several publications have also related Magnetofection[™]-induced gene expression using DNA plasmids which yield could be increased with the use of NeuroMag[™] associated to a non-permanent Magnetic field. In this way, Tickel JA. et al., used an oscillating magnetic field to

(1) evaluate the efficacy of NeuroMag[™] for dual reporter plasmid delivery and

(2) investigate the relationship between cellular nanoparticle load and transfection (% of transfected cells and reporter protein expression)³⁹.

In parallel, <u>Oligodendrocytes</u> have relatively small amounts of cytoplasm around the nucleus but have several processes which wrap themselves around axons to form myelin sheaths. These cells are a major transplant population to mediate repair of damage as occurs in spinal cord injury and neurological diseases such as Multiple Sclerosis. Using NeuroMag[™] coupled to oscillating magnetic field to modify Oligodendrocyte Progenitor Cells (OPC), Jenkins SI. et al., demonstrated that transfection efficacies obtained using Magnetofection[™] were "highly competitive with or better than current widely-used non-viral transfection methods" and moreover, the technics had "an additional critical advantage of high cell viability". As the authors stated "no adverse effects were found on the cells' ability to divide or give rise to their daughter cells "confirming that NeuroMag[™] keeps intact the capacity of progenitor cells to differentiate⁴⁰. Recently Cullen CL et al. transfected 8–10 DIV OPC cultures with NeuroMag[™] using a 1:1 ratio to determine whether primary cilia are dynamically assembled and disassembled on the surface of OPCs as they proliferate. As a matter of fact, this non harmful method authorized the authors to perform live imaging at high magnification confirming that magnetic nanoparticles do interfere neither with cell viability nor with high resolution imaging⁴¹.

41 Cullen, C. L. et al. Kif3a deletion prevents primary cilia assembly on oligodendrocyte progenitor cells, reduces oligodendrogenesis and impairs fine motor function. Glia 69, 1184–1203 (2021).

³⁹ Tickle, J. A. & Chari, D. M. Less is more: Investigating the influence of cellular nanoparticle load on transfection outcomes in neural cells. Journal of Tissue Engineering and Regenerative Medicine 13, 1732–1737 (2019).

⁴⁰ Jenkins, S. I., Pickard, M. R., Granger, N. & Chari, D. M. Magnetic nanoparticle-mediated gene transfer to oligodendrocyte precursor cell transplant populations is enhanced by magnetofection strategies. ACS Nano 5, 6527–6538 (2011).

Microglia

<u>Microglia</u> can be seen as the immunocompetent and phagocytic cells or macrophages of the nervous system; they are actually complex and multitasking cells with many diverse roles under physiological or disease conditions including Alzheimer's disease. Sentinels of the environment that surrounds neurons, microglia become reactive upon a wide array of stimuli by developing an appropriate inflammatory response that find its sources in virtually every process that occurs within the CNS. In parallel, **several studies have assigned a role for microglial in neurogenesis and blood vessel formation**.

BV2 Cell Line

The mouse **BV2 cells** is regularly used to **study microglia functions** due to their **high availability**, **easy manipulation** and **resemblance to primary microglial cells** and this has become a model of choice to study microglia activation and becomings.

In 2017, our team and collaborators have demonstrated for the first time that Magnetofection[™] was a method of choice to transfect this specific cell lineage. In the article from Smolders S et al., we compared the efficiency, toxicity and cell activation upon plasmid gene delivery in microglia cell line using a dedicated MNP formulation, Glial-Mag[™] to other commonly used chemical transfection methods in BV2 cells such as Calcium phosphate co-precipitation, X-tremeGENE and Lipofectamine 2000. We showed that Glial-Mag[™] yields functional gene expression with superior efficiency compared to competitors and that even if toxicity was above CaPo, it was far beyond Lipofectamine 2000⁴².

With the recent emergence of SARS-CoV-2 as a global pandemic, accumulating evidence suggests that neurological and neuropsychiatric symptoms are associated with the disease caused by the virus in addition to respiratory complications. Aiming at determining if the SARS-CoV-2 spike S1 glycoprotein activates BV-2 microglia to cause elevated release of pro-inflammatory mediators, Olajide OA et al. **transfected microglia cell line with Glial-Mag™ transfection reagent** at a 3:1 ratio before treating the cells with S1 glycoprotein for 6h. They further concluded that spike glycoprotein sub-unit S1 activated microglia cells to induce significantly increased release of inflammatory molecules confirming the link between infection and neurological and neuropsychiatric conditions⁴³.

43 Olajide, O. A., Iwuanyanwu, V. U. & Adegbola, O. D. SARS-CoV-2 spike glycoprotein S1 induces neuroinflammation in BV-2 microglia. 2020.12.29.424619 https://www.biorxiv.org/content/10.1101/2020.12.29.424619v1 (2020) doi:10.1101/2020.12.29.424619.

⁴² Smolders, S. et al. Magnetofection is superior to other chemical transfection methods in a microglial cell line. J Neurosci Methods 293, 169–173 (2018).

As emphasized in this application note, Magnetofection[™] with NeuroMag[™] and Glial-Mag[™] – the two main transfection reagents dedicated to neuronal applications – have been successfully used to transfect any kind of primary neurons and glial cells respectively.

However, since more than a decade now that this method was created and patented by OZ Biosciences, NeuroMag[™] was employed to genetically modify a variety of cell models, primary as well as cell lines, in close relationship to neurons or very far from this cellular lineage. Of the numerous applications for which Magnetofection[™] was employed, Genome Edition using CRISPR/Cas9 is the most representative to illustrate the potential of NeuroMag[™].

Primary Microglial Cells

Working with BV-2 cell lines removes the main limitation being the low yield of primary cells from each animal and their limited survival time period. However, in order to get closer to in vivo models, primary microglial cells remain the most relevant model even if they are difficult cells to transfect, providing low efficiency of gene expression or silencingand also vulnerability to apoptosis when using traditional methods of transfection. In their paper published in Frontiers in Cellular Neuroscience in 2018, Camillo-Jimenez et al. used Glial-Mag[™] to silence gene expression in murine primary microglia. They described the Magnetofection™ as "an easy, and effective method based on [...] magnetic nanoparticles and a magnet to successfully transfect primary microglia cells with different siRNAs. This method does not require specialist facilities or specific training and does not induce cell toxicity or inflammatory activation".

Measuring the efficiency using fluorescently labelled siRNA, they observed that Glial-Mag[™] induced 83-93% of positive transfected cells with a level of target mRNA decreased by about 60%, 48H after transfection. Of the numerous benefits the Magnetofection[™] methods provides, the authors pinpointed that "an additional advantage of this method is the low toxicity and non-priming effect over the inflammatory response in the culture"⁴⁴. Recently Grubman A et al. transfected primary human microglia in vitro with dox-inducible GFP-tagged constructs to genetically turn on two transcription factors (HIF1A or ELF3), using Glial-Mag[™]. This efficient method allowing genetic modification of primary microglial cells allowed them to provide insight into molecular mechanisms underpinning the functional diversity of microglia in Alzheimer's Disease⁴⁵.

45 Grubman, A. et al. Transcriptional signature in microglia associated with Aβ plaque phagocytosis. Nat Commun 12, 3015 (2021).

All these works along the years demonstrated the versatility of Magnetofection[™] to adapt to any cell culture format, nucleic acid and following applications such as for genome editing experiment using the CRISPR/Cas9 technology.

Application for Genome Edition

One story begins in 2013 with Welsbie DS' team that screened an arrayed library of 1869 siRNA against 623 kinases to cover the mouse kinome for the ability to promote the survival of primary Retinal Ganglion Cells (RGC). As noted by the authors "because traditional transcription procedures were either toxic or minimally effective with RGC, [they] adapted a magnetic nanoparticle-based reagent (NeuroMag) for high-efficiency, high throughput siRNA delivery." They screened the siRNA library at a final concentration of 20nM by developing a reverse-magnetofection protocol; meaning that complexes of nucleic acids/ **NeuroMag[™]** were added to the well before the addition of cells. **NeuroMag[™]-based transfection** resulted in consistent and efficient suppression of target gene expression and allow the research team to identify dual leucine zipper kinase as a key mediator of retinal ganglion cell death, providing a starting point for the development of more specific neuroprotective inhibitor for the treatment of glaucoma or other CNS neurodegenerations⁴⁶. Later on in 2017 the same team proceeded to genome edition using CRISPR/Cas9 system and reverse-transfected a pool of small RNAs (siRNAs, siPOOLs or sgRNAs) at the time of isolation in RGC from cas9 konckin mice using once again **NeuroMag**[™]. This leading research allowed them to increase the understanding of the Dual leucine zipper kinase pathway to better understand and potentially treat neurodegenerative diseases⁴⁷. Pursuing their Research, the authors in 2018 transfected sgRNA with **NeuroMag[™]** in primary RGC at the time of extraction from retinas isolated from postnatal day 0-3 Cas9-mice and seeded in 384-well plates⁴⁸. All these works along the years using Magnetofection[™] finally allowed Welsbie DS et al., to file a patent in 2020 describing methods for treating a retinal degeneration in a subject also altering expression of one or more gene product in a cell with help of NeuroMagTM. In this invention, RGC from cas9 mice are seeded in 384 well-plates and the library of siRNA is reverse-transfected with **NeuroMag[™]** at 30 ng per well. Using another model, the mouse neuroblastoma Neuro-2A cell line, Strong MD et al. transfected plasmid DNA encoding for cas9 and/or sgRNA using **NeuroMagTM**. The lack of toxicity allowed them to obtain clonal cell population after approximately 3 weeks of proliferation and CRISPR/cas9 genome editing resulted in impaired mitochondrial function, increased superoxide presence and detectable protein carbonylation⁴⁹.

⁴⁶ Welsbie, D. S. et al. Functional genomic screening identifies dual leucine zipper kinase as a key mediator of retinal ganglion cell death. Proc Natl Acad Sci U S A 110, 4045–4050 (2013).

⁴⁷ Welsbie, D. S. et al. Enhanced Functional Genomic Screening Identifies Novel Mediators of Dual Leucine Zipper Kinase-Dependent Injury Signaling in Neurons. Neuron 94, 1142-1154.e6 (2017).

⁴⁸ Role of SARM1 and DR6 in retinal ganglion cell axonal and somal degeneration following axonal injury - PubMed. https://pubmed.ncbi.nlm. nih.gov/29526794/.

⁴⁹ Role of zinc transporter ZIP12 in susceptibility-weighted brain magnetic resonance imaging (MRI) phenotypes and mitochondrial function - PubMed. https://pubmed.ncbi.nlm.nih.gov/32716562/.

Application for Cell Lines

The complete list of cells successfully transfected with **NeuroMag™**, can be found in our citation database: <u>https://www.ozbios-</u> <u>ciences.com/module/citationfinder/default</u>. <u>Primary cells</u> are challenging models given the brain is a very complex organ comprised of many diverse cell types, that engage intricate cell to cell interactions and whom the viability is directly related to their surroundings. However, in their quest for truly predictive neural models, scientists have created cell lines and mastered the art of tissues explant able to mimic physiology of the brain and to explore the origins of neurological disorder and diseases.

The use of immortalized cell lines has many advantages including the long-term growth and ease to cultivate. And besides its application in primary cells as previously demonstrated, Magnetofection[™] has also extensively been used to transfect a huge variety of cell lines. Remarkably, any nucleic acids of any length can virtually be transfected in any cell lines with NeuroMag[™], from neuronal cells to classic cell lines such as Neuroblastoma cell lines (Be(2)C, N2A, NG108-15, SHSY-5Y, SK-N-AS), glioma and glioblastoma (A172, C6, GL261, H4, LN229, U251), pituitary cells, motor neuron like, Pheochromocytoma (Adrenal), and even more classic cell lines such as HEK-293, HeLa, MEF, (...). The complete list of cells successfully transfected with NeuroMag[™], can be found in our citation database: https://www.ozbiosciences.com/module/citationfinder/default.

In vivo

As previously described, primary microglial and neuronal cultures present several advantages such as the possibility to observe, manipulate, and analyze single cells at a molecular level, a clear control of cellular environment and sustainability of culture conditions, as well as reproducibility, they have however some limitations. As soon as cells are dissected from animal tissue using proteolytic and collagenolytic enzymes, all the connections previously established between the cells are lost. Once seeded into a culture vessel, new contacts are engaged in vitro that poorly mimicks the in vivo situation; a crucial difference that is pressed by the lack of surrounding physiological influence. Finally, even if primary cell cultures present undeniable advantages in terms of ease of use, the major drawback lies in the fact that cell cultures are limited to studying phenomena restricted to single cells or small groups of cells.

In vivo

Therefore, results obtained from neuronal cultures cannot easily be extended to higher levels of function of nervous systems, especially not to complex processes such as cognition and consciousness and there is thus a need to study whole animal. To this end, it is crucial to have an effective and non-toxic tool allowing an efficient and/or targeted transfection for in vivo applications to produce significant results aiming at transferring results from Research to clinical phase I and II.

IN VIVO APPLICATIONS: Intracranial Injections MAGNETIC NANOPARTICLES MAGNET MAGNET External: focalized target IN VIVO VIROMAG NEUROMAG

OZ Biosciences has developed two categories of proprietary transfection reagents (Figure 4) dedicated to neuroscience applications in vivo: a polymer-based reagent for non-viral vectors (BrainFectIN[™], REF #IV-BF30500) and Magnetofection-based solutions for both viral (in vivo ViroMag[™], REF #IV-VM30500; AdenoMag[™], REF #AM7100) and non-viral carriers (NeuroMag[™]).

Figure 4. OZ Biosciences' solutions for in vivo applications. OZ Biosciences has developed MNP-based as well as polymeric transfection reagents for genetic modification in post-natal brain. When MNP are used, a magnetic device positioned outside the animal allows to enhance and target gene expression.

In Vivo: BrainFectIN

A couple of years ago, OZ Biosciences developed BrainFecIN™ a polymer-based transfection reagent dedicated to in vivo gene delivery within the Central Nervous System at postnatal age (Figure 5). To this end, **BrainFecIN[™]** was further used to deliver gene into different cell types in the rat brain after intra cranial injection by DiScala et al. As opposed to lipid-based methods currently used in vitro that present "limitation in vivo mainly due to inflammatory response", the authors described a "new polymer-based gene delivery system allowing persistent and robust in vivo transfection with low DNA amount, reduced inflammation and high diffusion" and evaluated the expression profile along the brain, the stability and the quantity of cells transfected. The remarkable point is that this powerful transfection reagent is adapted to reduced injection volumes as extremely low quantities of DNA complexed to small volumes of BrainFecIN[™] (ratio 2:1) induced a massive fluorescent protein expression 3 to 10 days after injection; using ideal condition, only a slight inflammatory response was observed compared to other methods. Moreover, with the intra cranial delivery of siRNA targeting the neuronal specific potassium chloride exporter KCC2 the authors demonstrated that this polymer-based non-viral delivery system could be "used as a tool to study the functionality of the entire network since the effect was not limited to the hippocampus"⁵⁰. Recently, Mets DG and colleagues used BrainFecIN™ to silence gene expression in Bengalese finch population. They injected siRNA targeting a zinc transporter (ZIP11) complexed to the polymer-based transfection reagent bilaterally into the High Vocal Center of adult birds and measured the impact on learned song tempo 48H after demonstrating that difference in ZIP11 levels could account for inter-individual differences in complex learned behaviors⁵¹.

Figure 5. Intracerebral transfection of neurons using BrainFecIN[™]. DNA encoding for GFP was complexed with BrainFecIN[™] according to the instructions manual and injected in brain of adult mice to transfect upper cortex (left) or CA3 (right).

BrainFectIN/DNA GFP complexes - Intracerebral injection in adult WT mice





Ipsilateral CA3

50 Di Scala, C. et al. A new polymer-based approach for in vivo transfection in postnatal brain. J Neurosci Methods 311, 295–306 (2019). 51 Mets, D. G., Mehaffey, W. H., Colquitt, B. M. & Brainard, M. S. Heritable differences in synaptic zinc-transporter levels drive variation in learned birdsong. 2021.05.01.442260 https://www.biorxiv.org/content/10.1101/2021.05.01.442260v1 (2021) doi:10.1101/2021.05.01.442260. 28

In Vivo: Magnetofection[™]

For many years now, OZ Biosciences have mastered the Magnetofection[™] technology for in vivo applications with successful applications in many animals and organs and of course, Central Nervous System is one of the many territories to which this technology is addressed. Briefly, Proof of concept was given by Plank et al. when MNP complexed with pDNA encoding for a report gene (luciferase) were injected in the ear vein of both right and left ears of pigs. A permanent magnet was placed in the proximity of the injection site only on the right ear. Following administration, only endothelial cells localized in the vicinity of the magnetic field were transfected while in the control no reporter gene expression was detected. This observation was extended also to other major organs underlining the feasibility of local, non-invasive gene therapy through Magnetofection^{™3}. Since then, In vivo gene delivery by Magnetofection[™] has been developed throughout the past several years using viral and non-viral vectors since the study by Jahnke et al. that explored the feasibility of gene-therapy with plasmid coding for feline GM-CSF in cats with fibrosarcomas⁵². Applied to Neurosciences, Magnetofection[™] has been used in vivo to deliver both viral and non-viral vector in brain of animals. Not only the overall efficiency is increased but the magnetic nanoparticles allow also to target and direct gene expression in focal areas of the brain following a magnetic field placed at the outside the animal.

Moreover, the interest of using the Magnetofection[™] technology *in vivo* when addressing viral vectors resides in several points:

(1) The increase in efficiency allows to limit the use of high doses of virus preventing an immune response.

(2) The magnetic nanoparticles favor the contact between cells and virus in a receptor-independent way enable to infect and transduce non-permissive cells. As an example as soon as 2012, Sapet et al. injected MNP complexed with adenovirus in the brain of rats where the expression of CAR is low or null. In this work the authors observed a magnetic-targeted boost of infection in the hippocampal neurons as compared to adenovirus alone where only limited GFP positive cells were observed⁵³.

(3) The democratization of its usage, had enabled Magnetofection[™] to found new applications for viral purpose such as "virus stamping". This very precise and recent technics was developed by Schubert R et

³ Scherer, F. et al. Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo. Gene Ther 9, 102–109 (2002 52 Jahnke, A. et al. Intra-tumoral gene delivery of feIL-2, feIFN-gamma and feGM-CSF using magnetofection as a neoadjuvant treatment option for feline fibrosarcomas: a phase-I study. J Vet Med A Physiol Pathol Clin Med 54, 599–606 (2007).

⁵³ Sapet, C., Pellegrino, C., Laurent, N., Sicard, F. & Zelphati, O. Magnetic nanoparticles enhance adenovirus transduction in vitro and in vivo. Pharm Res 29, 1203–1218 (2012).

In Vivo: Magnetofection[™]

al. that used lentiviral particle to drive expression of fluorescent markers in target cells in the brain of live mice with the help of in vivo ViroMag[™]. Viruses are reversibly bound to delivery vehicles – magnetic nanoparticle – that is brought into physical contact with the target cell using magnetic forces. The force of this application is that "different single cells in the same tissue can be infected with different viruses or individual cell can be simultaneously infected with different viruses". Moreover with the help of an external electromagnetic field, the authors directed infection in mouse cortex and performed in vivo two-photon calcium imaging of virus-stamped cells⁵⁴,⁵⁵.

Even if Magnetofection[™] would allow lowering the dose of viral vector, some issues still remain such as non-desired side effect linked to inflammation or genetic insertion. This is why non-viral vectors have gained such interest over the years and NeuroMag[™] was indeed used in brain in vivo to deliver nucleic acids. In 2015 Soto-Sanchez C et al. showed for the first time the capacity of this magnetoparticles to deliver nucleic acids in vivo. They "demonstrate[d] the successful long-term transfection in vivo of a DNA plasmid vector in rat visual cortex neurons using the Magnetofection[™] technique [NeuroMag[™]] for optogenetic applications". Even if this reagent is routinely used for in vitro applications as extensively depicted before, it is also amenable for genetic modifications in vivo with a huge impact for following experiments as "the transfection rates reached values of up to 97% of the neurons after 30 days, comparable to those achieved by viral vectors but avoiding their safety concerns" as the authors added. They further concluded that their results "suggest that NeuroMag[™] particles can be used to introduce genes into the mature mammalian brain in vivo and be efficiently expressed at least up to 30 days after pDNA injection administration. [...] Thus, this technique allows a safer optogenetic application to activate selective regions of the brain through genetically modified cells which express channelrhodopsin proteins on the cell membrane"⁵⁶.

56 Sapet, C., Pellegrino, C., Laurent, N., Sicard, F. & Zelphati, O. Magnetic nanoparticles enhance adenovirus transduction in vitro and in vivo. Pharm Res 29, 1203–1218 (2012).

⁵⁴ Scherer, F. et al. Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo. Gene Ther 9, 102–109 (2002 55 Jahnke, A. et al. Intra-tumoral gene delivery of feIL-2, feIFN-gamma and feGM-CSF using magnetofection as a neoadjuvant treatment option for feline fibrosarcomas: a phase-I study. J Vet Med A Physiol Pathol Clin Med 54, 599–606 (2007).

In Vivo: Magnetofection[™]

3 years later the same team led by Titze de Almeida, in its efforts to deciphering the critical role that aberrant expression of miRNAs play in the pathogenesis of many disorders including Parkinson's disease, aimed at examinating **NeuroMag™** for delivery of synthetic miRNA inhibitors in the rat CNS. To this end, they injected miRNA inhibitors complexed with **NeuroMag™** into the lateral ventricle next to the striatum by stereotaxic surgery. The addition of **Magnetofection™** strategy with **NeuroMag™** enhanced by magnetic plate was effective in driving the oligos accross the ependymal cells and for the subsequent delivery in striatal cells. The authors noted that "after injection, the antimiRNA were efficiently biodistributed across the ependymal cell layer, reaching neurons and glial cells in the striatum" and that injected mRNA could suppress target miRNA in striatum for at least 7 days. This relevant study concluded that "microRNAs inhibitors complexed with **NeuroMag™** are **capable of decreasing the content of targeted miRNA overexpressed in brain diseases including PD, in which the striatal degeneration is a key pathological hallmark"⁵⁷.**

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OZ Biosciences provides rising generations of research reagents based on molecular delivery systems to serve and assist the life science community in its mission.

Know-how

Transfection Solutions

In vivo Delivery

Transduction Enhancers

High Quality mRNAs

Vaccine Adjuvants

Cellular Assay Kits

mRNA (Ready-to-Use & Custom)

Conclusion

In an effort to address Neurosciences Field aiming at offering efficient solutions for genetically modifying cells, OZ Biosciences has devoted lot of effort in formulating, synthesizing and developing specific proprietary reagents to transfect and transduce any neural cell with any vector in vitro and also and moreover in vivo. Since its development more than 15 years ago, Magnetofection[™] has shown countless successes for neuroscience that were validated by hundreds of scientific publications illustrating the efficiency as well as the versatility of this method. First **NeuroMag[™]** is, as described in this application note, the most efficient transfection reagent to date for neurosciences applications. Originally dedicated to hippocampal and cortical neurons, it has rapidly positioned itself as the ideal reagent for any type of culture; Neural Stem cells, motor neurons, striatal, DRG, amygdal, cerebellar granules, (...), can now be efficiently transfected with any nucleic acid. Moreover, its biodegradable formulation appeared not to have influence on the becoming of the cells nor on the differentiation potential of cells bearing pluripotent capacity. These characteristics are also shared by Glial-Mag that next to Neuro-Mag[™], is gradually gaining ground thanks to its ability to transfect microglia cells that were known to be really hard-to-transfect before opening the way to new axis of research. When taking a step forward to gene therapy, the company offers also solutions for neuroscience applications in vivo, both for viral and non-viral vectors based or not on MNP formulations. Considering viral vectors, in vivo ViroMag[™] is able to target and enhance infection in brain and the intrinsic magnetic formulation authorizes new applications such as virus stamping. Non-viral vectors (DNA, siRNA, miRNA...) which are less prone to immune response and integration in the genome can also be delivered in the brain thanks to **BrainFecIN**[™], a dedicated polymer-based reagent and also **NeuroMag[™]**.

All these solutions open new pathways for future studies aiming at modifying cell populations within the CNS and offer perspectives for an efficient gene delivery in the brain with promising outlooks to study brain function *in vivo*.

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