

A novel *ex vivo* application of RNAi for neuroscience.

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Introduction

A large number of patients worldwide suffer from neuronal disorders such as Alzheimer's, Parkinson's, and Huntington's diseases. At present, the fundamental molecular mechanisms underlying these and other neuronal diseases are still unclear. Harnessing RNA interference (RNAi) to elucidate the mechanisms leading to such neuronal disorders may provide insights leading to the development of efficient treatments.

Using organotypic slice cultures for studies to analyze molecular pathways in neurons is a means of bridging the gap between *in vitro* cell cultures and *in vivo* models. In contrast to cultures of primary neurons, *ex vivo* slice cultures allow the study of primary neurons within the context of an intact cellular network of supporting cells and other neurons, thereby more closely mimicking the *in vivo* situation. In addition, it is possible to manipulate the cells in slice cultures to optimize parameters and monitor results - all of which is very challenging in rodent *in vivo* models.

RNAi in neuronal cells

Up until now the use of small interfering RNA (siRNA) in neuronal cell models was limited by delivery efficiency of siRNA to neuronal cells *in vitro* and *in vivo*. Using lipid-based transfection reagents to transfect neuronal cells with siRNA traditionally has been problematic; primarily due to low efficiency of delivery or lipid-induced effects that lead to toxicity. Lowering the lipid concentration to ensure reasonable cell viability does not lead to sufficient transfection rates for effective gene silencing. Investigators using lipids mostly have to work with transfection efficiency as low as 30% under optimized conditions.



Transfection methods based on electroporation require additional equipment and also may not result in reliably sufficient transfection efficiencies and cell viability.

In contrast to conventional RNAi delivery methods, Dharmacon Accell™ siRNA reagents allow the delivery of siRNA independent of transfection reagents or instrument-based procedures that may result in loss of cell viability, thus overcoming a major obstacle in working with neuronal cells. By applying Accell siRNA in organotypic mouse brain slices, unwanted non-specific delivery effects commonly caused by transfection reagents or instruments are excluded. In addition, long-term effects such as cell differentiation can be easily monitored by repeated addition of Accell siRNA reagents to the cultured slice without detrimental effects to the overall structure or physiology of the tissue.

Workflow for delivery of Accell siRNA to cultured brain slices

Cerebella dissected out of embryonal day 17 and postnatal day 8 C57/Bl6 pups were placed carefully into cold modified Hank's solution (137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 5 mM glucose, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 4.17 mM NaHCO₃, pH 7.2) without removing the meninges. After all cerebella were collected, the tissue was embedded in 5 mL (20% wt/vol) liquid gelatin (from porcine skin, type A) dissolved in Hank's solution for 20 minutes at 37 °C in a CO₂ incubator. Subsequently, cerebella were mounted onto a sterile specimen disc, and sagittal sections of 250 μM thickness were prepared using a McIlwain tissue chopper.

The slices were gently placed in cold Accell Delivery Medium (Cat #B-005000) and individual sections were separated under a dissection microscope (Zeiss, Stemi 2000 C). Slices were then recovered from the medium and transferred in a small volume of liquid onto membranes of tissue culture inserts (Millicell-CM, Millipore, Bedford, MA; 12 mM, pore size 0.4 μM) placed in a 24-well dish with 300 μL Accell Delivery mix (Accell Delivery Medium supplemented with 1x B-27 (Gibco) and 1 μM Accell siRNA) using the broad end of a Pasteur pipette. A small amount of medium underneath the membrane promoted attachment of the sections.

Wells not containing culture inserts were filled with sterile water to increase humidity inside the culture dish. Cultures were kept in a standard cell incubator (37 °C, 5% CO₂ and 95% humidity) and were harvested after 48, 72 and 96 hours of incubation. Cultures were each rinsed twice with ice-cold PBS. Slices were collected into 40 μL RIPA buffer supplemented with 1x protease inhibitor cocktail (Complete, Roche) and sonicated for 1 minute to lyse the tissue. Western blot analysis with 50 μg of tissue lysate per lane was incubated with anti-Cyclophilin B (Abcam, 1:2,000) and goat-anti-rabbit-HRP (Dako, 1:30,000) antibodies and detected by chemiluminescence (Thermo Scientific Super Signal West Femto, Cat #34095, 34096).

Observation of cellular penetration by Accell siRNA

To assess uptake of Accell siRNA, 250 μM cerebellar sections were prepared and cultured as described above. After 3 and 72 hours of incubation with Accell Red Non-targeting siRNA, slices were inspected with a Leica DM IRE2 microscope (filter TX2).

Uptake of Accell siRNA was visible after 3 hours of incubation (Figure 1). The most prominent fluorescence was observed in cells of the Purkinje and granule cell layer. After 72 hours in culture, fluorescence in the slices was more intense, indicating an increased uptake of Accell siRNA with extended incubation. The addition of B-27 neuronal medium supplement enhanced the overall cellular viability and had no observed effect on siRNA uptake (data not shown).

Demonstration of knockdown of Cyclophilin B

To demonstrate target gene knockdown in cultured brain slices using Accell siRNA, the workflow described above was employed to target the housekeeping gene PPIB (Cyclophilin B, Accession #NM_011149). Cultured brain slices were assessed following Accell siRNA incubation for three time points (48, 72, 96 hours) and the level of remaining protein was evaluated using western blot analysis. The results of these experiments are presented in Figure 2 and demonstrate the efficacy of Accell technology in providing effective target silencing.

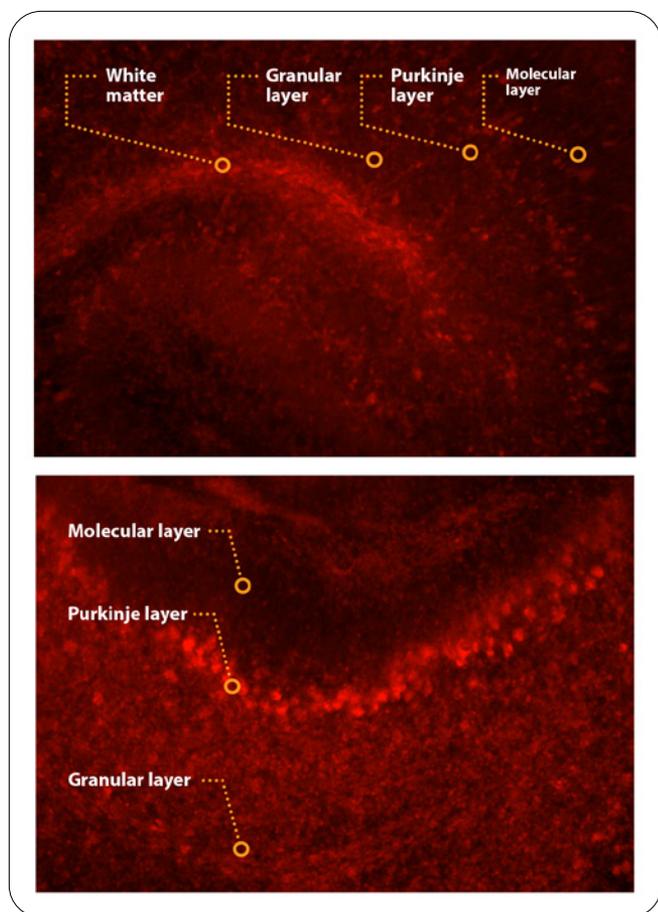


Figure 1: Three hours and 72 hours post-Accell application. Shutter time 150 ms.

For more information:

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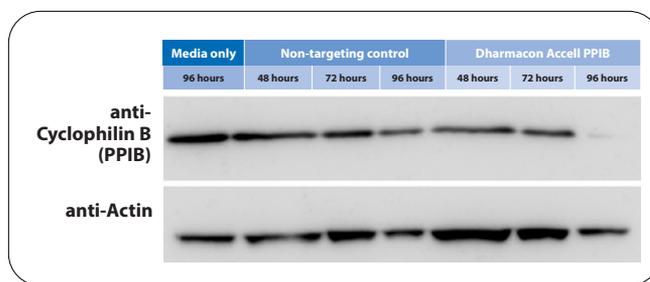


Figure 2: Accell effectively silenced a target gene in cultured brain slices. 250 μ M fresh P8 mouse cerebellum slices were incubated in Accell Delivery Media (Cat #B-005000) supplemented with Gibco® B-27 serum-free supplement containing 1 μ M Accell siRNA targeting Cyclophilin B (Accell PPIB; Cat #D-001920-02) or Accell Non-targeting siRNA #1 (NTC; Cat #D-001910-01). Slices were cultured for 48, 72, or 96 hours prior to knockdown detection by western blot analysis. Tissue was harvested using RIPA-buffer, 50 μ g of protein lysate were analysed per sample.

Summary

These preliminary studies demonstrated that Accell siRNA can be delivered into cells of a brain slice culture allowing for successful RNAi in neurons. Furthermore, the ease and convenience of this method will enable scientists to use Accell siRNA reagents to empower a variety of applications for the study of neuronal mechanisms and diseases, including:

1. Medium-to-high throughput RNAi screens to assess gene silencing in neurons.
2. Knockdown of a gene's expression and monitoring the resulting phenotype to evaluate the experimental potential of a knockout mouse of the same gene.
3. The ex vivo model of the cultured brain slice can also serve as a framework to address the discrepancy between anticipated and actual *in vivo* results, addressing phenotypes such as cellular organization and differentiation.

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