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TITLE:

Nuclear Transport Assays in Permeabilized Mouse Cortical Neurons

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KEYWORDS:

nucleocytoplasmic transport, neurons, neurodegeneration, nuclear pore complex, nuclear import, high content microscopy

SUMMARY:

We have developed a reliable method of selective plasma membrane permeabilization of primary mouse cortical neurons for high content automated analysis of neuronal nucleocytoplasmic transport.

ABSTRACT:

Disruption of nucleocytoplasmic transport is increasingly implicated in the pathogenesis of neurodegenerative diseases. Moreover, there is a growing recognition of cell-specific differences in nuclear pore complex structure, prompting a need to adapt nuclear transport methods in neurons. Permeabilized cell assays, in which the plasma membrane is selectively perforated by digitonin, are widely used to study passive and active nuclear transport in immortalized cell lines but have not been applied to neuronal cultures. In the initial attempts, the rapid loss of nuclear membrane integrity was observed in primary mouse cortical neurons exposed to even low concentrations of digitonin. It is hypothesized that neuronal nuclear membranes might be uniquely vulnerable to the loss of cytoplasmic support. After testing multiple approaches to improve nuclear stability, optimal nuclear integrity was observed following hypotonic lysis in the presence of concentrated Bovine serum albumin (BSA) cushion. Neuronal nuclei prepared by this approach reliably import recombinant fluorescent cargo in an energy-dependent manner, facilitating analysis of nuclear import by high content microscopy with automated analysis. It is anticipated that this method will be broadly applicable to studies of passive and active nuclear transport in primary neurons.

INTRODUCTION:

Disruption of nucleocytoplasmic transport, the regulated trafficking of proteins and RNA between the nucleus and cytoplasm, is increasingly implicated in the pathogenesis of

neurodegenerative diseases (recently reviewed[1]). Researchers have reported structural and functional disruption of the nucleocytoplasmic transport apparatus in postmortem tissue and animal models of *C9orf72* amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), Alzheimer's disease, and Huntington's disease[2–5]. The mechanisms and functional consequences of nucleocytoplasmic transport disruption for neurodegeneration, and approaches for therapeutic rescue, are areas of an ongoing investigation.

Nuclear pores are large transmembrane complexes of ~30 nucleoporin proteins that permit diffusion of small molecules across the nuclear membrane but increasingly restrict the passage of cargoes >40 kD via a permeability barrier of phenylalanine-glycine (FG)-rich nucleoporins in the central channel[6]. Larger cargoes containing nuclear localization signal (NLS) or nuclear export signal (NES) sequences undergo active, receptor-mediated transport across the pore via nuclear transport receptors (importins and exportins) and a steep gradient of the small GTPase Ran across the nuclear membrane (recently reviewed[7]). A wide array of methods has been developed to analyze nuclear transport dynamics in cultured cells, including the trafficking of endogenous cargoes and tagged reporter constructs that serve as substrates for the major subclasses of transport receptors. Such approaches have been readily adapted to neurons[2,5,8] and provide a readout of nuclear transport perturbations in the context of an intact, living cell. However, in live cell assays the ability to directly manipulate nuclear transport reactions or investigate them in isolation from other cellular processes is limited.

In permeabilized cell assays, the plasma membrane is selectively perforated, and the cytoplasm is released, leaving the nuclear envelope and nuclear pore complexes intact and able to perform either passive or energy-dependent bidirectional transport[9,10]. Such transport reactions can be readily reconstituted by adding whole-cell lysates, cytoplasmic fractions, or purified recombinant nuclear transport proteins and their cargos. Thus, permeabilized cell assays permit a broad range of biochemical or biophysical investigations, including delivery of recombinant or synthetic proteins and RNAs relevant to the study of neurodegenerative diseases.

Given reports of cell-specific differences in nuclear pore complex structure and transport dynamics[11,12], the study aimed to adapt the permeabilized cell assay for use in primary neuronal cultures. Although widely used to analyze nuclear transport in immortalized cell lines, despite exhaustive literature search, no published reports of neuronal plasma membrane permeabilization were found that verified preservation of nuclear membrane integrity. Most protocols rely on digitonin, a detergent that targets the unique cholesterol composition of the plasma membrane, to perforate the nuclear membrane while leaving the nuclear membrane intact[13]. The initial attempts using digitonin in primary mouse cortical neurons showed immediate loss of nuclear membrane integrity, evidenced by diffusion of a 70 kD fluorescent dextran into the nucleus. It was hypothesized that nuclear envelope rupture might be caused by mechanical perturbation from loss of cytoplasmic support, and multiple methods of optimization were tested, including molecular crowding, cytoskeletal stabilization, and alternate methods of cell lysis. Here, the protocol details a method of rapid hypotonic permeabilization using a concentrated Bovine serum albumin (BSA) cushion to protect neuronal nuclei and facilitate downstream analysis of neuronal nuclear import. This method was recently used to evaluate the mechanism of dipeptide repeat protein disruption

in *C9orf72*-ALS/FTD[14] and anticipate that it will be broadly applicable to future studies of passive and active nuclear transport in primary neurons.

PROTOCOL:

First, the protocol describes the generation of primary neuronal cultures (step 1) and preparation of materials for the transport assay (step 2), followed by the transport assay itself (steps 3–4) and image acquisition and analysis (step 5). All methods described here were approved by the Animal Care and Use Committee (ACUC) of Johns Hopkins University.

1. Primary mouse cortical neuron cultures

1.1. Prepare the stock solutions.

1.1.1. Dissection buffer: Combine 50 mL of 10x HBSS, 15 mL of 1 M glucose (in dH_2O), 5 mL of 1 M HEPES, 5 mL of 100 mM sodium pyruvate, and 5 mL of 100x penicillin-streptomycin. Bring to volume to 500 mL with dH_2O. Sterile filter and store at 4 °C.

1.1.2. Papain: Dilute papain to 30 mg/mL with dissection buffer. Store at 4 °C.

1.1.3. DNase: Dilute DNase I to 10 mg/mL with dissection buffer. Aliquot and store at -20 °C.

1.1.4. Plating medium: Add 5% fetal bovine serum (FBS), 2% B-27, 1% Glutamax, and 1% penicillin-streptomycin to neurobasal medium. Sterile filter and store at 4 °C.

1.1.5. Feeding medium: Add 2% B-27, 1% Glutamax, and 1% penicillin-streptomycin to Neurobasal medium. Store at 4 °C.

1.2. Coat the plates.

1.2.1. Coat the optical glass-bottom 96-well plates with poly-D-lysine (20 μg/mL) and laminin (1:100) in sterile dH_2O. Place the plate in an incubator overnight.

1.2.2. On the day of dissection, rinse the wells three times with sterile dH_2O and replace them with the neurobasal medium. Equilibrate in an incubator for at least 30 min.

1.3. Dissect and digest the cortices.

NOTE: Carry out dissection on a clean bench using autoclaved tools to prevent culture contamination.

1.3.1. Euthanize the E15-16 C57BL/6J pregnant female by cervical dislocation. Spray abdomen with 70% ethanol. Work quickly. Use scissors and toothed forceps to make a midline abdominal incision, excise the uterus, and then transfer the embryos from the amniotic sac into a Petri dish. Decapitate the embryos with scissors and collect heads in a 10 cm Petri dish containing dissection buffer.

1.3.2. Under a dissecting microscope, use fine forceps to remove the skull and peel away the

meninges. Transfer whole brains to a fresh 10 cm Petri dish containing dissection buffer. Separate the hemispheres and remove the subcortical structures.

1.3.3. Chop the cortices with a sterile razor blade and transfer them to a conical tube containing 5 mL of dissection buffer. Add 100 µL of papain (stock 30 mg/mL, final concentration 0.6 mg/mL) and 50 µL of DNase (stock 10 mg/mL, final concentration 0.1 mg/mL). Digest in a 37 °C water bath for 10 min.

NOTE: Digestion beyond 10 min is detrimental to cell survival.

1.3.4. Remove the supernatant and wash twice with 10 mL of plating media to stop digestion. Allow the tissue to settle by gravity in between each wash.

1.3.5. Triturate gently in 2 mL plating media with P1000 pipette until large pieces of tissue disappear (approximately 20 times). Add another 8 mL of plating media to bring the volume to approximately 10 mL and pass the cell suspension through a 40 µm cell strainer.

1.4. Plate and maintain neurons.

1.4.1. Centrifuge at 200 x *g* for 5 min. Resuspend the cells in a prewarmed plating medium. Count the cells and dilute to 500,000 cells/mL in the plating medium. Gently invert to mix and transfer to a sterile multichannel reservoir.

1.4.2. Aspirate the neurobasal media from the wells and immediately add 100 µL of cells per well with a multichannel pipette (50,000 cells/well). Do not allow the coated wells to dry.

1.4.3. After 24 h of dissection, change 50% of medium with the feeding medium. Continue 50% medium change every other day until day 5–7 to perform the *in vitro* assays.

2. Preparation of the nuclear transport assay components

2.1. Transport buffer (TRB, adapted from[15])

2.1.1. To prepare 5x TRB, dissolve 100 mM HEPES, 550 mM KOAc, 10 mM $Mg(OAc)_2$, 25 mM NaOAc, 2.5 mM EGTA, and 1.25 M sucrose in dH_2O. Adjust the pH to 7.3 and store the buffer at 4 °C.

2.1.2. On the day of the transport experiment, prepare 1x TRB-BSA. Dilute 5x TRB in dH_2O, add EDTA-free protease inhibitor cocktail, 2 mM of DTT, and 50 mg/mL of BSA.

2.2. Energy regeneration mix (ERM, adapted from[9,16])

2.2.1. To prepare 20x ERM, dissolve 2mM ATP lithium salt, 2 mM GTP lithium salt, 80 mM Creatine phosphate, and 400 U/mL Creatine kinase in 1x TRB.

2.2.2. Freeze the single-use aliquots at -20 °C.

2.3. Preparation of Whole cell extract (WCE, source of transport receptors, and Ran cycle proteins).

NOTE: Cytoplasmic or whole cell extract (WCE) may be used, depending on experimental goals. Here, the preparation of WCE is described as used in the initial neuron transport assays[10].

2.3.1. Grow HEK293T cells to confluence in fifteen 150 mm culture dishes. Detach with trypsin-EDTA and spin down as if passaging cells. Resuspend the cells in 10 mL of ice-cold 1x TRB with a freshly added protease inhibitor cocktail.

2.3.2. Sonicate on ice, 3 x 10 pulses, using a hand-held probe sonicator. Clarify by centrifugation at 14,000 x *g* for 15 min at 4 °C.

2.3.3. Measure the protein concentration (goal 8–10 mg/mL) and snap-freeze single-use aliquots in liquid nitrogen prior to storage at -80 °C.

2.4. Recombinant nuclear import cargo

NOTE: The transport protocol in steps 3–5 may be adapted for any fluorescent nuclear transport cargo, to interrogate the active or passive nuclear transport pathway of interest. Here, the protocol describes the expression and nuclear import of Rango (Ran-regulated importin β cargo), which consists of the importin β-binding domain of importin α1 flanked by the fluorescent proteins CyPet and YPet[14,17]. Rango is a versatile sensor that can be used for FRET as well as nuclear import assays, where it functions as a direct importin β cargo.

2.4.1. Transform *E. coli* BL21(DE3) cells with the Rango plasmid (containing an N-terminal 6-His tag) by heat-shock as follows. Thaw a 50 µL aliquot of BL21(DE3) cells on ice. Combine with 100–200 ng plasmid. Mix by tapping the tube 4–5 times and then incubate on ice for 30 min before placing on a 42 °C plate for 40 s. Transfer immediately back to ice for another 2–5 min.

2.4.2. Add 900 µL of SOC media at room-temperature and incubate on a shaker at 37 °C for 1 h before spreading the cells on a prewarmed agar plate (1.5% agar, LB media, 100 µg/mL Ampicillin). Incubate the plate overnight at 37 °C. Store the plate at 4 °C for up to 1 week or use it immediately for protein production.

2.4.3. Start the protein production by inoculating three 25 mL of starter cultures in 50 mL tubes with increasing number of colonies (~3–10) from the BL21(DE3) plate. After incubation overnight at 37 °C on a shaker, select the cultures with $OD_{600\ nm} < 1.0$ to inoculate a 1L culture in LB media using a 2.8 L baffle-free Fernbach flask. Grow at 37 °C until reaching $OD_{600\ nm} = 0.1–0.3$.

NOTE: The use of older BL21(DE3) LB agar plates or high density of starter cultures ($OD_{600\ nm} >1.0$) could reduce the yield and purity of the protein preparation.

2.4.4. Cool to room temperature (22–25 °C) by placing on ice. Induce protein expression with

0.3 mM IPTG (final concentration) and incubate on a shaker (80–120 rpm) at room temperature for 12–14 h.

2.4.5. Collect the cells by centrifugation (6,000 x *g*, 15 min, 4 °C) and resuspend in 30 mL of ice-cold 10 mM imidazole in PBS (pH 7.4), supplemented with EDTA-free protease inhibitor cocktail. Lyse by two passes through an ice-cold French pressure cell and clarify the lysate by centrifugation (16,000 x *g*, 40 min, 4 °C).

2.4.6. Incubate the lysate with high flow Nickel affinity agarose resin (30–60 min, 4 °C), using 1 mL of resin per 1 L of *E. coli* culture. Place the resin into the chromatography columns, wash with 10–20 volumes of ice-cold 10 mM imidazole/PBS (pH 7.4). Elute Rango with 25 mM imidazole/PBS increments (25–200 mM, pH 7.4), using a 5-fold bed volume in each step.

2.4.7. Use an SDS-PAGE to select and pool the batches with the highest purity.

2.4.8. Prepare XB buffer by dissolving 10 mM HEPES, 100 mM KCl, 10 mM $CaCl_2$, 1 mM $MgCl_2$, and 50 mM Sucrose in dH_2O. Adjust the pH to 7.7.

2.4.9. Dialyze the protein in XB buffer and concentrate by centrifugation (7500 x *g*, 4 °C) on the ultrafiltration device with a 30 kD molecular weight cutoff.

2.4.10. Measure the protein concentration by the Bradford or other preferred method, and snap-freeze single-use aliquots in liquid nitrogen before storage at -80 °C.

3. Determination of the optimal plasma membrane permeabilization conditions

NOTE: Due to variations between each batch of neurons, optimize the permeabilization conditions for each batch of neurons. Perform this on the same day before performing nuclear transport assays.

3.1. In a plastic 96-well staging plate, prepare a serial dilution of Tris-HCl pH 7.5 (to cause osmotic swelling), at 0 µM, 10 µM, 20 µM, and 40 µM in each of three BSA concentrations: 50 mg/mL, 100 mg/mL, and 150 mg/mL (for molecular crowding/mechanical support). Prewarm the solutions to 37 °C.

3.2. Rinse the neurons once in prewarmed PBS. Remove the PBS and transfer Tris/BSA from the staging plate. Return to the incubator for 4 min.

3.3. Remove from the incubator and place the culture plate on ice. Rinse 2 x 5 min in 1x TRB-BSA (as prepared in step 2.1.2).

3.4. After the final rinse, add 70 kD of Texas Red-labeled dextran (0.6 mg/mL) and Hoechst (1:10,000) in 1x TRB-BSA. Leave the plate on the bench at room temperature for 15 min, protected from light.

3.5. Image using a confocal microscope to identify the buffer and the BSA concentration in which the plasma membrane permeabilization percentage is the highest, but the nuclear

membrane restricts entry of the 70 kD dextran (goal ≥ 80%). Use these conditions for subsequent experiments.

4. Nuclear import assay

4.1. Prepare the transport reactions

NOTE: To enable all reactions in the plate to be started simultaneously, prepare reaction mix in a plastic 96-well staging plate on ice, prior to neuron permeabilization. Carry out a minimum of two technical replicates per condition. Include controls on each plate.

4.1.1. Prepare the base reaction mix consisting of 2.5 mg/mL of WCE, 1x ERM, fluorescent cargo (200 nM Rango), and Hoechst 33342 (10 mg/mL, 1:10,000) in 1x TRB-BSA, sufficient for 50 µL per well. OPTION: Include 70 kD Texas red-labeled dextran to permit monitoring of nuclear pore integrity throughout the transport reaction, keeping in mind that confocal imaging is needed to appropriately image nuclear dextran restriction.

NOTE: The WCE concentration provided is a reasonable starting point for assay optimization. Perform empiric testing of each batch of WCE to determine the optimal concentration required for efficient nuclear import of the desired cargo. Avoid combining data from replicates prepared with different batches of WCE since import efficiency may vary.

4.1.2. Prepare the controls including (1) Cargo alone: Rango, but no WCE or ERM and (2) Inhibitor: fluorescent cargo, ERM, WCE, and 100 µM importazole (IPZ, a small molecule inhibitor of importin β[18]). As an Alternate, use 0.8 mg/mL of wheat germ agglutinin (WGA)[19].

NOTE: Equilibrate the inhibitors in the assay mix containing WCE for at least 30 min before initiation of transport. For maximal inhibition, post-permeabilization rinse steps in corresponding wells should also include the inhibitor.

4.2. Permeabilize and rinse the neurons

4.2.1. Permeabilize the neurons according to the optimal, batch-specific protocol identified in step 3.

4.2.2. Remove the culture plate from the incubator and immediately place it on ice. Rinse 2x for 5 min in 1x TRB-BSA. Include an inhibitor in the rinses for designated wells.

4.3. Run the transport reaction.

4.3.1. Following the final rinse, remove 1x TRB-BSA. Working quickly, use a multichannel pipette (50 µL/well) to transfer the premixed transport reactions onto the permeabilized cells.

4.3.2. Immediately load the plate in high content imager at room temperature to initiate time-lapse imaging (see step 4.4) or place plate on the bench, protected from light, and allow the reaction to proceed for the desired time period (e.g., 30–120 min) prior to fixation and imaging.

4.3.3. If desired, fix in 4% paraformaldehyde/PBS for 15 min, rinse 2x for 5 min in PBS, and transfer to 50% glycerol/PBS for later imaging. Keep the fixed plates protected from light at 4 °C (stable for up to 1 week or more).

4.4. Live imaging

NOTE: Apply any preferred image acquisition and analysis method at this stage.

4.4.1. Load the plate in a high content microscope. Run an initial test plate to optimize and save imaging parameters, including magnification, focus, exposure times (Hoechst and FITC), and intervals prior to running experimental samples. Save the parameters to reload with each subsequent plate.

NOTE: For quantitative analysis, set the exposure time for the fluorescent import cargo (FITC) well below saturation at steady state or the latest timepoint to be used. Establish this using the test plate, targeting half-maximal saturation to permit variations in intensity across experimental conditions. Collect the Hoechst images for each frame and timepoint to permit nuclear identification during subsequent automated analysis.

4.4.2. Adjust the focus offset, if needed, using Hoechst as the focus wavelength and run the imaging protocol. Collect the images every 5 min for 30 min. Adjust the interval and reaction time as needed based on the imaging speed and kinetics of the import reaction of interest.

5. Image analysis

5.1. In the microscope **Review Plate** dialog, open Hoechst and FITC images from a representative frame. Under **Run Analysis** tab, select **Translocation-Enhanced** module, and configure the settings. Set Hoechst as the compartment image and FITC as the translocation probe image.

5.2. Under compartments, adjust approximate nuclear width, intensity above local background, and minimum/maximum area. Activate **Auto Separate Touching Compartments** as the neurons tend to cluster. View results by selecting **Test Run**. Adjust the settings to maximize the accuracy of neuronal nuclei identification while limiting erroneous inclusion of non-neuronal nuclei or debris.

NOTE: Glial contamination, if present, can typically be excluded at this step based on nuclear size. Determination of optimal compartment identification settings is a trade-off between accuracy and cell number and must be set empirically depending on the assay, cell type, and culture density. Stringent parameters will omit numerous cells but include fewer errors, while liberal parameters will be more inclusive but contain more errors. If different cell populations (i.e., disease mutants) or treatments are included that may affect nuclear size, we recommend setting size/intensity parameters that are broad enough to accommodate all conditions to avoid the need for multiple parameter sets within an experiment.

5.3. To avoid artifacts from the nuclear edge, define regions for measuring several pixels

inside and outside the compartment edge defined by Hoechst staining. Depending on magnification and binning, set 2–4 pixels for inner region distance, and 1–2 pixels for outer region distance. Adjust the outer region width (1–2 pixels).

5.4. Select the desired background estimation method (default of **Auto Constant** is recommended). In **Configure data log**, select the desired output parameters, including mean inner intensity, mean outer intensity, and inner/outer mean intensity (the nuclear to cytoplasmic (N/C) ratio). Save the analysis parameters.

NOTE: The inclusion of internal positive and negative controls within each experiment is strongly encouraged to ensure that parameters maximize sensitivity to detect the biological event of interest.

5.5. Use the **Plate Utilities** dialog to run analysis on the desired plate(s) using the saved parameters and export the measurements.

5.6. Review and summarize the data by the treatment condition. OPTION: Apply filters to remove non-physiologic data, such as probe intensity = 0 and extremes of N/C ratio (i.e., <0.1 and >100). Equally apply any filters across all conditions and experiments.

5.7. As an additional correction for baseline fluorescence, normalize the data to the control wells in which lysate and ER were omitted. To facilitate comparisons across biological replicates (i.e., independent neuronal culture preparations), express the final nuclear import data as a percent of time 0.

REPRESENTATIVE RESULTS:

Selective permeabilization of the plasma membrane (**Figure 1A**) is the most critical step in the protocol and must be verified prior to proceeding with the analysis of nuclear import. Due to variations between each culture preparation, an initial titration plate is routinely run to identify the optimal, batch-specific concentrations of hypotonic Tris-HCl buffer and BSA cushion, as described in step 3. Under- and over-permeabilized cells are readily identified by confocal microscopy (**Figure 1B**) due to lack of penetration of 70 kD dextran into the cytoplasmic compartment or presence of dextran within both the cytoplasmic and nuclear compartments, respectively. In optimal conditions, the majority of cells (≥80%) show 70 kD dextran surrounding but not crossing the nuclear membrane.

Following optimization of permeabilization, the next critical phase, described in step 4, is to establish that nuclear import of fluorescent cargo is energy- and transport receptor-dependent (**Figure 2A**). Controls lacking ERM and WCE (source of importins and Ran cycle proteins) should show only a trace, if any, of nuclear accumulation of fluorescent cargo (**Figure 2B**,**C**). Import should be blocked by the relevant nuclear import inhibitors. These controls must be validated for each cargo to verify that facilitated nuclear import is occurring by the intended mechanism. Once the nuclear transport assay is validated, experimental perturbations can be applied depending on the question of interest.

FIGURE AND TABLE LEGENDS:

Figure 1: Selective permeabilization of the plasma membrane in mouse primary cortical

neurons. (**A**) Schematic of neuronal permeabilization using the hypotonic Tris-HCl with BSA cushion, followed by the validation with Texas red-labeled 70 kD dextran. (**B**) Confocal images of under-permeabilized neurons in which the plasma membrane remains intact, restricting entry of fluorescent dextran (arrows); over-permeabilized neurons in which dextran freely enters into the nucleus (asterisks); and optimally-permeabilized neurons in which dextran surrounds but does not enter the nucleus. Scale bars = 10 μm.

Figure 2: Validation of energy- and importin β-dependent nuclear import of Rango in permeabilized mouse primary cortical neurons. (**A**) Schematic of nuclear import reaction in permeabilized neurons. (**B**) Automated analysis of nuclear to cytoplasmic ratio (N/C) of Rango. Mean ± SEM is shown for n = 3 biological replicates (independent neuronal cultures), each containing two technical replicates (wells) per condition or approximately 400 neurons. (**C**) Confocal images of Rango nuclear import showing requirement for whole cell extract (WCE) and energy regeneration mix (ERM), and import blockade by importazole (IPZ, 100 μM). Confocal acquisition parameters were kept constant across all conditions. Scale bar = 10 μm.

DISCUSSION:

The protocol detailed above provides a reliable and reproducible method for selectively permeabilizing the plasma membrane of primary mouse cortical neurons for nuclear import analysis. Here, an application of the method for nuclear import analysis of a direct importin β cargo (Rango)is shown, but this same approach can be used to analyze the passive and active import of a wide range of fluorescent cargoes. Permeabilization enables precise manipulation of the transport reaction in ways that are not feasible in intact cells, as we recently described for mutant *C9orf72* dipeptide repeat proteins (DPRs) implicated in ALS and FTD[14]. Synthetic DPR proteins were preincubated with transport lysates prior to initiation of the nuclear import reaction, facilitating analysis of concentration- and length-dependence of the nuclear import blockade by arginine-containing DPRs. In fractionation studies, DPR-induced aggregates were included or excluded from the transport lysate to analyze functional consequences for nuclear import. DPRs were also preincubated with nuclei to ascertain effects on nuclear pore permeability. These are a few examples of variations of the assay that can be applied to the study of disordered and aggregation-prone proteins, commonly implicated in neurodegeneration. Since this is an artificial *in vitro* system, the findings should be carefully interpreted within the limitations of the assay.

Success of the protocol critically depends on selective permeabilization of the plasma membrane, to the extent that careful optimization of conditions for each batch of primary neurons is recommended. Importantly, this protocol is specific to primary mouse cortical neurons. Preliminary successful testing with primary mouse spinal motor neurons were also carried out, but caution that the hypotonic permeabilization method is not reliable in human-induced pluripotent stem cell-derived spinal neuron cultures (not shown). If active nuclear import is not observed, despite optimal permeabilization, troubleshooting efforts should focus on ensuring high quality, concentrated WCE and fluorescent cargo preparations. Due to the potential for batch-to-batch variation, it is best to prepare and freeze large stocks of single-use aliquots and avoid comparing results across different batches. The use of automated high content imaging and analysis permits rapid imaging of nuclear import kinetics across hundreds of neurons per well, with minimal investigator input. If high throughput imaging is not available, nuclear import reactions containing Rango and related cargoes are

fixable[14] for alternate imaging and analysis methods. If manual imaging and analysis are pursued, investigators should be blinded to treatment condition to avoid bias.

This method will be broadly applicable to studies of passive and active nuclear transport in primary neurons. As described above, permeabilized cells are a useful model for the study of the effect of disordered and aggregation-prone proteins, commonly implicated in neurodegeneration and increasingly suspected to disrupt nucleocytoplasmic transport[20–22]. Comparison between different cell types may also improve the understanding of potential differences in transport dynamics related to cell-specific differences in nuclear pore complex composition[11,12]. Thus far, no alteration in the concentrations of components was needed in the transport reaction mix for permeabilized neurons versus HeLa cells[14]. However, no formal comparisons were made with the minimal transport requirements or attempted comparisons with permeabilized glia. In addition to varying the permeabilized cell type, utilization of neurons or even CNS tissue as the source of cytoplasmic or whole cell extract, thus varying the source of soluble transport factors, may provide an additional approach for testing disease-relevant questions.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

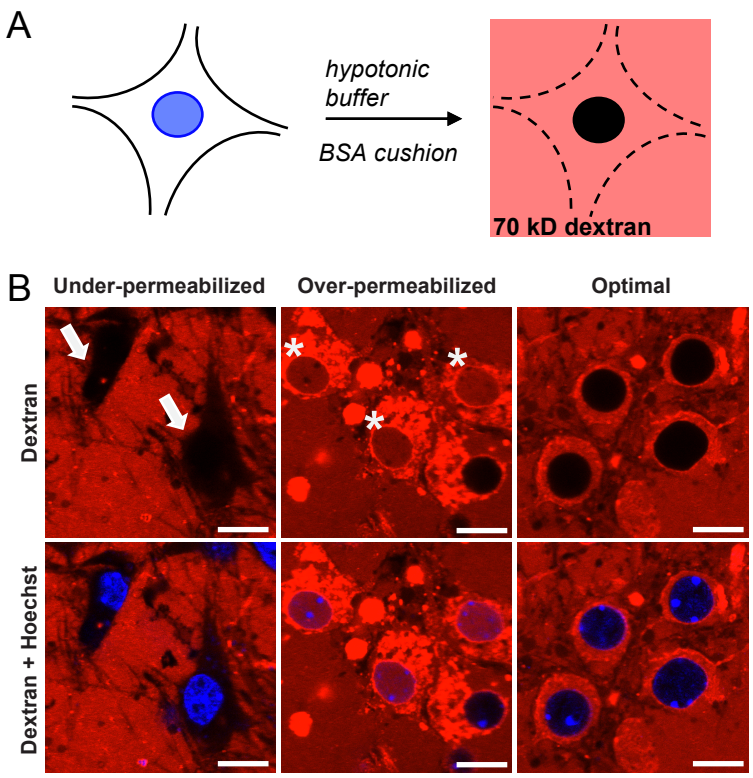
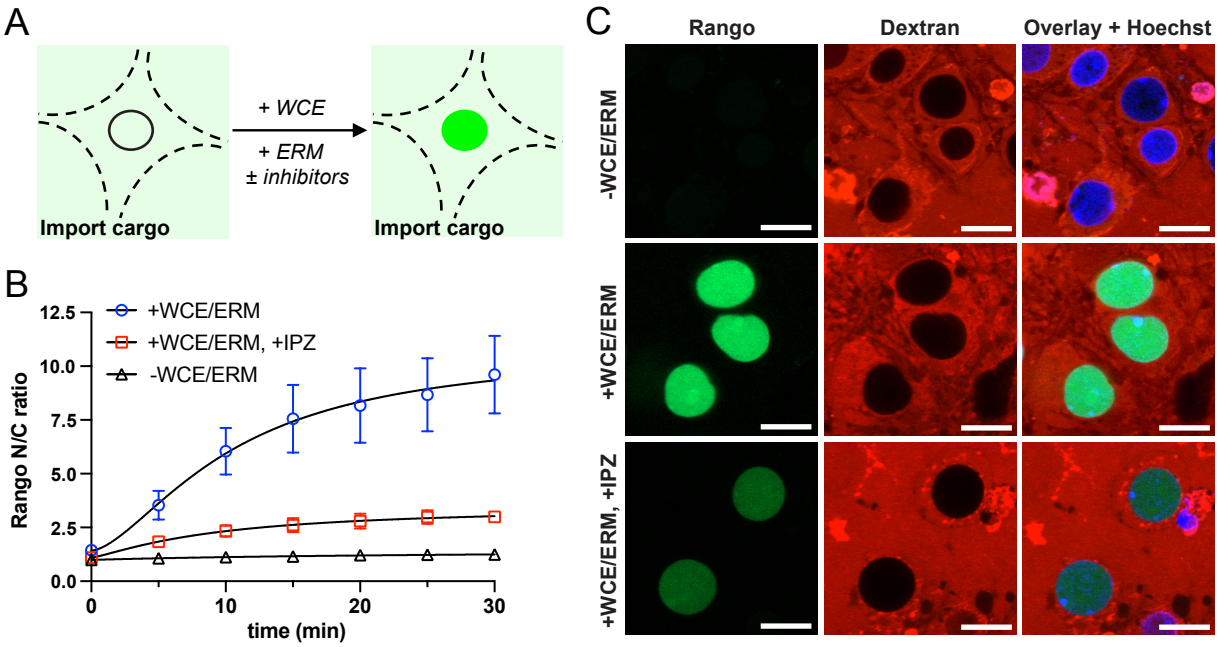


Figure 2



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 M HEPES	Gibco	15630-080	
10x HBSS	Gibco	14185-052	
32% paraformaldehyde	Electron Microscopy Sciences	15714-S	
96-well optical glass plates	CellVis	P96-1.5H-N	
ATP lithium salt	Millipore Sigma	11140965001	
B27	Gibco	17504-044	
Bio-Rad Protein Assay Kit II	Bio-Rad	5000002	
BL21(DE3) *E. coli*	NEB	C2527H	
Bovine serum albumin fraction V, heat shock, fatty acid free	Sigma-Aldrich	3117057001	
Chromatography columns	Bio-Rad	7311550	
Creatine kinase	Millipore Sigma	10127566001	
Creatine phosphate	Millipore Sigma	10621722001	
Dextran, Texas Red, 70,000 MW	Thermo Fisher	D1864	
DNase I	Sigma-Aldrich	DN25	
E15-16 timed pregnant C57BL/6J female mice	Jackson Laboratory	000664	
Excel	Microsoft	N/A	
Fetal bovine serum	Hyclone	SH30070.03	
Glutamax	Gibco	35050-061	
Glycerol	Thermo Fisher	15514011	
GTP lithium salt	Millipore Sigma	11140957001	
HALT protease inhibitor (100x)	Thermo Fisher	78439	
HEK293T cells	ATCC	CRL-3216	
HIS-Select HF Nickel affinity gel	Sigma-Aldrich	HO537	
Hoechst 33342	Thermo Fisher	H1399	
ImageExpress Micro Confocal High-content Imaging System	Molecular Devices	N/A	Used for time-lapse imaging

Imidazole	Millipore	I3386	
Importazole	Sigma-Aldrich	SML0341	
IPTG	Corning	46-102-RF	
Laminin	Sigma-Aldrich	L2020	
LB broth	Grainger	31FZ62	
LSM800 confocal microscope	Zeiss	N/A	Used for dextran imaging
MetaXpress High Content Image Analysis Software	Molecular Devices	N/A	
Neurobasal medium	Gibco	21103	
Papain	Worthington	LS003126	
Penicillin-streptomycin	Gibco	15140-122	
Poly-D-Lysine	Sigma-Aldrich	P6407	
Protease inhibitor cocktail	Millipore Sigma	11873580001	
Rango Plasmid (pRSET Rango2/a1 + linkers)	N/A	N/A	pK44, containing N-terminal 6-His tag
SOC (super optimal broth with catabolite repression) media	Quality Biological	340-031-671	
Sodium pyruvate	Gibco	11360-070	
Spin-X UF concentrators (30K MWCO)	Corning	CLS431484	
Trypsin-EDTA (0.05%)	Gibco	25300054	

Thank you for the detailed and thoughtful review of our manuscript. We have carefully reviewed each comment and have incorporated the changes into the document as described below. We sincerely appreciate the reviewers' and editors' time in helping to improve the manuscript and ensure that it will be useful to our colleagues in the field.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Completed.

2. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Confirmed. All uses of "should" or "could" phrases are within "Note" comments and not within the protocol itself.

3. For water symbol, please use O instead of 0 so H2O and not H20.

This has been verified and edited where needed.

4. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

We have added additional details to multiple steps in the protocol including primary cultures, recombinant protein production, and others as requested by the reviewers and discussed further below.

5. 1.3.1: is only cervical dissection enough for euthanasia- do you perform CO2 asphyxiation? Do you wipe with 70% ethanol? How do you remove embyros?

For our studies, pregnant dams utilized for primary culture studies are euthanized by cervical dislocation alone, in compliance with AVMA guidelines and approved by the JH ACUC. This is the preferred method as there is no exposure of the embryos to an anesthetic agent.

We have added details about ethanol and embryo removal into the manuscript in step 1.3.1.

6. 1.3.4: Please include stock concentration for papain and DNase.

Stock concentrations for these reagents were provided in section 1.1.2: "Dilute papain to 30 mg/mL with dissection buffer. Store at 4 °C. Dilute DNase I to 10 mg/mL with dissection buffer. Aliquot and store at -20 °C." We have now added a reminder of those stock concentrations in step 1.3.3 where they are used, as well as the final concentrations.

7. 2.4.1. How do you perform transformations.

These details have been added to step 2.4.1.

8. For the imaging and analysis steps please include button clicks and knob turns if any.

Our microscope and imaging system is operated entirely by computer interface, so there are no buttons or knobs. All steps are detailed in the protocol.

9. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Excel, Metaexpress, Molecular Devices ImageXpress Micro Confocal, etc.

Thank you for clarifying. We were concerned that the terminology in our image acquisition and analysis section would not make sense to readers without first naming the equipment and software being used. Per request, we have now removed all references to the brand of microscope and software from protocol sections 4-5, but kept the terms from specific menus or commands in the software modules as they seem essential to following the protocol.

We've tried to consistently mark the software terms with a single apostrophe and have kept the note at the beginning of section 4 that any preferred method of analysis can be used. We will defer to the editors' guidance as to whether this is a reasonable/effective approach.

10. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

3 pages including headings and spacing are highlighted in yellow.

11. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Verified as completed, all aspects (a) through (e) are included in our discussion section.

12. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.

Confirmed. For some newer journals, no page numbers exist. In those cases, the DOI has been provided.

Reviewers' comments:
Reviewer #1:

Major Concerns:

1. The Authors did not provide essential background and the recent progress in the measurement of nucleocytoplasmic transport in neurons. For example, the measurement of nucleocytoplasmic transport of both protein and mRNA cargoes in cultured neurons has been reported (PMID: 32317929). In this report, both protein import and export activities could be measured with a dual reporter in both mouse primary neurons and iPSC-derived human neurons. This could be discussed in discussion section how the current method and the previous approaches compensate each other in the measurement of neuronal nucleocytoplasmic transport.

The reviewer is quite correct, there are a wide array of methods for measuring nucleocytoplasmic transport in intact/living cells including neurons. However, since this manuscript focuses on permeabilized cell assays, a detailed discussion of nucleocytoplasmic transport measurement in living neurons is beyond the scope.

To clarify the emphasis on permeabilization, we have added a section to the introduction (lines 61-75) that discusses live cell approaches but clarifies the role of permeabilized cell assays and why one might select this approach.

2. It will be clearer if the authors could provide tables that summarize the recipe of critical buffers used in this protocol, such as 5X transport buffer (TRB), 20x energy regeneration mix, etc.

Tables for the more complicated buffers have now been inserted into the text. We ask the editors' guidance in determining if this is an acceptable means of formatting.

Minor Concerns:
3. Page 5, Line 158, "2.3.1 Grow HEK293T cells to confluence in 15, 15 cm dishes." The first "15" is a typo or want to express 15 of 15 cm dishes?

We routinely grow large batches of 15 dishes (some protocols use more!). Perhaps it will be clearer if we spell out the quantity of fifteen to distinguish it from the measurement and use 150 mm instead of 15 cm. Text now reads "**Grow HEK293T cells to confluence in fifteen 150 mm culture dishes**."

4. In Image Analysis, "5.2 Under compartments, adjust approximate nuclear width, intensity above local background and minimum/maximum area." "5.4 Select desired background estimation method (default of 'Auto Constant' is recommended)". If this method is used to analyze the protein important under different conditions, such as healthy and diseased neurons, the neuronal density, size, morphology, and the nuclear size and morphology could be very different. How to set up these settings during image analysis to avoid any bias and systematically errors? For example, "Adjust settings until the desired population of neuronal nuclei is selected." What percentage of desired population should be covered, 80%, 90% or 95% (based on Hoechst or neuronal markers)? The same thing for other settings. It's better to clearly describe these in protocol or discussed in discussion section.

All good points. We have edited 5.2 to specify "Adjust settings to **maximize the accuracy of neuronal nuclei identification while limiting erroneous inclusion of non-neuronal nuclei or debris**." It is not possible to provide a percentage range for investigators for their purpose, suggesting one might lead to improper "blind" attempts to select too many or too few cells. The parameters must be empirically determined. We added to our note following this step to try to make this as clear as possible:

"**Determination of optimal compartment identification settings are a trade-off between accuracy and cell number and must be set empirically depending on assay, cell type, and culture density. Stringent parameters will omit numerous cells but include fewer errors, while liberal parameters will be more inclusive but contain more errors. If different cell populations (i.e. disease mutants) or treatments are included that may affect**

nuclear size, we recommend setting size/intensity parameters that are broad enough to accommodate all conditions, to avoid the need for multiple parameter sets within an experiment.

We also added a note to reiterate that the analysis stage is where including positive and negative controls within each experiment becomes really critical. That is essential for checking that the analysis parameters are appropriate.

Reviewer #2:

Minor Concerns:
1) The title seems a bit confusing. How about "Nuclear transport assays in permeabilized primary mouse cortical neurons" or similar?

The title has been changed as suggested to clarify any confusion.

2) A flow chart outlining the main steps would be helpful. (Not sure if this is commonly done for JoVE).

Since we already have diagrams of the key transport assay steps in figures 1-2, we have added an introductory statement at the beginning of the protocol (line 99) explaining the order and relationship of the steps:

"First, we will describe the generation of primary neuronal cultures (step 1) and preparation of materials for the transport assay (step 2), followed by the transport assay itself (steps 3-4), and image acquisition and analysis (step 5)."

Hopefully this helps the reader orient effectively.

3) Aside from digitonin mentioned here as a failed approach for neurons, bacterial toxins have been employed as an alternative strategy to form transient pores in the plasma membrane of mammalian cells.

Thank you for the suggestion. Yes, pore-forming bacterial toxins (reviewed here: https://doi.org/10.1038/nrmicro.2015.3) have been shown to perforate the plasma membrane of mammalian cells, although we could not find examples in the literature of their use for subsequent nuclear transport assays, nor did we personally test this approach. Under typical working concentrations, one group found that the functional pore size induced by a subset of these toxins is quite small (<8 kDa), although at very high concentrations, pores could be induced that permitted entry of GFP or other larger proteins (doi: 10.1128/mcb.19.12.8604). We'd be open to including in our discussion but would need to ask the reviewer to provide a suitable reference if possible related to nucleocytoplasmic transport assays, as we were not able to locate one.

4) The main issue is that it is written mainly for experienced nuclear transport assay users, who are familiar with many general principles omitted from the protocol. A brief explanation of the principles of nuclear transport assays at the beginning for non-experts would be helpful. This should include a brief explanation why 70kD fluorescent molecules are being used to demonstrate the integrity of the nuclear envelope.

Basic nuclear transport concepts and recommended reviews have been added to the introduction (paragraph starting at line 55), including the size of the nuclear pore selectivity filter.

5) Some acronyms are confusing and change throughout the article and figures. This includes "concentrated cell extract", otherwise more appropriately (since there is no concentration step) called "lysate" or "L" in the

figure. Why not stick with one term and acronym, e.g. whole cell extract (WCE) throughout the manuscript and figures? Another example is "energy regeneration mix (ER)", called "E" in the figures. Why not avoid the potentially confusing "ER" and use e.g. either "energy regeneration mix (ERM)" or "energy regeneration buffer (ERB)" throughout the manuscript and figures?

We have changed all instances in the text and figures to whole cell extract (WCE) and energy regeneration mix (ERM) as requested.

6) 2.4. "Here, we will describe the expression and transport of Rango, a direct importin beta cargo (10,13). 2.4.1 Transform E. coli BL21(DE3) cells with Rango (pK44)." This is incomprehensible unless you read the associated literature. Rango is not just a cargo. "Cargo" could be a natural cargo protein of importin beta, whereas Rango is an engineered reporter construct/biosensor that increases its fluorescence resonance energy transfer signal when released from importin-beta by RanGTP, and needs to be briefly described as such. "pK44" is perhaps some internal identifier? "

Thank you for this helpful observation. To make this clearer without referring to our previous publications, that section (starting line 202) now reads:

"NOTE: This transport protocol may be adapted for any fluorescent nuclear transport cargo, to interrogate the active or passive nuclear transport pathway of interest. Here, we will describe the expression and nuclear import of Rango ('Ran-regulated importin β cargo'), which consists of the importin β-binding domain of importin α1 flanked by the fluorescent proteins CyPet and YPet[10,13]. Rango is a versatile sensor that can be used for FRET as well as nuclear import assays, where it functions as a direct importin β cargo."

We removed the internal plasmid designator pK44 from the text and instead inserted it along with a description in the spreadsheet of materials.

7) 2.4.4 Incubate lysate with Ni-NTA agarose resin". This would suggest Rango has a 6His tag? Needs more information.

We have added additional description of this step and the previous transformation step as requested above to add clarity.

8) 1.3.3 "Avoid overdigestion." Mentioning signs of overdigestion (perhaps viscous cell suspension due to cell lysis and release of DNA?) would be helpful.

Text has been changed to, "**Digestion beyond 10 min. is detrimental to cell survival**."

9) "In addition to varying the permeabilized cell type, utilization of neurons or even CNS tissue as the source of concentrated lysate, thus…". Concentrated how? See suggestion for using "cell extract" of "lysate" above.

Text in line 475 has been changed to, "**as the source of cytoplasmic or whole cell extract**" to keep the nomenclature consistent throughout the manuscript.