CURRENT PROTOCOLS
The Fine Art of Experimentation

TEMPLATE for PROTOCOL UNIT

Magnetic nanoparticle-mediated gene delivery to two- and three-dimensional neural stem cell cultures: magnet-assisted transfection and multifection approaches to enhance outcomes

Mark R. Pickard[2], Christopher F. Adams[1], and Divya M. Chari[1]*

[1]Keele University, Keele, United Kingdom; [2]University of Chester, Chester, United Kingdom

Contact information

MRP: Chester Centre for Stress Research, Institute of Medicine, University of Chester, Bache Hall, Countess Way, Chester CH2 1BR, UK. Tel: +44-1244-513718; E-mail: m.pickard@chester.ac.uk

CFA: Cellular and Neural Engineering Group, Institute for Science and Technology in Medicine, Keele University, Keele, Staffordshire ST5 5BG, UK. Tel: +44-1782-733678; E-mail: c.adams@keele.ac.uk

DMC: Cellular and Neural Engineering Group, Institute for Science and Technology in Medicine, Keele University, Keele, Staffordshire ST5 5BG, UK. Tel: +44-1782-733314; Fax: +44-1782-734637; E-mail: d.chari@keele.ac.uk.

Significance Statement

Genetic engineering of transplant cells has significant clinical application in achieving combinatorial therapy (i.e. cell replacement and therapeutic biomolecule delivery). Multimodal magnetic nanoparticles (MNPs) offer a key translational platform to achieve this by providing safe and scalable alternatives to viruses, with unique advantages in non-invasive cell tracking and localization. To further increase MNP utility, we have developed state of the art magnetofection protocols (application of static/oscillating magnetic fields) to enhance MNP mediated transfection of neural stem cells (NSCs; a key cell transplant population). Crucially, magnetofection is both safe and effective for NSCs propagated by two clinically relevant formats (neurospheres/monolayers), thereby offering an attractive methodology for translation involving therapeutically important stem cell populations.

ABSTRACT

Neural stem cells (NSCs) have high translational potential in transplantation therapies for neural repair. Enhancement of their therapeutic capacity by genetic engineering is an important goal for regenerative neurology. Magnetic nanoparticles (MNPs) are major non-viral vectors for safe bioengineering of NSCs, offering critical translational benefits versus viral vectors (safety, scalability and ease of use). This unit describes protocols for production of suspension (neurosphere) and adherent (monolayer) murine NSC cultures. Genetic engineering of NSCs with MNPs and application of 'magnetofection' (applied magnetic fields)/'multifection' (repeat transfection) approaches to enhance gene delivery are described. Magnetofection of monolayer cultures achieves optimal transfection, but neurospheres offer key advantages for neural graft survival post-transplantation; a further protocol is presented which allows the advantageous features of each approach to be combined into a single procedure for transplantation. The adaptation of these protocols for other MNP preparations is considered, with emphasis on the evaluation of procedural safety.

Keywords:

Magnetic nanoparticles, neural stem cells, gene delivery, magnetofection, transplantation

INTRODUCTION

Neural stem cells (NSCs), a major transplant population for neural cell therapies (Kim and de Vellis, 2009), are routinely cultured as suspension (three-dimensional) neurosphere and as adherent (two-dimensional) monolayer cultures; each culture system has its own merits and disadvantages with respect to cell replacement therapies (Conti and Cattaneo, 2010). However, both culture formats are amenable to non-viral mediated gene delivery using magnetic nanoparticles (MNPs) (Pickard et al., 2011; Adams et al., 2013; Pickard et al., 2015), as described in this unit. A protocol for the preparation of NSC cultures from murine brain is firstly described (Basic Protocol 1), involving their initial derivation as neurospheres and followed by their propagation as either neurosphere or monolayer cultures. This is accompanied by an ancillary protocol for the preparation of polyornithine/laminin-treated culture surfaces for the latter (Support Protocol 1).

We then describe protocols for MNP-mediated gene delivery to NSCs. Notably this process can be enhanced by the application of high gradient static/oscillating magnetic fields—the so-called 'Magnetofection Technology'—and/or multifection (repeat transfection) (Pickard et al., 2011; Adams et al., 2013; Pickard et al., 2015). Magnetofection of monolayers yields the highest transfection efficiencies, and a protocol for this is first described (Basic Protocol 2). Magnetofection and multifection also enhance MNP-mediated gene delivery to neurospheres, which is next considered (Alternate Protocol 2). Neurospheres offer key advantages in terms of neural graft survival post-transplantation (Mothe et al., 2008); a final protocol (Support Protocol 2) is presented, involving the formation of neurospheres from magnetofected

monolayers, thereby allowing the advantageous features of each system to be combined into a single procedure for the transplantation of genetically modified NSCs.

While we have focused here on MNP-mediated gene delivery to NSCs using a particular commercial formulation of transfection-grade MNPs, termed NeuroMag, these protocols and approaches can be readily adapted to other MNP formulations, including those with primary application in magnetolabelling (for non-invasive cell tracking by MRI, for example). Key considerations that need to be addressed when adapting the presented protocols are highlighted throughout the unit. Procedural safety is a primary concern when manipulating NSC transplant populations; ways in which this can be evaluated are discussed in the final section of the unit (Commentary: Critical Parameters/Troubleshooting).

BASIC PROTOCOL 1

PREPARATION OF NEURAL STEM CELL SUSPENSION AND MONOLAYER CULTURES

The protocol here describes methods to establish and maintain both neurosphere and monolayer cultures of NSCs. Firstly, the subventricular zone (SVZ) is dissected from neonatal mouse cerebral cortex and cells are mechanically dissociated and sieved to obtain a single cell suspension. Cells are then seeded into low adherence culture flasks in culture media containing EGF and FGF to stimulate stem cell proliferation, resulting in the formation of neurospheres. This suspension culture system is a more convenient format to deal with the relatively high level of cell death (largely of non-NSCs) that occurs immediately after the initial establishment of cultures, and which would otherwise cause detachment of colonies in monolayer culture formats. Cell death normally resolves within the first 2 – 3 days of culture, resulting in the emergence of characteristic balls of cells or neurospheres. These are enzymatically dissociated at 7 days post-seeding, then reseeded either as neurosphere cultures for routine propagation of NSC stocks/transfection or as monolayer cultures for transfection.

NOTE: All reagents/solutions and equipment (including that used for dissection) that comes into contact with tissue/cells must be sterile.

NOTE: All incubations are conducted in a humidified 5% CO_2 incubator at 37 °C unless specified otherwise.

Materials

Mice; CD1 strain; at 1 – 3 postnatal days

Phosphate-buffered saline (PBS; 10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4)

NS-M medium (see recipe)

DNase I (10x; see recipe)

Accutase/DNase I (see recipe)

Trypan blue (0.4%)

Stereomicroscope or dissecting microscope

Standard microscope

Inverted microscope

Dissection equipment: Watchmaker forceps (110 mm); scalpel blades no.10

Kimberley Clark paper hand towels

Universal tubes

50 ml Falcon-type tubes

Eppendorf tubes (1.5 ml)

Neubauer counting chamber and coverslip

Cell sieves (40 µm mesh size)

Petri dishes; 10 cm and 3.5 cm diameter

Manual pipettors

Transfer pipettes

Untreated Nunc T-25 flasks for suspension cultures (Thermofisher, code TKT-300-010J)

Untreated Nunc 24-well plates for suspension cultures (Thermofisher, code TKT-220-024P)

Cell culture treated Nunc 24-well plates for monolayer cultures (Thermofisher, code 142475)

Dissection and culture preparation

1. Euthanize mouse pups by a humane (Schedule 1) method. Aseptically dissect out the whole brain and transfer this to a Universal tube containing ice-cold PBS (*ca*. 5 ml/brain).

2. In a laminar flow cabinet, transfer the brains to a Petri dish (10 cm diameter), and replace PBS with fresh. Gently transfer each brain in turn to a fresh hand towel, and carefully roll to remove meninges.

Use curved forceps to lift brains and avoid applying excessive pressure as early postnatal brain tissue has a relatively high water content and can be easily damaged.

3. Using a scalpel, remove and discard olfactory bulbs plus a 1 mm band of forebrain tissue caudal to the bulbs. Collect a 2 – 3 mm band of tissue caudal to this cut, and carefully transfer this to individual Petri dishes (3.5 cm diameter) containing a small amount of PBS (*ca*. 3 ml) with the more caudal portion facing upwards.

4. Under a stereo-microscope or dissecting microscope, bisect cortical tissue into its two halves and, using a fresh scalpel blade, carefully remove tissue surrounding the SVZ (**Figure 1A**).

   With each cut of the blade, push the extraneous tissue to the edge of the Petri dish, leaving only the two SVZs in the centre of the dish.

5. Using a transfer pipette, transfer SVZs into an Eppendorf tube; remove any PBS carry-over using a manual pipettor.

6. Add NS-M (0.5 ml/pair of SVZs) followed by 0.1 volume of DNase I (10X).

   The DNase I will digest any DNA released from cells during the subsequent mechanical dissociation steps, which would otherwise cause cell clumping.

7. Dissociate the tissue mechanically using a P1000 followed by a P200 manual pipettor.

8. Centrifuge (1000 rpm, 5 min) to collect cells. Resuspend the pellet in fresh NS-M (*ca*. 1 ml/brain) and pass this through a cell sieve (40 µm) into a 50 ml Falcon-type tube, followed by a further 1 ml NS-M wash through.

9. Perform a viable cell count, and adjust the density of the main stock to 10^5 cells/ml by adding fresh NS-M. Add 4 - 5 ml of this per T25 flask and incubate.

   For the cell count, dilute a sample of cell suspension 1:1 with trypan blue solution (0.4%) and load the mix into a Neubauer chamber – non-viable cells take up the dye and appear blue, while viable cells exclude the dye and appear transparent.

10. Observe cultures daily; discourage neurosphere adherence to plastic by regularly giving culture vessels a sharp tap. Feed cultures every 2 – 3 days by performing a 50% medium change.

    Use a ruler to give culture vessels a sharp tap laterally to dislodge any neurospheres which are attaching to the culture flask. There will be many dead or dying cells in the

*first two days after establishing cultures. However, these and cell debris will start to clear after 2 - 3 days resulting in the emergence of balls of cells which will increase in size as cells proliferate over the ensuing days (**Figure 1B**).*

11. Cells should be passaged after 1 week (or when spheres reach *ca*. 150 µm in diameter). To do this, collect the neurospheres by centrifugation (1000 rpm, 5 min) of the entire flask contents and wash the pellet once with PBS. Then gently resuspend neurospheres in accutase/DNase I solution. After incubation for 10 min at 37 °C, mechanically dissociate the spheres, as per step 7, and centrifuge (1000 rpm, 5 min) to collect cells.

12. For secondary neurosphere cultures, resuspend cells at a density of 10^5 cells/ml NS-M and re-plate in non-treated, polystyrene T25 flasks (4 – 5 ml/flask; for main stock) and/or 24-well plates (0.5 ml per well; for transfection), as desired. For monolayer cultures for transfection, resuspend cells at a density of 3 x 10^5 cells/ml ML-M and re-plate (0.4 ml per well) in 24-well plates pre-treated with polyornithine-laminin (see Support Protocol 1).

SUPPORT PROTOCOL 1

COATING OF CULTURE SURFACES AND COVERSLIPS WITH POLYORNITHINE-LAMININ

Coating of culture substrates promotes the adherence of NSCs in monolayer cultures and a mixture of polyornithine-laminin is normally used for this purpose, though other coatings are possible. For experiments involving a range of cellular microscopic analyses, it is convenient to place sterile acid-washed coverslips into wells of 24-well plates and to coat these *in situ* before seeding monolayer cultures.

Materials

PBS (Basic Protocol 1)

Poly-ornithine (0.01% solution; Sigma-Aldrich P4957); diluted 1 in 5 in PBS just before use

Laminin (1 mg/ml; Sigma-Aldrich code L-2020); stored as 25 µl aliquots at -20 °C; diluted 1 in 200 in PBS just before use

Coverslips (circular, 13 mm diameter); acid-washed before use and sterilised

Cell culture treated Nunc 24-well plates (Thermofisher, code 142475)

Coating procedure

1. Add coverslips to wells, if required.

2. Add 0.25 ml diluted poly-ornithine per well and incubate at 37 °C for 1 h.

3. Aspirate and wash wells x1 with 0.5 ml PBS.

4. Add 0.25 ml diluted laminin per well. Incubate at 37 °C for 1 h.

5. Aspirate and wash wells x3 with PBS (0.5 ml).

Do not remove the final PBS wash until just before cell seeding to prevent the wells from drying out.

BASIC PROTOCOL 2

MAGNETOFECTION OF NSC MONOLAYER CULTURES

Here protocols are described for the magnetofection of NSC transplant populations using a commercial transfection-grade MNP preparation, NeuroMag. These are iron oxide containing MNPs which carry a positive charge and for which the average particle size is 160 nm (range 140 – 200 nm). When developing and optimizing these protocols, several commercial MNP preparations were tested and NeuroMag, which was originally developed for the transfection of neurons, consistently yielded the highest transfection efficiency with minimal toxicity, irrespective of the culture format. Note that to completely avoid toxicity, the concentration of NeuroMag used in the protocol is much lower than that recommended by the manufacturer for the transfection of neurons. The application of magnetic fields enhances gene delivery by this reagent, with oscillating magnetic fields outperforming static magnetic fields in both adherent and suspension culture formats. However higher transfection efficiencies are consistently obtained with monolayer *versus* neurosphere cultures and, for this reason, the main protocol in this section focusses on procedures for MNP-mediated gene delivery to NSC monolayers, while magnetofection/multifection protocols for neurospheres are described as an alternate protocol.

Materials

ML-M medium (as per recipe but minus antibiotics)

DMEM: Ham's F12 medium (1:1)

Plasmid (0.5 µg/µl water; stored at -20 °C in aliquots; vortex before use)

NeuroMag transfection reagent (Oz Biosciences; stored at -20 °C in aliquots; 30 second vortex before use)

Magnefect-Nano oscillating magnetic array system (nanoTherics Ltd), with a 24-magnet array (NdFeB, grade N42; field strength of 421 ± 20 mT)

Magnetofection of NSC monolayers

1. After plating monolayers in 24-well plates (Basic Protocol 1, step 11), incubate overnight (18 - 24 h) to allow cell attachment to the substratum.

2. Replace medium with 0.225 ml fresh ML-M minus antibiotics.

3. After 2 h, prepare transfection complexes. The following is per well; prepare multiples of this according to the number of wells required. Firstly, dilute 176 ng plasmid (i.e. 0.352 µl of a 0.5 µg/µl stock) with 75 µl base medium (1:1 mix of DMEM:Ham's F12 with no additions), and mix by vortexing. Secondly, add all of this to an Eppendorf tube containing 0.62 µl NeuroMag (or water for plasmid only controls). Carefully mix by pipetting up and down 10 times using a P1000 manual pipettor. Incubate at room temperature for 20 min exactly.

Dynamic light scattering approaches have shown that binding of plasmid (size 3.5 kb) by NeuroMag under the above conditions increases particle size by ca. 35% (Pickard and Chari, 2010)*; extended incubations for complex formation result in marked aggregation of particle-plasmid complexes and are to be avoided. A ratio of 3.5 µl NeuroMag/µg DNA is used here, at which particle binding of plasmid is maximal* (Pickard and Chari, 2010)*.*

4. Add 75 µl complexes (or plasmid only controls) drop-wise to cells whilst gently swirling the plate. Immediately place the culture plate on the desired magnetic field in the incubator.

Gently pipette complexes to avoid disrupting these. For optimal transfection efficiency, an oscillating magnetic field (of frequency 4 Hz and amplitude 0.2 mm) is recommended, yielding values that are more than treble those of basal (no magnetic field) conditions. If an oscillating magnetic field system is not available, then a static magnetic field can be used, but this will produce only a doubling of basal transfection efficiency.

5. After 30 min, remove the plate from magnetic field.

6. After a further 30 min, aspirate medium from cells and replace with 0.4 ml fresh ML-M (minus antibiotic).

7. Culture cells as normal.

As judged from experiments with plasmids encoding fluorescent reporter proteins or growth factors tagged with the latter, transgene expression is maximal at 48 h. If longer incubation times are required, then it will be necessary to maintain cultures (i.e., feeding every 48 h) and to passage these before they become confluent.

8. Transfection can be increased by repeating steps 2-6, 24 h after the initial transfection.

Magneto-multifection in monolayers has been shown to increase transfection efficiency from ca. 43 to 52% with a concomitant doubling in mean cell fluorescence intensity when introducing Green Fluorescent Protein (GFP) encoded by minicircles (plasmid DNA without the bacterial backbone) suggesting higher levels of expression compared to single transfections (Fernandes and Chari, 2016a).

9. If required, neurospheres can be reformed from transfected monolayers, as detailed in Support Protocol 2.

ALTERNATE PROTOCOL 2

MAGNETOFECTION AND MULTIFECTION OF NSC SUSPENSION CULTURES

MNPs can also be used to safely deliver genes to the widely studied neurosphere NSC culture model (Pickard et al., 2011; Adams et al., 2013). While this can also be enhanced by the application of magnetic fields (specifically oscillating magnetic fields), transfection efficiencies are lower than for NSC monolayers (Adams et al., 2013; Pickard et al., 2015). However, an alternative approach to enhance transfection, termed multifection, and involving the repeat administration of transfection complexes (Pickard et al., 2011), can be safely employed to attain efficiencies which exceed those obtained using oscillating magnetic fields (Adams et al., 2013). Here we describe protocols for the magnetofection and multifection of neurosphere cultures using NeuroMag MNPs.

Materials

NS-M medium (as per recipe but minus antibiotics)

DMEM: Ham's F12 medium (3:1)

Plasmid (0.5 µg/µl water; stored at -20 °C in aliquots; vortex before use)

NeuroMag transfection reagent (Oz Biosciences; stored at -20 °C in aliquots; 30 second vortex before use)

Magnefect-Nano oscillating magnetic array system (nanoTherics Ltd), with a 24-magnet array (NdFeB, grade N42; field strength of 421 ± 20 mT)

Transfection of neurospheres

1. After plating of dissociated neurospheres in untreated 24-well plates (10^5 cells/ml NS-M; 0.5 ml per well; Basic Protocol 1, step 11), culture overnight (20 – 24 h).

Transfections have been conducted at passages 2 – 4 without any appreciable loss in transfection efficiency.

2. Prepare complexes. The following is per well; prepare multiples of this according to the number of wells required. Firstly, dilute 125 ng plasmid (i.e. 0.25 µl of a 0.5 µg/µl stock) with 50 µl base medium (3:1 mix of DMEM:F12 with no additions), and mix by vortexing. Secondly, add all of this to an Eppendorf tube containing 0.435 µl NeuroMag (or water for plasmid only controls). Carefully mix by pipetting up and down 10 times using a P1000 manual pipettor. Incubate at room temperature for 20 min exactly.

Note that neurosphere size influences the extent of transfection; extended culture of NSCs prior to transfection will result in larger sized neurospheres and reduced transfection efficiency (Pickard et al., 2011)*. NeuroMag exhibits dose-dependent toxicity in neurosphere cultures; the dose employed here is non-toxic* (Pickard et al., 2011)*. Furthermore, the NeuroMag:DNA ratio of 3.5 µl/µg used here is that at which particle binding of plasmid is maximal* (Pickard and Chari, 2010)*.*

3. Add 50 µl complex or control mix dropwise to neurospheres whilst gently swirling the plate and immediately return to the incubator. For magnetofection, incubate the plate with an oscillating magnetic field (of frequency 4 Hz and amplitude 0.2 mm) for the first 30 min.

Gently pipette complexes to avoid disrupting these. Static magnetic fields have negligible effects on the transfection efficiency of NeuroMag in neurospheres.

4. At 24 h after addition of complexes, a further transfection can be performed to safely enhance transfection efficiency, by simply repeating steps 2 and 3.

Static magnetic fields also have negligible effects on the multifection of neurospheres with NeuroMag (Pickard et al., 2011; Adams et al., 2013)*. Magneto-multifection approaches employing oscillating magnetic fields have so far not been tested on NSC neurospheres.*

5. Culture cells as normal.

Transgene expression will be maximal at 48 h after complex application i.e. 72 h post-plating for single transfections or 96 h post-plating for multifections. If longer incubation times are required, then it will be necessary to maintain cultures (i.e., feeding every 48 – 72 h) and to passage these before they become confluent. Periodic sharp tapping of culture vessels is recommended to prevent neurosphere adherence to culture surfaces.

SUPPORT PROTOCOL 2

FORMATION OF NEUROSPHERES FROM TRANSFECTED MONOLAYER CULTURES

Magnetofection of NSC monolayer cultures yields higher transfection efficiency than that of NSC neurosphere cultures, but transplantation of neurospheres can yield better graft survival

than that of dissociated cells. Since magnetofected NSC monolayers can form neurospheres, even after transfection with a functional gene (FGF2) (Pickard et al., 2015), it is possible to combine the advantageous features of both systems using the protocol described here.

Materials

Magnetofected NSC monolayers (generated by Basic Protocol 2)

PBS (as per Basic Protocol 1)

Accutase/DNase I (see recipe)

NS-M medium (see recipe)

Trypan blue (0.4%)

Neubauer counting chamber and coverslip

Untreated Nunc T-25 flasks and/or Nunc 24-well plates for suspension cultures (Thermofisher, codes TKT-300-010J and TKT-220-024P, respectively)

Standard microscope

Inverted microscope

Formation of neurospheres from magnetofected monolayers

1. Remove and discard medium from transfected monolayers, and carefully wash these once with PBS (0.5 ml well) so as not to disturb the monolayers. Discard this wash.

2. Add accutase/DNase I solution (0.5 ml/well) and incubate at 37 °C. Regularly observe wells microscopically, until cells start to detach from wells (*ca.* 5 min).

Avoid overexposing cells to accutase-DNAse I solution; regularly observe wells on an inverted microscope during the dissociation process and collect the well contents as soon as cells start to detach.

3. Immediately transfer the cell suspension to a centrifuge tube. Wash wells twice with 0.5 ml PBS per wash (to maximize cell collection) and combine washes with detached cells.

4. Centrifuge (1000 rpm, 5 min) to collect cells.

5. Resuspend cells at a density of 10^5 cells/ml NS-M and re-plate in non-treated, polystyrene tissue culture flasks or wells (i.e. 4 – 5 ml/T25 flasks or 0.5 ml/well of a 24-well plate).

REAGENTS AND SOLUTIONS

Neurosphere medium (NS-M)

A 3:1 mix of DMEM and Ham's F12 (both media from Life Technologies) containing:

2% B27 supplement (Life Technologies)

20 ng/ml bFGF (see recipe)

20 ng/ml EGF (see recipe)

5 ng/ml Heparin (Sigma-Aldrich; prepared from a 5 µg/ml working solution in DMEM/Ham's F12 [3:1])

1X Penicillin/streptomycin (added from 100x stock)

Store at 4 °C for ≤1 week

Monolayer medium (ML-M)

A 1:1 mix of DMEM and Ham's F12 (both media from Life Technologies) containing:

1% N2 supplement (Life Technologies)

20 ng/ml bFGF (see recipe)

20 ng/ml EGF (see recipe)

5 ng/ml Heparin (Sigma-Aldrich; prepared from a 5 µg/ml working solution in DMEM/Ham's F12 [3:1])

1X Penicillin/streptomycin (added from 100x stock)

Store at 4 °C for ≤1 week

Human recombinant basic fibroblast growth factor (bFGF)

Aseptically dissolve 25 µg bFGF (Sigma-Aldrich, product F-0291) in 0.625 ml sterile 20 mM Tris HCl, pH 7 for a 40 µg/ml solution. Store aliquots at -80 °C.

Human recombinant epidermal growth factor (EGF)

Aseptically dissolve 200 µg EGF (R&D Systems, product 236EG) in 5 ml sterile 10 mM acetic acid containing 0.1% BSA. Store aliquots at -80 °C.

DNAse I

Dissolve 100 mg DNAse I (Roche; product 1284932) in 20 ml DMEM/F12 (3:1) to make a 10x stock solution and store aliquots at -20 °C.

Accutase/DNase I

To 1 volume accutase (Sigma A6964; aliquots stored at -20 °C), add 0.1 volume DNase I (10x) and use fresh.

COMMENTARY

Background Information

NSCs are capable of continuous self-renewal and can differentiate into the three major cell types of the central nervous system (CNS), namely neurons, astrocytes, and oligodendrocytes (Conti and Cattaneo, 2010). Furthermore, they exhibit low immunogenicity, are non-tumorigenic, can migrate long distances in the CNS—especially towards pathological foci—and can enhance endogenous pro-regenerative activity (Meng et al., 2003; de Filippis, 2011). Consequently, they are a major transplant population for cell therapies to promote repair in neuropathological conditions. Their therapeutic capacity can be enhanced by genetic engineering; for example, to increase the delivery of regeneration-promoting molecules to sites of pathology (Phillips and Tang, 2012). Traditionally, this has been accomplished with viral vectors, which have several drawbacks, notably safety issues and scale-up limitations (Mintzer and Simanek, 2009; Elsabahy et al., 2011).

In this context, nanomaterials, especially MNPs, are emerging as major non-viral gene delivery vectors for the safe bioengineering of NSCs. MNPs typically comprise magnetic cores overcoated with biocompatible materials that can be functionalized for the binding of a range of molecules, including nucleic acids (i.e. DNA plasmids, oligonucleotides and siRNA) (Plank et al., 2011; Yiu, 2011). Cellular internalisation of the resulting transfection complexes (and consequently gene delivery) is mediated via endocytotic uptake mechanisms; this step can be markedly enhanced by the application of static or oscillating magnetic fields (so called ‘magnetofection’ or ‘magnet-assisted transfection’ approaches), which can enhance particle interaction with the cellular endocytotic machinery. Notably, amongst conventional viral and non-viral vector-based genetic modification approaches, MNPs uniquely can offer a combination of key benefits for experimental and clinical cell therapies, especially: i. non-

invasive tracking of transplanted cells *in vivo* (since MNPs are routinely used as MRI contrast agents (Berman et al., 2011)); and ii. magnetic cell localization strategies (i.e. the application of magnetic fields over sites of pathology to localize systemically administered transplant cells (Polyak et al., 2008; Carenza et al., 2014)).

Two culture systems are routinely used for the propagation of NSCs i.e., neurosphere (three-dimensional; suspension) and monolayer (two-dimensional; adherent) cultures; each has distinct features (Conti and Cattaneo, 2010). Relative to monolayer cultures, neurospheres yield a high density of cells within a small culture surface area; an important consideration for clinical translation, which often requires large numbers of cells. Furthermore, the transplantation of neurospheres as opposed to dissociated cells is thought to be more beneficial for graft survival (Mothe et al., 2008). On the other hand, neurosphere cultures yield a more heterogeneous population of cells than monolayer cultures, with a tendency towards the presence of differentiated cells within the neurosphere core (Jensen and Parmar, 2006). Although cells in neurosphere cultures retain multipotentiality with progressive subculture, neurogenic potential tends to decline relative to gliogenic potential (Conti and Cattaneo, 2010). In contrast, cells in monolayer cultures exhibit more homogeneous behaviours and generally retain their neurogenic potential even upon prolonged passage (Conti and Cattaneo, 2010). The latter can be a key consideration for cell therapies for which the intended purpose is the replacement of damaged neurons. Both systems are amenable to MNP-mediated gene delivery, which can be increased by several approaches, including multifection (as demonstrated for neurospheres) (Pickard et al., 2011) and magnetofection, especially employing an oscillating magnetic field (as demonstrated for both neurospheres and monolayers) (Adams et al., 2013; Pickard et al., 2015). Overall, the highest levels of transfection are obtained using monolayer NSCs and magnetofection. Magnetofected monolayer NSCs can, in turn, reform neurospheres, allowing the advantages of this system in terms of transplant survival to be exploited.

Critical Parameters and Troubleshooting

Critical reagent and protocol parameters

These protocols were devised for the genetic engineering of NSC transplant populations with clinical translation in mind, and as an alternative to widely used viral transduction and other non-viral methods (i.e., electroporation and lipofection-based approaches). Safety was therefore of primary concern when developing these methods, and the MNP doses used and exposure times to NeuroMag/magnetic fields have been carefully optimized to minimize cytotoxicity. It is recommended that the NeuroMag particles are aliquoted upon receipt, to avoid excessive freeze-thawing, and that only endotoxin-free preparations of pure plasmid are used; the concentration of the latter should be carefully verified for each preparation. The formation of MNP-plasmid complexes is a critical step to determining a successful outcome. It is important to mix reagents in serum-free medium (serum inhibits complex formation), to allow complexes to form for only the specified time (to prevent time-dependent aggregation) and to

carefully pipette complexes (to avoid their disruption). Complex formation will therefore need to be carefully staggered when performing magnetofection of multiple plates on a single device. Excessive sequential use of the Magnefect-Nano at high frequencies and/or for prolonged periods of time may increase the temperature within tissue culture plates (at least for the original model of the device) and this can be periodically checked using a mock culture with determination of media temperature.

Determining optimal plasmid:DNA ratios and plasmid vectors

Maximal DNA binding capacity of MNPs can be determined by conducting a DNA binding curve. This is achieved by complexing a constant amount of DNA to various amounts of particles as in Basic Protocol 2. After the 20 minute incubation, centrifuge the Eppendorf to remove complexes. Free DNA can then be assessed by absorbance at 260 nm and presented as a percentage of a no particle control (Pickard and Chari, 2010). The ratio of NeuroMag: plasmid used here (i.e. 3.5 µl/µg) is equivalent to the maximal DNA binding capacity of the MNPs; consequently, increasing the amount of plasmid will have no beneficial effect on transfection, while reducing the amount of plasmid will lower transfection efficiencies. For similar reasons, alterations in plasmid size will also affect transfection efficiency, due to variation in plasmid copy number complexed with MNPs; increasing plasmid size will decrease—and decreasing plasmid size will increase—transfection efficiency (Pickard et al., 2015). For functional gene delivery in particular, it is recommended that insert size and/or extraneous plasmid sequences are kept to the minimum required to achieve the desired experimental goal. Strong evidence suggests that the use of minicircle vector systems may be beneficial in this regard. These vectors are further suited to translation as they contain no bacterial backbone elements (e.g. antibiotic resistance genes and origin of replication) meaning they are safer than conventional plasmids but also demonstrate sustained gene expression due to reduction of mammalian transgene silencing mechanisms (Fernandes and Chari, 2016a). Minicircle DNA encoding brain-derived neurotrophic factor has been successfully delivered to NSCs using magnetofection technology, with functional in vitro effects observed from secreted BDNF, including enhanced NSC proliferation and differentiation into neurons (Fernandes and Chari, 2016b).

Application of protocols to other transfection grade particles: Initial optimization and subsequent safety analyses

When transferring these protocols to other preparations of transfection-grade MNPs (or MNPs intended for applications other than gene transfer), it will be important to carefully optimize a number of parameters. Optimal DNA binding should be determined as above. The MNP concentration used together with the duration of exposure of particles to cells are of prime importance, if toxicity is to be minimized. Such preliminary experiments should be conducted using a range of MNP concentrations complexed with the nucleic acid of choice, since this addition can influence surface charge of the particles and consequently, their cellular uptake

and gene delivery potential. Furthermore, it is judicious to include cells exposed to a magnetic field (static at least), since the latter influences particle interaction with cells and consequently, cytotoxicity. Initial screening can then be conveniently performed by phase-contrast microscopic observation of treated and control (no particle) cultures; cytotoxicity will be evident by the rounding and detachment of NSCs in monolayer cultures and by friable appearing spheres with dead/dying cells in neurosphere cultures, which are also of smaller size than control suspension cultures (**Figure 2**).

Qualitative assessment of NSC health can then be supplemented with quantitative measures of cell number/cell viability post-harvesting of cells/neurospheres. Cells from both culture systems can be re-plated on polyornithine/laminin-coated coverslips and either cultured in ML-M for staining with antibodies to SOX2 and nestin by immunocytochemistry to assess stem cell state or cultured in differentiation medium (i.e. ML-M minus growth factors but supplemented with 1% fetal bovine serum) for staining with antibodies to β-tubulin III (neuronal marker), glial fibrillary acidic protein (astrocytic marker) and myelin basic protein (oligodendrocytic marker) to assess differentiation potential, as detailed elsewhere (Pickard et al., 2011; Adams et al., 2013; Pickard et al., 2015). It is important to note that we primarily use a microscopic based approach to assess both transfection efficiency, cellular morphology and staining profiles simultaneously. This enables data generation with respect to multiple safety readouts (including cell morphology, numbers of cells, staining profile, proportions of stem or daughter cells and pyknotic nuclei) to be performed on the exactly the same cells that are used for transfection analysis.

Anticipated Results

Using plasmids that encode fluorescent proteins, transgene expression is usually evident by approximately 6 hours post-complex application and increases over time to become maximal by approximately 40 – 48 h (**Figure 3**). To assess transfection efficiency we use a microscopic based approach which allows for simultaneous safety and transfection analyses on the same samples. This is routinely performed using fixed monolayers although it can also be achieved using dissociated cells from neurospheres. The protocols described here are for transient gene expression; in the longer term, transgene expression declines but is usually observable up to 14 days post-transfection. Typical transfection efficiencies for a 3.5 kb plasmid encoding GFP in the various protocols are summarized in **Table 1**; the transfection efficiencies obtained are usually highly reproducible under the conditions specified here. While transfection efficiencies are lower for larger plasmids, functionally relevant levels of expression of FGF2 (plasmid size 7.5 kb) (Pickard et al., 2015) and BDNF (minicircle size 4.1 kb) (Fernandes and Chari, 2016b) have been observed in bioassays. It should be noted that whilst MNP-mediated transfection does not affect NSC differentiation potential, irrespective of the culture system under study (Pickard et al., 2011; Adams et al., 2013), transgene expression is largely confined to astrocytic progeny for reasons which are not currently understood (**Figure 4**).

Time Considerations

Preparation of the initial neurosphere culture takes approximately 2 – 3 hours, depending on the numbers of dissections to be performed, and these need to be cultured for one passage (usually one week) before MNP-mediated gene delivery can be attempted. Secondary neurosphere and monolayer cultures for transfection can be established within 60 - 90 min, but it is important to start the coating of monolayer culture surfaces beforehand, as this will take approximately 2.5 hours. Irrespective of the culture format, cells need to be cultured for 18 – 24 h prior to transfection. Magnetofection of monolayer cultures usually takes approximately 3.5 h from start to finish for a single plate/magnetic field condition; when applying a range of magnetic field conditions, then the whole process can be staggered at 30 min intervals. The procedure time for magnetofection of neurosphere cultures is shorter at around 90 min per plate, however for multifection the procedure will need to be repeated on two consecutive days. Allow a further 48 h after the final application of complexes to cells for maximal gene expression. The procedure for the formation of neurospheres from monolayer cultures takes approximately 1 h and, if required, this can be performed at 24 h (rather than at 48 h) post-complex addition with no adverse effects. If assessing differentiation potential of transfected NSCs, cells are normally detached/dissociated 48 h after transfection then cultured in differentiation medium for 1 week.

ACKNOWLEDGEMENT (mandatory for NIH, optional for all others)

The work was supported by the Biotechnology and Biological Sciences Research Council, UK (by a research grant) and the Engineering and Physical Sciences Research Council, UK (via the Doctoral Training Centre in Regenerative Medicine and an Engineering:Tissue Engineering and Regenerative Medicine Fellowship).

LITERATURE CITED

Adams, C.F., Pickard, M.R. and Chari, D.M. 2013. Magnetic nanoparticle mediated transfection of neural stem cell suspension cultures is enhanced by applied oscillating magnetic fields. *Nanomedicine.*, 9: 737–741. doi:10.1016/j.nano.2013.05.014

Berman, S.M.C., Walczak, P., Bulte, J.W.M. 2011. Tracking stem cells using magnetic nanoparticles. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.*, 3: 343–355. doi:10.1002/wnan.140

Carenza, E., Barceló, V., Morancho, A., Levander, L., Boada, C., Laromaine, A., Roig, A.,

Montaner, J. and Rosell, A. 2014. In vitro angiogenic performance and in vivo brain targeting of magnetized endothelial progenitor cells for neurorepair therapies. *Nanomedicine.*, 10: 225–34. doi:10.1016/j.nano.2013.06.005

Conti, L. and Cattaneo, E. 2010. Neural stem cell systems: physiological players or in vitro entities? *Nat. Rev. Neurosci.* 11: 176–87. doi:10.1038/nrn2761

Elsabahy, M., Nazarali, A. and Foldvari, M. 2011. Non-viral nucleic acid delivery: key challenges and future directions. *Curr. Drug Deliv.* 8: 235–244. doi:10.2174/156720111795256174

Fernandes, A.R. and Chari, D.M. 2016a. Part I: Minicircle vector technology limits DNA size restrictions on ex vivo gene delivery using nanoparticle vectors: Overcoming a translational barrier in neural stem cell therapy. *J. Control. Release*. doi:10.1016/j.jconrel.2016.06.024

Fernandes, A.R. and Chari, D.M. 2016b. Part II: Functional delivery of a neurotherapeutic gene to neural stem cells using minicircle DNA and nanoparticles: Translational advantages for Regenerative Neurology. *J. Control. Release*. doi:10.1016/j.jconrel.2016.06.039

de Filippis, L. 2011. Neural stem cell-mediated therapy for rare brain diseases: perspectives in the near future for LSDs and MNDs. *Histol. Histopathol.,* 26: 1093–109. doi:10.14670/HH-26.1093

Jensen, J. and Parmar, M. 2006. Strengths and limitations of the neurosphere culture system. *Mol. Neurobiol.*, 34: 153–161. doi:10.1385/MN:34:3:153

Kim, S.U. and de Vellis, J. 2009. Stem cell-based cell therapy in neurological diseases: a review. *J. Neurosci. Res.*, 87: 2183–2200. doi:10.1002/jnr.22054.

Meng, X.-L., Shen, J.-S., Ohashi, T., Maeda, H., Kim, S.U. and Eto, Y. 2003. Brain transplantation of genetically engineered human neural stem cells globally corrects brain lesions in the mucopolysaccharidosis type VII mouse. *J. Neurosci. Res.*, 74: 266–77. doi:10.1002/jnr.10764

Mintzer, M.A. and Simanek, E.E. 2009. Nonviral vectors for gene delivery. *Chem. Rev.,* 109: 259–302. doi:10.1021/cr800409e

Mothe, A. J., Kulbatski, I., Parr, A., Mohareb, M. and Tator, C.H. 2008. Adult spinal cord stem/progenitor cells transplanted as neurospheres preferentially differentiate into oligodendrocytes in the adult rat spinal cord. *Cell Transplant.,* 17: 735–51. doi:http://dx.doi.org/10.3727/096368908786516756

Phillips, M.I. and Tang, Y. 2012. Genetic modification of stem cells for cardiac, diabetic, and hemophilia transplantation therapies. *Prog. Mol. Biol Transl Sci.,* 111: 285–304. doi:10.1016/B978-0-12-398459-3.00013-7

Pickard, M.R., and Chari, D.M. 2010. Enhancement of magnetic nanoparticle-mediated gene transfer to astrocytes by “magnetofection”: effects of static and oscillating fields.

Nanomedicine (Lond.), 5: 217–232. doi:10.2217/nnm.09.109

Pickard, M.R., Adams, C.F., Barraud, P. and Chari, D.M. 2015. Using magnetic nanoparticles for gene transfer to neural stem cells: stem cell propagation method influences outcomes. *J. Funct. Biomater.* 6: 259–76. doi:10.3390/jfb6020259

Pickard, M.R., Barraud, P. and Chari, D.M. 2011. The transfection of multipotent neural precursor/stem cell transplant populations with magnetic nanoparticles. *Biomaterials*, 32: 2274–2284. doi:10.1016/j.biomaterials.2010.12.007

Plank, C., Zelphati, O. and Mykhaylyk, O. 2011. Magnetically enhanced nucleic acid delivery. *Adv. Drug. Deliv. Rev.*, 63: 1300–1331. doi:10.1016/j.addr.2011.08.002

Polyak, B., Fishbein, I., Chorny, M., Alferiev, I., Williams, D., Yellen, B., Friedman, G. and Levy, R. J. 2008. High field gradient targeting of magnetic nanoparticle-loaded endothelial cells to the surfaces of steel stents. *Proc. Natl. Acad. Sci. U. S. A.*, 105: 698–703. doi:10.1073/pnas.0708338105

Yiu, H.H.P. 2011. Engineering the multifunctional surface on magnetic nanoparticles for targeted biomedical applications: a chemical approach. *Nanomedicine (Lond.)*, 6: 1429–1446. doi:10.2217/nnm.11.132

KEY REFERENCE (optional)

INTERNET RESOURCES (optional)

FIGURE LEGENDS

Figure 1. SVZ dissection and NSC culture as neurospheres. (A) Schematic of SVZ location in a coronal brain slice highlighted by red boxes alongside representative light microscope image of coronal slice with SVZ appearing as lighter region in the centre (yellow box and arrow). (B) Representative phase image of NSC cultures five days post SVZ dissociation. (B, inset) Representative phase image of NSCs starting to form neurospheres (arrows) two days post SVZ dissociation. Note the presence of debris from dead and dying cells, mostly non-NSCs, absent from the older cultures.

Figure 2. Microscopic detection of MNP mediated toxicity in live cultures. (A) Representative phase image of NeuroMag toxicity in neurosphere cultures which is evident by friable neurospheres which also appear smaller than controls (depicted in the inset). (B) Representative phase image of toxicity in NSC monolayers with high numbers of rounded and detached cells compared to controls (depicted in inset). Images in A reproduced from Pickard et al. (2011), with permission, and in B (inset) from Pickard et al. (2015).

Figure 3. MNP mediated transfection of NSCs in monolayers and neurospheres. (A-B) Representative fluorescent images of NSC monolayers transfected using NeuroMag under (A)

No field and (B) a 4 Hz oscillating magnetic field with increased numbers of cells expressing the reported protein, GFP. (C) Phase and (D) counterpart fluorescence image depicting neurospheres reformed from NSCs transfected under the 4 Hz oscillating field condition. (E-F) Representative fluorescent images of NSC neurospheres transfected with NeuroMag using (E) single transfection and (F) multifection which results in a higher number of GFP expressing cells. (E-F, insets) Counterpart phase images to the main images. Images in A – D, reproduced from Pickard et al., 2015; images in E & F reproduced from Pickard et al. (2011), with permission.

Figure 4. Magnetofected cells can differentiate into their daughter cells. Representative triple merged images of NSCs transfected as neurospheres under a 4 Hz oscillating magnetic field which differentiate into (A) astrocytes, (B) neurons and (C) oligodendrocytes. Note that the majority of daughter cells expressing the transgene GFP are astrocytes with GFP expressing, GFAP positive astrocytes in (A) and GFP expressing cells with the morphological appearance of astrocytes in (B) and (C). Images A – C reproduced from Adams et al. (2013) with permission.

TABLES

System	n	Transfection (% cells)	Total cell no. (10^5)	% Viability
Neurospheres/multifection				
Day 1	8	13.7 ± 1.8	1.49 ± 0.20	97.6 ± 0.5
Days 1 + 2	8	22.6 ± 2.4	1.59 ± 0.20	96.2 ± 1.4
Monolayers/magnetofection				
No field	4	9.4 ± 1.0	2.42 ± 0.27	96.3 ± 0.3
Static field	4	18.4 ± 1.3	2.39 ± 0.44	95.7 ± 0.7
Oscillating (4 Hz) field	4	32.2 ± 1.5	2.37 ± 0.41	95.9 ± 1.0

Table 1. *Expected transfection efficiencies, cell number and culture viability for NeuroMag-mediated multifection of neurospheres and magnetofection of monolayers. Data are from studies with the pmaxGFP plasmid* (Pickard et al., 2011; Pickard et al., 2015).

Additional instructions:

The following should be submitted as individual files, NOT as part of the main document:

- Figures
- **COPYRIGHT PERMISSION** (if required)
- **VIDEOS** (optional)

If you have any questions about your manuscript, or formatting, or submitting it, consult the Contributor's Style Guide (For Authors page) or contact the Developmental Editor listed for your title.

For questions about our submissions site (ScholarOne Manuscripts), your Contributor Agreement, or copyright permissions, please contact our Editorial Program Coordinator at cpsubmissions@wiley.com.