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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol to image and quantify nucleocytoplasmic transport in cultured cells using fluorescent in situ hybridization and a dual reporter system

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SUMMARY
Nucleocytoplasmic transport (NCT) plays critical roles in maintaining cellular homeostasis. Here, we present a protocol to measure NCT for both transcript and protein cargos in cultured cells. We first describe the fluorescent in situ hybridization (FISH) assay to measure the nuclear mRNA export. We then detail a dual reporter system to measure the protein NCT. This protocol also includes image analysis and data output using CellProfiler™. The combined approach can be used to unbiasedly analyze NCT activities in cultured cells. For complete details on the use and execution of this protocol, please refer to Ding et al. (2020, 2021).

BEFORE YOU BEGIN
Institutional permissions

1. Obtain the permission from Institutional Biosafety Committee for working on lentivirus in a BSL2 laboratory. This protocol has been approved and the BSL2 laboratory has been certified by the Institutional Biosafety Committee at LSU Health Sciences Center at Shreveport (Project number: B22-008).
2. Obtain the permission from institutional animal care and use committee (IACUC) for the animal study. This protocol has been approved by IACUC at LSU Health Sciences Center at Shreveport (Proposal number: P-22-037).

Preparation of research materials

3. Acquire all critical reagents mentioned in the key resources table.
4. Acquire cell lines to be used in this protocol and prepare enough frozen stocks for the future research.
5. Acquire and prepare the high-quality lentiviral vectors to be used in this protocol.
6. Prepare media using recipes described in this protocol.

△ CRITICAL: All procedures are performed in a BSL-2 certified laboratory equipped with biosafety cabinet using standard aseptic techniques. Cultures are grown and maintained in a humidified incubator at 37°C with 5% CO₂.
## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Sheep anti-DIG, polyclonal (1:100) | MilliporeSigma | Cat# 1133089001, RRID: AB_514496 |
| FITC conjugated Donkey anti-Sheep, polyclonal (1:200) | Abcam | Cat# ab6896, RRID: AB_955329 |
| Rabbit anti-FITC, polyclonal (1:700) | Thermo Fisher Scientific | Cat# 71-1900, RRID: AB_2533978 |
| Mouse anti-FITC, polyclonal (1:700) | Thermo Fisher Scientific | Cat# MA5-14696, RRID: AB_627695 |
| Mouse anti-GFP, monoclonal (1:200) | Santa Cruz Biotechnology | Cat# sc-9996, RRID: AB_627695 |
| Chicken anti-MAP2, polyclonal (1:10,000) | BioLegend | Cat# 822501, RRID: AB_2564858 |
| Mouse anti-TUBB3 (1:3000) | Covance | Cat# MM5-435P, RRID: AB_2313773 |
| Rabbit anti-TUBB3 (1:3000) | Covance | Cat# PRB-435P, RRID: AB_291637 |
| Mouse anti-LMNA/C (1:500) | Abcam | Cat# ab40567, RRID: AB_775967 |
| Alexa Fluor® 488- Donkey anti-Mouse IgG (1:250) | Jackson ImmunoResearch | Cat# 715-545-150, RRID: AB_2340846 |
| Alexa Fluor® 488- Donkey anti-Rabbit IgG (1:250) | Jackson ImmunoResearch | Cat# 711-545-152, RRID: AB_2313584 |
| Alexa Fluor® 594- Donkey anti-Rabbit IgG (1:250) | Jackson ImmunoResearch | Cat# 711-585-152, RRID: AB_2340621 |
| Alexa Fluor® 594- Donkey anti-Chicken IgG (1:250) | Jackson ImmunoResearch | Cat# 703-585-155, RRID: AB_2340377 |
| Alexa Fluor® 568- Donkey anti-Mouse IgG (1:250) | Thermo Fisher Scientific | Cat# A10037, RRID: AB_2534013 |
| **Bacterial and virus strains** |        |            |
| One Shot™ Stbl3™ Chemically Competent E. coli | Invitrogen | Cat# C737303 |
| **Experimental models: Cell lines** |        |            |
| Human embryonic kidney (HEK) 293T cells (passage number <20) | American Type Culture Collection (ATCC) | ATCC Cat# CRL-3126, RRID: CVCL_0063 |
| Human fibroblast cells (passage number <15) | Coriell Institute For Medical Research | GM03211, RRID: CVCL_0063; GM03204, RRID: CVCL_0063; GM00024, RRID: CVCL_7269; Coriell Cat# GM03652, RRID: CVCL_7397; GM04506, RRID: CVCL_7413; AG07473, RRID: CVCL_2C33 |
| Reprogrammed human neurons | (Ding et al., 2020, 2021; Sepehrimanesh et al., 2021; Sepehrimanesh and Ding, 2020) | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Dulbecco’s Modified Eagle Medium (DMEM) | Gibco | Cat# 11995065 |
| Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (DMEM-F12) | HyClone | Cat# SH30023.02 |
| Neurobasal Media | Thermo Fisher Scientific | Cat# 21103049 |
| Fetal bovine serum (FBS) | Gibco | Cat# 16140071 |
| Penicillin/Streptomycin (Pen/Strep) | Thermo Fisher Scientific | Cat# 15140122 |
| N2 supplement (100X) | Thermo Fisher Scientific | Cat# 17502-048 |
| B27 supplement (50X) | Thermo Fisher Scientific | Cat# 17504-044 |
| Rho kinase (ROCK) inhibitor (Y-27632), 10 mM | MilliporeSigma | Cat# 129830-38-2 |
| MEM Non-Essential Amino Acids Solution (NEAA) | Thermo Fisher Scientific | Cat# 11400050 |
| Glutamax (100X) | Thermo Fisher Scientific | Cat# 35050 |
| Brain Derived Neurotrophic Factor (BDNF) | PeproTech | Cat# 450-02 |
| Glial Derived Neurotrophic Factor (GDNF) | PeproTech | Cat# 450-10 |
| Neurotrophic factor 3 (NT3) | PeproTech | Cat# 450-03 |
| Heat Stable Recombinant Human Basic Fibroblast Growth Factor (bFGF) | Gibco | Cat# PHG0367 |
| Human EGF recombinant protein | PeproTech | Cat# PHG0311L |
| 2-Mercaptoethanol (b-ME) | Thermo Fisher Scientific | Cat# 21985023 |

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### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Forskolin (FSK)     | MilliporeSigma | Cat# F6886 |
| Matrigel Matrix     | Corning | Cat356234  |
| Gelatin             | Thermo Fisher Scientific | Cat# G7-500 |
| Trypsin             | Thermo Fisher Scientific | Cat# 25200056 |
| Nuclease-Free Water* | Thermo Fisher Scientific | Cat# AM9930 |
| Ambion™ Recombinant RNase A* | Fisher | Cat# AM2269 |
| Yeast tRNA*         | Thermo Fisher Scientific | Cat# AM7119 |
| Hoechst 33342 (HST)* | Thermo Fisher Scientific | Cat# 62249 |
| Fisher Bioreagents™ Bovine serum albumin (BSA) DNase- and Protease-free Powder* | Thermo Fisher Scientific | Cat# BP9706100 |
| Digoxigenin (DIG)-labeled oligo-dT (30 nt)* | Synthesized at IDT | N/A |
| Digoxigenin (DIG)-labeled oligo-dA (30 nt)* | Synthesized at IDT | N/A |
| Triton X-100 (TX-100)* | Thermo Fisher Scientific | Cat# 85111 |
| Paraformaldehyde (PFA)* | Sigma-Aldrich | Cat# 158127 |
| Formamide* | Sigma-Aldrich | Cat# FX0420-6 |
| Ribonucleoside-vanadyl complex (RVC)* | New England Biolabs | Cat# S14025 |
| Sodium citrate* | Thermo Fisher Scientific | Cat# BP327-1 |
| Dextran sulfate* | Sigma-Aldrich | Cat# D-8906 |
| ProLong Diamond Antifade Mountant* | Thermo Fisher Scientific | Cat# P36970 |
| LB Broth | Thermo Fisher Scientific | Cat# 611875000 |
| Ampicillin | Thermo Fisher Scientific | Cat# BP1760-5 |
| Ethidium bromide solution | Roth | Cat# 2218.2 |
| Polyethyleneimine (PEI) | Polysciences | Cat# 23966-2 |
| Leptomycin B (LMB) | Alfa Aesar | Cat# J63784 |
| psPAX2 | Addgene | RRID: Addgene_12259 |
| pCMV-VSV-G | Addgene (Stewart et al., 2003) | RRID: Addgene_8454 |
| pLVX-EF1alpha-2×GFP.NES-IRES-2×RFP.NLS (2G2R dual reporter) | Addgene (Mertens et al., 2015) | RRID: Addgene_71396 |

### Critical commercial assays

| Plasmid DNA Midiprep Kits | Fisher | Cat# K0481 |
| DNA gel loading dye, 6× | Thermo Scientific™ | Cat# R0611 |
| GeneRuler 1 kb DNA ladder | Thermo Scientific™ | Cat# SM0312 |
| DNAse/RNase-free water | Invitrogen | Cat# 10977049 |

### Software and algorithms

| ImageJ | NIH (Schneider et al., 2012) | ImageJ RRID: SCR_003070 |
| CellProfiler | Broad Institute of MIT and Harvard | CellProfiler RRID: SCR_007358 |

### Other

| Laminar Culture Hood | Thermo Fisher Scientific | Cat# SHKE435HP |
| CO₂ Incubator | VWR | Cat# 10810-888 |
| Shaker incubator | Thermo Fisher Scientific | Cat# SHKE436HP |
| Microbiological Incubator | Thermo Fisher Scientific | Cat# 50125590 |
| Centrifuge 5420 | VWR | Cat# 76404-386 |
| Centrifuge 5418R | VWR | Cat# 97058-928 |
| Centrifuge MEGAFUG | VWR | Cat# 89511-734 |
| NanoDrop | Thermo Fisher Scientific | Cat# ND-2000 |
| ChemiDoc™ Touch Imaging System | Bio-Rad | Cat# 1708370 |
| Freezer – 20°C | Fisher Scientific | FBV20FPSA |
| Freezer – 80°C | VWR | Cat# 76318-814 |
| 100 mm tissue culture disc | Thermo Scientific | Cat# 130182 |
| Microscope slides | Fisher Scientific | Cat# 12-550-15 |
| 6-well plate | Corning | Cat# 490007-412 |
| 12-well plate | Corning | Cat# 83-3336 |
| 24-well cell culture plates | Thermo Fisher Scientific | Cat# 142485 |
| Fisherbrand™ Petri Dishes | Thermo Fisher Scientific | Cat# FB0875713 |

(Continued on next page)
CRITICAL: Adjust pH to 5.0 with citric acid and make volume up to 1,000 mL with nuclease-free water. The 20× SSC solution should be stored at RT for up to 2 years. For making 4×, 2×, and 1× SSC, just dilute 20× SSC with nuclease-free water as 1:5, 1:10, and 1:20, respectively.
△ CRITICAL: Aliquot and store stocks of formamide in −80°C for up to 2 years. If it is liquefied at −20°C, deionization is required. Use a single stock aliquot to prepare 20% formamide solution freshly in the day of use.

**Note:** Formamide is carcinogenic and need to wear protective clothing and mask.

### Hybridization buffer (2×HB in 4×SSC)

| Reagents                          | Final concentration | Amount |
|----------------------------------|---------------------|--------|
| Dextran sulphate                 | 120 mM              | 2 g    |
| Ribonucleoside Vanadyl Complex (RVC) | 20 mM               | 1 mL   |
| 20× SSC                          | 4×                  | 2 mL   |
| Nuclease-free water              | N/A                 | ~ 7 mL |
| Total                            | N/A                 | 10 mL  |

△ CRITICAL: Use a 50 mL conical tube to add 2 g of dextran sulfate and then add 5 mL of nuclease-free water slowly. Heat the mixture in a 37°C water bath for 30 min. Add 2 mL of 20× SSC and then 1 mL of RVC and continue incubation in a 37°C water bath to become light gray. Then make volume up to 10 mL with nuclease-free water. Store at −80°C for up to 1 year.

**Note:** RVC is irritating. Users need to wear safety goggles and protect skin and body by wearing appropriate protective cloths.

### 1× HB without probe

| Reagents                          | Final concentration | Amount |
|----------------------------------|---------------------|--------|
| 2× HB                            | 1×                  | 10 mL  |
| Formamide                        | 20%                 | 4 mL   |
| Nuclease free water              | N/A                 | ~ 6 mL |
| Total                            | N/A                 | 20 mL  |

**Note:** This solution is used to briefly equilibrate samples before hybridization with probes. It needs to be made fresh.

### 1× HB with probe (Oligo-dT working solution)

| Reagents                          | Final concentration | Amount |
|----------------------------------|---------------------|--------|
| 2× HB                            | 1×                  | 10 mL  |
| Formamide                        | 20%                 | 4 mL   |
| Oligo-dT (1000×)                  | 1×                  | 20 μL  |
| Nuclease free water              | N/A                 | ~ 6 mL |
| Total                            | N/A                 | 20 mL  |

△ CRITICAL: Heat a 50 mL conical tube contain ~6 mL nuclease-free water and 4 mL of formamide at 100°C, indirectly. After adding oligo-dT, heat for 10 min at 100°C and then put on ice. After cooling, add 10 mL of 2×HB and aliquot and store in −80°C for up to 1 year.

**Note:** Formamide is carcinogenic and need to wear protective clothing and mask.
CRITICAL: After adding RVC to 4× SSC, heat the solution in in 37°C water bath till gray. Then add antibody. Scale up and down according to samples. Recycled Sheep anti-DIG can be stored in −80°C for up to 1 year and reused directly but do not reuse more than 3 times because the signals will be gradually diminished.

Note: RVC is irritating and need to wear safety goggles and protect skin and body by wearing appropriate protective cloths.

### Sheep anti-DIG solution

| Reagents                                    | Final concentration | Amount  |
|---------------------------------------------|---------------------|---------|
| Ribonucleoside Vanadyl Complex (RVC)        | N/A                 | 80 μL   |
| 4× SSC                                      | N/A                 | 800 μL  |
| Sheep anti-DIG, polyclonal (1:100)          | N/A                 | 20 μL   |
| **Total**                                   | N/A                 | 900 μL  |

△ CRITICAL: It is better to prepare freshly. You can use this as blocking buffer and diluting solution for primary and secondary antibodies.

### Blocking buffer

| Reagents          | Final concentration | Amount  |
|-------------------|---------------------|---------|
| BSA               | 30 mg/mL            | 300 mg  |
| Triton X-100      | 0.1%                | 10 μL   |
| 1× PBS            | N/A                 | ~10 mL  |
| **Total**         | N/A                 | 10 mL   |

△ CRITICAL: Always prepare freshly before use. Store complete medium at 2°C–8°C for up to 2 weeks.

### HEK293T culture medium

| Reagents                        | Final concentration | Amount  |
|---------------------------------|---------------------|---------|
| Dulbecco’s Modified Eagle Medium (DMEM) | N/A              | 45 mL   |
| Fetal bovine serum (FBS)        | 10%                 | 5 mL    |
| Pen/Strep (100x)                | 1×                  | 500 μL  |
| **Total**                      | N/A                 | ~50 mL  |

△ CRITICAL: Always prepare freshly before use. Store complete medium at 2°C–8°C for up to 2 weeks.

### Neuronal maturation medium

| Reagents          | Final concentration | Amount  |
|-------------------|---------------------|---------|
| DMEM:F12          | N/A                 | 32 mL   |
| Neurobasal        | N/A                 | 16 mL   |
| Pen/Strep (100x)  | 1×                  | 500 μL  |
| N2 (100x)         | 1×                  | 500 μL  |
| B27 (50x)         | 1×                  | 1 mL    |
| FSK (20 mM)       | 5 μM                | 12.5 μL |
| BDNF (20 μg/mL)   | 10 ng/mL            | 25 μL   |
| GDNF (20 μg/mL)   | 10 ng/mL            | 25 μL   |
| NT3 (20 μg/mL)    | 10 ng/mL            | 25 μL   |
| **Total**         | N/A                 | ~50 mL  |

△ CRITICAL: Always prepare freshly before use. Store complete medium at 2°C–8°C for up to 2 weeks.
CRITICAL: Always prepare freshly before use. Store complete medium at 2°C–8°C for up to 2 weeks.

**Neural progenitor cell medium (NPC Medium)**

| Reagents                  | Final concentration | Amount |
|---------------------------|---------------------|--------|
| DMEM:F12                  | N/A                 | 32 mL  |
| Neurobasal                | N/A                 | 16 mL  |
| Pen/Strep (100×)          | 1×                  | 500 μL |
| N2 (100×)                 | 1×                  | 500 μL |
| B27 (50×)                 | 1×                  | 1 mL   |
| Glutamax (100×)           | 1×                  | 500 μL |
| NEAA (100×)               | 1×                  | 500 μL |
| b-ME (50 mM, 1000×)       | 50 μM               | 50 μL  |
| bFGF (200 μg/mL)          | 10 ng/mL            | 2.5 μL |
| EGF (100 μg/mL)           | 10 ng/mL            | 5 μL   |
| Rock inhibitor (Y-27632, 10 mM) | 10 μM         | 50 μL  |
| **Total**                 | N/A                 | ~50 mL |

△ CRITICAL: Always prepare freshly. The medium is ready to add to cells.

**Leptomycin B stock**

| Reagents                  | Final concentration | Amount |
|---------------------------|---------------------|--------|
| HEK293T culture medium    | N/A                 | 50 mL  |
| Leptomycin B (1 mM)       | 50 nM               | 2.5 μL |
| **Total**                 | N/A                 | ~50 mL |

△ CRITICAL: Always prepare freshly. The medium is ready to add to cells.

**PEI solution stock**

| Reagents                  | Final concentration | Amount |
|---------------------------|---------------------|--------|
| PEI                       | 1 μg/μL             | 50 mg  |
| MilliQ water              | N/A                 | 50 mL  |
| **Total**                 | N/A                 | ~50 mL |

△ CRITICAL: To facilitate dissolving PEI, use autoclaved nuclease-free water with pH ~2 (adjusted with HCl). Stir until all the PEI dissolved, adjust pH to 7 using 2 N NaOH. Filter the solution through a 0.22 μm filter. Aliquot 1 mL of PEI solution into each 1.5 mL microtubes. Store them in −80°C for up to 2 years.

**Note:** Inhalation of PEI may cause irritation in respiratory tract. Use a fume hood.

**Transfection mixture**

| Reagents                  | Final concentration | Amount |
|---------------------------|---------------------|--------|
| DMEM                      | N/A                 | 10 mL  |
| PEI stock solution (1 μg/μL) | 4 μg/mL         | 40 μL  |
| 2Gi2R dual reporter       | 1 μg/mL             | 10 μL  |
| **Total**                 | N/A                 | ~10 mL |

△ CRITICAL: Add PEI at last step, and mix it well by vortexing or quickly pipetting. Scale up and down according to the total amount of cells to be transfected.
STEP-BY-STEP METHOD DETAILS

This protocol starts with cultured cells and focuses on three sections, 1) the fluorescent in situ hybridization (FISH) assay to measure the nuclear mRNA export, 2) a dual reporter system to measure the protein NCT, and 3) image analysis and data output using CellProfiler/C228 Software (Figure 1). For details on cell culture and reprogramming of human neurons, please refer to our previous publications (Akter et al., 2022; Ding et al., 2020, 2021; Sepehrimanesh et al., 2021; Sepehrimanesh and Ding, 2020).

Section 1: Nuclear mRNA export measurement by FISH assay

© Timing: 3 days

This section describes the protocol of measuring nuclear mRNA export using the FISH assay with oligo-dT probes, which specifically bind the poly(A) tails of mRNAs. Importantly, the FISH assay could be combined with immunocytochemistry (ICC) for detecting specific markers, which will be used to define the nucleus and the cytoplasm of target cells. This experiment consists of three major steps. 1) Cultured cells on coverslips are fixed and then hybridized with Digoxigenin (DIG)-labeled oligo-dT probes. 2) Following the standard ICC, mRNA signals are to be detected using the anti-DIG antibody, and the nucleus and/or cell identity markers will be co-stained with other compatible antibodies or dyes. 3) Based on confocal images, the subcellular distributions of nuclear and cytoplasmic fractions for total mRNAs will be analyzed, and the nuclear mRNA export activity will be evaluated by the ratio of the oligo-dT signal in the nucleus to the cytoplasm. The higher ratio of dT\textsubscript{Nuc}/dT\textsubscript{Cyt} indicates compromised nuclear mRNA export.

Day 1. In situ hybridization

© Timing: 2–4 h

In this step, DIG-labeled oligo-dT is used to probe mRNAs. To detect specific transcripts, the probes could be designed with the complementary sequence to the target RNAs and labeled with DIG. The following procedure is described based on cultured cells on coverslips in a 24-well plate.

1. Set up in situ hybridization to detect mRNAs in cultured cells.
a. Remove the culture media and briefly wash once with ~250 μL per well of 1× PBS.
b. Add 250 μL of 4% paraformaldehyde (PFA) per well and fix the cells at room temperature (RT) for 30 min.

**Note:** The volume of 250 μL is based on the 24-well plate. Please adjust the volume if you use different culture plates or containers to perform this assay.

**Note:** If samples cannot be processed immediately, replace PFA with 500 μL per well of 70% ethanol (diluted with nuclease-free water). Seal the plate well to avoid drying out. It is not harmful to keep coverslips at −20°C for a few weeks without an obvious decrease in oligo-dT signals. Once you are ready, continue the process from step c).

c. Remove PFA (or alternatively 70% ethanol from step b), wash with 1× PBS three times, each time 250 μL per well for 5 min.
d. For each well, add 250 μL of 1× HB without probe and incubate for 3 min at 37°C to equilibrate samples.
e. Remove 1× HB without probe, add 250 μL of hybridization solution (1× HB with probe) that contains oligo-dT (or oligo-dA for negative control), yeast tRNA (to minimize the non-specific binding), and 20% formamide in 1× HB.
f. Incubate overnight (10–16 h) at 37°C.

**Note:** It is better to keep plates in a wet chamber during incubation to avoid drying out.

### Day 2. ICC to detect probes and specific cellular markers

**Timing:** 8–10 h

In this step, antibodies are added sequentially and washed to detect oligo-dT probes. Some steps need to be performed at 37°C and others are performed at RT. The use of fresh buffer and high-quality antibodies in this step is very important.

2. To detect probe and cellular markers.
   a. Thaw a formamide aliquot from −80°C to prepare a proper volume of wash buffer containing 20% formamide in 2× SSC.
   b. Remove hybridization solution.

   **Note:** Hybridization solution can be reused for up to 3 times.

c. Add 250 μL of 2× SSC containing 20% formamide and incubate for 15 min at 37°C.

   **Note:** For each wash step, carefully add/remove liquid from the edge of a well to avoid detaching cells from coverslips.

d. Wash with 250 μL of 2× SSC per well for 15 min at 37°C.
e. Remove 2× SSC and wash with 250 μL of 1× SSC per well for 15 min at 37°C.
f. Prepare antibody mixture containing Sheep anti-DIG and RVC in 4× SSC.
g. Remove 1× SSC, add 200 μL of Sheep anti-DIG solution (1:100) and incubate for 2 h at 37°C.
h. Remove (and alternatively recycle) the Sheep anti-DIG solution.

   **Note:** After this step, the 37°C incubator is not required, and all the following steps will be performed at RT.
i. Wash with 250 μL of 4x SSC per well for 10 min at RT.

j. Remove 4x SSC and wash with 250 μL of 4x SSC containing 0.1% Triton X-100 for 10 min at RT.

k. Wash again with 250 μL of 4x SSC per well for 10 min at RT.

l. Remove 4x SSC and fix cells with 250 μL of 4% PFA per well for 10 min at RT.

m. Remove fixing buffer and wash with 250 μL of freshly prepared permeabilization buffer 1x PBS containing 0.1% Triton X-100 (0.1% PBS-T) for 10 min at RT.

**Note:** The following steps are the same as the standard ICC protocol (Ding et al., 2013, 2016, 2018).

n. Remove permeabilization buffer and wash with 250 μL of 1x PBS for 10 min at RT.

o. Remove 1x PBS and add the FITC-labeled Donkey anti-Sheep antibody (1:200) and incubate for 2 h at RT.

**Note:** The FITC-labeled antibody is light sensitive and thus samples need to be kept from light in the following steps, e.g., by covering a plate with an aluminum foil.

p. Remove the antibody solution and wash with 1x PBS three times at RT, 10 min for each time.

**Note:** After this step, coverslips could be mounted on a glass slide and oligo-dT signals could be visualized based on FITC. Since the FITC signal is usually weak, perform the following steps to amplify the signal:

q. Remove 1x PBS and incubate samples with 250 μL of blocking buffer (3% BSA in 1x PBS) for 10 min at RT.

r. Add Rabbit anti-FITC antibody (1:700) and Chicken anti-MAP2 (1:10,000), and incubate overnight (10–16 h) at 4°C.

**Note:** Antibodies should be diluted in 3% BSA in 0.1% PBST to minimize non-specific binding.

**Note:** Use different combinations of antibodies for specific samples to distinguish mRNA signals and other protein markers with different colors. If you will use a new secondary antibody that the specificity has not been verified, you need to set up a secondary antibody control.

---

**Day 3. mount coverslips and visualization**

© Timing: 4–6 h

3. This step describes how to visualize mRNA probed with a primary antibody using fluorescent conjugated secondary antibodies. Using secondary antibodies with different colors to distinguish the oligo-dT signals and specific protein markers, imaging is recommended to be performed under a confocal microscope.

a. Remove primary antibodies and wash four times with 250 μL of 1x PBS at RT, each time for 10 min.

b. Remove 1x PBS of the last wash, add corresponding secondary antibodies, and incubate for 2 h at RT in dark. Here, we used AF488 Donkey anti-Rabbit (1:250) and AF594 Donkey anti-Chicken (1:250).

**Note:** Antibodies should be diluted in 3% BSA in 0.1% PBS-T to minimize non-specific binding.
Note: Usually, we use Alexa Fluor 488 for dT signals since it is brighter. If samples contain a GFP reporter, then use Alexa Fluor 594 for dT signals.

c. Remove the secondary antibody solution and wash three times with 250 µL of 1× PBS at RT, and each time for 10 min.
d. Remove 1× PBS from the last wash. Add the 250 µL of nuclei dye HST or DAPI (1 µg/mL in PBS) and incubate for 5 min at RT.
e. Remove HST solution and wash with 250 µL of 1× PBS for 2 min at RT.
f. Briefly wash with 250 µL of nuclease-free water for 1 min at RT.
g. Air dry coverslips at RT (in dark) for 20 min or use a paper towel to carefully remove liquid from the edges of coverslips.
h. Mount coverslips on slides with a mounting medium. Allow mounting medium to be fully hardened at RT (about 30 min to a few hours).

Note: To preserve fluorescent signals, the slides should be kept at 4°C for short-term storage or at ~20°C for long-term storage. If the mounting medium is not fully hardened, the cells could be detached from coverslip when visualizing samples.

i. Visualize the FISH samples under a confocal microscope.

Note: In this protocol, we used the Leica DMI 6000 CS Inverted Confocal Microscope with motorized stage, condenser, objective, and filter turrets. The objectives were used are 40x air lens (HCX PL APO CS 40x/0.75U-V-I 0.17) or 63x oil lens (HCX PL APO CS 63x/1.4–0.6NA) and the resolution of images is 1024 × 1024 pixels. Any equivalent confocal microscope with similar settings should be good to produce confocal images for later quantification analysis.

j. Confocal image analysis (see section 3: image analysis using CellProfiler software of this protocol).

Section 2: Protein NCT measurement by a dual reporter system

© Timing: 4 weeks

In this section, we will describe the procedure to examine protein nuclear transport using a dual reporter system, in which GFP-NES and RFP-NLS are co-expressed (Mertens et al., 2015). Therefore, both protein export and import activities can be analyzed in individual cells. Direct transfection of a reporter plasmid could be employed for rapid-growing cells, such as HEK cells. For slow-growing postmitotic cells, such as primary and reprogrammed neurons, it is required to prepare appropriate lentivirus to deliver the reporter vector. To achieve high transduction efficiency and the proper expression of the dual reporter, it is critical to prepare a high-quality lentivirus.

This part consists of five major steps. 1) The dual reporter plasmid preparation, including the transformation of a plasmid into Stbl3 competent cells and plasmid midi preparation. 2) The validation of the dual reporter system in HEK cells with the treatment of Leptomycin B (LMB), a specific nuclear export inhibitor (Jang et al., 2003). 3) The dual reporter lentivirus preparation. This dual reporter system was constructed with the second-generation lentiviral vector. 4) Transduction of the dual reporter lentivirus to fibroblast cells or cultured neurons. 5) Dual reporter analysis. Depending on cell subtypes and the developmental stages to be analyzed, infected cells could be visualized at 1–4 wpi (weeks post-viral infection). Once the reporter system has been validated, only repeating steps 3–5 to measure the protein transport in cultured cells are needed.
Preparation of a lentiviral vector

© Timing: 3–5 h

4. This step describes the purification of a lentiviral vector via plasmid Midi prep columns.
   a. Transform a lentiviral vector to Stbl3™ chemical competent cells (or equivalent) and culture them in 50 mL of LB medium with 100 μg/mL Ampicillin.

   Note: For lentiviral plasmid preparation, the culture temperature between 30°C–35°C is better than 37°C in the maintenance of plasmid stability. The culture time to reach saturation varies according to the temperature, from overnight (10–16 h) to 30 h.

   b. The next morning, collect the bacteria at 1,000 g for 5 min.
   c. Purify the plasmid via Midi prep column according to the manufacturer’s instructions. For detailed information, please see User Guide: Plasmid Midiprep Kit (thermofisher.com).
   d. Examine the plasmid concentration by using a NanoDrop and evaluate the plasmid quality by running an agarose gel.

   Note: A good yield of a plasmid midi prep is no less than 100 μg from a 50 mL culture. The quality of newly purified plasmids could be evaluated by the ratio of the absorbance at 260 and 280 nm (A260/280 ≥ 1.8) and by running an agarose gel. The desired plasmid DNA is the supercoiled form, which migrates faster than predicted (based on size) in an agarose gel due to its conformation. The major band on the agarose gel should be the supercoiled form.

The validation of the reporter system

© Timing: 7 days

5. In this step, the dual reporter system is validated in HEK cells with the treatment of Leptomycin B (LMB), a specific nuclear export inhibitor (Jang et al., 2003). In the presence of LMB, nuclear export of GFP-NES will be disrupted, while the distribution of RFP-NLS won’t be influenced.
   a. Culture HEK cells in DMEM containing 10% FBS and 1× Pen/Strep in a CO2 incubator at 37°C.
   b. When cells reach about 80% confluency (~2 or 3 days), collect cells by trypsinization with 0.05% Trypsin in PBS.
   c. Seed HEK cells onto gelatin coated-coverslips in a 12-well plate at the cell density of about 5 × 10⁴ cells/cm². After seeding, shake a plate well to reach homogenous cell density.

   Note: For coating coverslips, incubate coverslips with 0.2% gelatin at 37°C for more than 30 min.

   d. The next day, HEK cells were transfected with reporter plasmid using PEI solution. For each well of the 12-well plate, add 1 μg plasmid and 4 μg PEI to 50 μL of serum-free DMEM.

   Note: It is better to prepare the transfection mixture for all wells together.

   e. Immediately mix the transfection mixture after the addition of PEI by either vortexing for 15 s or quickly pipetting up and down five times.
   f. Incubate the transfection mixture for 15 min at RT.
   g. Add 50 μL of transfection mixture to each well of the 12-well plate and put it back into a CO2 incubator.
   h. The next day, change the whole volume of HEK culture medium (1 mL/well).
Note: 48–72 h post-transfection, distinct GFP and RFP signals can be noticed under a fluorescent microscope. Treat cells with Leptomycin B (LMB) as follows to validate the dual reporter system:

i. Prepare a culture medium that contains 50 nM LMB.

j. Fully change the culture medium containing 50 nM LMB (1 mL/well).

k. Take coverslips at 0-, 30-, 60- and 90-min post-exposure to LMB and directly mount them on slides.

l. Visualize the cells by confocal microscope.

m. Confocal micrographs will be analyzed by CellProfiler software as described in section 3: image analysis using CellProfiler software of this protocol.

Note: The effectiveness of the dual reporter system can be demonstrated by the gradual accumulation of nuclear GFP signal, while the distribution of RFP will not be influenced.

Lentivirus preparation

© Timing: 4 days

6. Preparation of lentivirus using HEK293T cells.

Note: To examine the protein transport in post-mitotic cells such as cultured neurons, preparation of lentivirus expressing the dual reporters is necessary. This dual reporter system was constructed with the second-generation lentiviral vector. Two extra helper plasmids (psPAX2 and pCMV-VSVG) are required for the assembly of the viral particles. For more information about lentivirus preparation using the second-generation system, please refer to our previous report (Ding and Kilpatrick, 2013).

a. Culture HEK293T cells as described in step 5.

b. For transfection, seed about $8 \times 10^6$ HEK cells in each 10 cm plate in a 10 mL HEK culture medium.

Note: The next day, HEK cells should be around 80% confluency before transfection.

c. Prepare the lentivirus transfection mixture in a 15 mL conical tube according to the recipe.

d. For each 10 cm plate, add 15 µg of a dual reporter plasmid, 10 µg of psPAX2 and 5 µg of pCMV-VSVG to 1 mL serum-free DMEM and mix well.

Note: Scale up if more than one plate will be prepared.

e. Add 90 µg of PEI (90 µL of 1 µg/µL PEI stock) and immediately mix by vortexing for 15 s.

f. Leave the transfection mixture for 15 min at RT.

g. Gently add 1 mL transfection mixture to each 10 cm plate and mix well to distribute it evenly.

h. Incubate cells in a 37°C incubator.

i. After overnight (~16 h) exposure to transfection reagents, fully change the HEK culture medium.

j. Collect viral supernatant at 24 h post medium change and add back 10 mL of the HEK culture medium to each plate. Put the plates back into the incubator and store the viral supernatant at 4°C.

k. After another 24 h, collect the viral supernatant for the second time and discard the plate in a biohazard bag.
**Note:** All lentivirus-contaminated materials (e.g., plates and pipettes) will be trashed in a container labeled with biohazard and processed according to the institutional biosafety protocol. Any spill of the lentivirus in the biosafety hood or other devices needs to be immediately wiped with 70% ethanol or 10% bleach.

I. Pool these two collections together. Filter the viral supernatants with 0.45 μm vacuum filters.

m. Store viral supernatants at 4°C until the immediate use within two weeks or freeze them at −80°C for long-term storage.

**Note:** The titer of the virus could be estimated by the transduction of HEK cells with a series of dilutions of the virus as previously reported (Ding and Kilpatrick, 2013). A good titer of lentiviral supernatants should be no less than $1 \times 10^6$ IFU/mL. (IFU, Infectious Unit).

**Transduction of the lentivirus to cultured cells**

© Timing: 2 days

7. This step describes the transduction of the dual reporter lentivirus to human fibroblast cells or cultured neurons.

△ CRITICAL: To achieve a reasonable infection efficiency, the dosage of the virus should be optimized to transduce different cell types. Too much virus may cause more cell death.

**Note:** The transduction efficiency and the cell survival should be well balanced. Generally, about 60% transduction efficiency is good enough to examine the protein transport in most samples. We usually employ 5 MOI (multiplicity of infection) to infect fibroblast cells, and around 2 MOI to transduce primary or iPSC-derived neural progenitor cells (NPCs). For transduction of post-mitotic and differentiated neurons, we suggest using 3 or 4 MOIs.

**Note:** In this step, iPSC-derived NPCs are taken as an example to show the procedure of transduction and examination of protein NCT using the dual reporter system. For more information about NPC generation and induction of specific neuronal subtypes, please refer to (Akter et al., 2022; Ding, 2021; Ding et al., 2021; Sepehrimanesh and Ding, 2020).

a. Prepare a Matrigel-coated 6-well culture plate and incubate it for more than 30 min at 37°C.

**Note:** For detailed to prepare Matrigel-coated plates, please refer to our previous report (Akter et al., 2022).

b. Quick thaw an NPC frozen stock in a 37°C water bath and transfer cells into a 15 mL tube containing 5 mL of warm NPC medium.

c. Spin down cells at 200 × g for 3 min.

**Note:** For one frozen stock with $1 \times 10^6$ cells, seed onto 3 wells of a 6-well plate.

d. Gently resuspend cell pellets into a proper volume of NPC medium and seed NPCs onto Matrigel-coated plates at a density of $3 \times 10^5$ cells/cm².

e. When NPCs reach ~60% confluence, add the proper amount of lentivirus to each well.

**Note:** For lentivirus transduction, the proper NPC density (50%–60% confluence) is critical to obtain neurons with high yield and purity.
f. NPCs are exposed to lentivirus for about 6 h to overnight (10–16 h). Replace the culture medium with Neuronal Maturation Medium.

g. Change the half volume of Neuronal Maturation Medium every other day till analysis or replating.

**Note:** The age of induced neurons (iNs) is counted from the lentiviral transduction as dpi (day post-viral infection). NPCs become neuron-like in morphology after 3 dpi.

**Note:** Both GFP and RFP signals can be noticed at 3 dpi and the signal density increases gradually.

h. The protein transport could be analyzed by imaging living cells under an inverted fluorescence microscope with the focus on dual reporter positive cells at desired time points.

i. To analyze the protein transport in mature neurons (more than 14 dpi), replate neurons onto coverslips with a monolayer of astrocytes at 5 dpi as previously reported (Akter et al., 2022). Change the half volume of Neuronal Maturation Medium every other day till analysis.

j. At desired time point, take the coverslips from the culture plate.

k. Incubate coverslips with Hoechst 33342 (HST) (1 μg/mL in PBS) for 10 min at RT.

l. Briefly rinse the coverslips twice with PBS and mount the coverslips on slides.

m. Image samples with a confocal microscope.

n. Confocal micrographs will be analyzed by CellProfiler software as described in section 3: image analysis using CellProfiler software of this protocol.

o. The ratio of nuclear to cytoplasmic GFP (Nuc/Cyt) will be used to evaluate protein export, and the ratio of cytoplasmic to nuclear RFP (Cyt/Nuc) will be used to evaluate protein nuclear import.

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**Section 3: Image analysis using CellProfiler software**

**Timing: hours to days**

Confocal images of FISH assay or reporter assay could be analyzed using software such as ImageJ or CellProfiler. At single cell levels, the subcellular distributions of dT signal or fluorescent protein levels will be measured. For each cell, as large an area as possible was measured within the nucleus or cytoplasm. An unbiased approach to data collection is recommended (Ding et al., 2020). For example, the individual performing the analysis using CellProfiler is completely blinded to sample information.

Images of nuclear markers are needed to properly designate nuclear boundaries before beginning. Additionally, images of the stained translocated objects of interest are needed. Overlaying images are not required for quantification but can be used as controls to ensure the correct subcellular areas are selected for the measurement. The mean values are used to quantify an average signal density. For FISH assays, the average of oligo-dT signals in the soma (both nucleus and cytoplasm) was used to evaluate the overall mRNA expression levels. Nuclear mRNA export was measured by the ratio of nuclear to cytoplasmic oligo-dT signals (dT (Nuc/Cyt)). For the dual reporter assay, GFP and RFP signals will be separately measured in the nucleus and cytoplasm. The ratio of nuclear to cytoplasmic GFP (Nuc/Cyt) is used to evaluate the protein export activity, while the ratio of cytoplasmic to nuclear RFP (Cyt/Nuc) is used to evaluate protein import activity. In this section, the confocal images of the FISH assay in fibroblast cells are used as an example to demonstrate the procedure of quantification.

**Import confocal images to CellProfiler**

**Timing: 5–10 min**
8. This step describes the import of confocal images using CellProfiler software.
   a. Upload images to be analyzed into the CellProfiler/C228 application images tab. This can be accomplished by dragging and dropping files into the designated box for files (Figure 2).

   **Note:** Take note of the image names, as they will serve as initial tags for the files.

   b. Under the NameAndTypes tab assign names to image matching rules. This allows the assignment of different names to images with specific criteria/alterations in downstream applications.

   c. As the images are 2-dimensional, select no for “Process as 3D”.

   d. Select “All” for matching rules of the image.

   e. Select the rule criteria: This option states what the image contains. I.e., if the image contains images of chromatin staining, you will want to enter the following settings: “File” “Does” “Contain regular expression” “Title of photo uploaded that stains for chromatin”.

   f. Enter the name you wish to assign to the image.

   g. For this experiment, color images are uploaded before converting them to grayscale. Because of this, you will set “Select the image type” as “Color image”.

   h. Select “Image metadata” for “Set intensity range from”. This will rescale fluorescence intensities for oversaturated areas of the image.

   i. Repeat the previous steps to include additional needed images such as overlayed images or other stained targets. At any time, you may enter test mode to run portions of your pipeline to ensure images are being processed properly.

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**Convert color images to grayscale**

© Timing: 5–10 min
9. This step describes the procedure of converting color images to grayscale.
   a. Insert a new module using the "+" button on the bottom left-hand side of the pipeline. This will open the module search tool.
   b. Type in “ColorToGray”.
   c. Add the Coloratura module to the pipeline by selecting the module and clicking "+ Add to Pipeline".
   d. Select the image you would like to input to convert to grayscale. This will be the name you’ve entered in the NamesAndTypes tab. Depending on your signal, you can select split or combine as your conversion method. For this experiment, channels were split into red and green channels. For overlayed images, channels were combined to convert to grayscale.
   e. Select RGB for image type as .tif images are used.
   f. Depending on the color of the stain, select what channel you would like to convert to gray; Red to gray, green to gray, blue to gray.
   g. Repeat the previous 6 steps for each image you are using e.g., nucleoplasm signal image, cytoplasmic signal image, and overlayed image (Figure 3).

*Note:* Optimal settings will depend on signal strength and crosstalk.

   e. Select RGB for image type as .tif images are used.
   f. Depending on the color of the stain, select what channel you would like to convert to gray; Red to gray, green to gray, blue to gray.
   g. Repeat the previous 6 steps for each image you are using e.g., nucleoplasm signal image, cytoplasmic signal image, and overlayed image (Figure 3).

**Settings to define subcellular boundaries**

- **Timing:** 10–30 min

10. This step describes the settings to define subcellular boundaries based on nuclear markers. These settings will allow the software to distinguish the nucleus and the cytoplasm of each cell. The first step is to set the primary object (nuclei) based on nuclear markers, such as nuclear Lamins or DAPI/HST signals.
**Note:** If fluorescence is too sharp compared to the background signal, smoothing may be needed for the identification of edges. This can be accomplished using the “Smooth” module if needed.

**Note:** Although standard settings may be adequate for identifying nuclei, advanced settings are good for reducing false positives and nuclei that may not be completely in focus. Upon opening advanced settings, select the image you will be used for primary object (nuclei) identification (Figure 4A).

a. Assign a name to the primary objects identified.

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**Figure 4. Settings for identification of Primary Object (nuclei)**

(A) Identify Primary Objects Module for setting parameters to identify nuclei in confocal micrographs of the FISH assay in fibroblast cells.

(B) Identified nuclei as designated primary objects for analysis. Scale bar: 20 μm.

(C) The primary object nuclei area decreased to avoid overlap with cytoplasmic mRNA signals. Scale bar: 20 μm.
b. Depending on the nuclei, adjust the maximum and minimum diameter of the primary object to be possessed.

Note: Depending on magnification and cell type, this setting may need adjustment to get the most accurate counts.

c. To avoid inaccurate signal measurements, select to discard images outside the diameter parameters as well as those touching the edges of the image.

d. For threshold strategy, select “Global”. This identifies pixels as foreground or background signals.

e. Select “Manual” as the thresholding method. Depending on the image, the threshold smoothing scale and manual threshold settings may need to be increased or decreased to best properly identify objects.

f. For the method of distinguishing both clumped objects and dividing lines between clumped objects, select “No”.

Note: Depending on cell type, quality of image, and staining quality, mixing and matching the methods of identifying clumped objects may be needed to produce the most accurate results.

g. Select “No” for log transforming before thresholding.

h. Automatically calculating the size of the smoothing filter may be sufficient for discerning different objects. If not, select “No” to automatically calculate the size of the smoothing filter.

Note: If many nuclei are being clumped together and counted as single objects, the value of the smoothing filter may need to be higher to increase the accuracy of counts. As with adjusting the smoothing filter, automatic calculation of the minimum allowed distance between intensity maxima may be sufficient in differentiating objects. If you find incorrect clumping of objects, select “No” for this option.

i. Lower values for the suppression of local maxima will help in properly splitting nuclei that may still be clumped together as a single object. Select “No” for the options to speed up the processing using a lower resolution image to find local maxima and “No” for the option to display local maxima. If you would like to view local maxima, you can select “Yes.”

j. Select “After both thresholding and declumping for the option to fill holes in identified objects and select “Continue” for the handling of objects if an excessive number of objects are identified (Figure 4B).

k. For adjusting nuclei pixel area and getting more accurate signals, open the module search tool as mentioned in the methods of converting images to grayscale. The module is titled “ExpandOrShrinkObject”. Select the input objects you will be shrinking and name the resulting output objects (e.g., the resulting shrunken nuclei).

l. Select to shrink the objects by a specified number of pixels.

Note: Depending on the magnification, the number of pixels to shrink can be adjusted, with a higher number being indicative of a greater degree of shrinking of the object (Figure 4C).

11. This step describes the settings for identifying the secondary object (cell body) based on Oligo-dT signals.

Note: Depending on cell type, different images may be used for identifying secondary objects as a lack of signal in the nuclei can produce errors in object identification. The input objects will be the nuclei identified in the previous step. This will be the structure which the secondary objects will be built around and associated with.
a. After assigning a name to the secondary objects, select “Watershed-Gradient” as the method of identifying secondary objects.

*Note:* The thresholding strategy will be adaptive for the secondary object with the thresholding method being minimum cross-entropy. As with the primary object, settings such as threshold smoothing, and thresholding correction factor can be adjusted to reduce improper clumping of objects.

*Note:* A value of 0.1 for the threshold smoothing scale and correction factor was found to produce accurate object identification in most cases. Adjusting the lower and upper bounds on thresholds will, after calculating threshold values, allow the program to automatically throw out objects that are outside the calculated threshold. 0.1–1 was found to produce accurate counts in most cases for associated stains.

b. For the size of an adaptive window, use a multiple of the largest expected object size you estimate will be present. This can vary based on magnification and cell type.

c. Select “No” for: Log transforming before thresholding and filling holes in identified objects.

d. Select “No” for discarding secondary objects touching the edges of the image.

*Note:* Depending on cell type and object areas, you may elect to remove cells that are not 100% in the frame. If done, removing the associated primary objects will also be required to ensure the correct secondary objects are associated with the correct primary objects (Figure 5).

12. This step describes the settings to designate a tertiary object (cytoplasm). In this case (the FISH assay of fibroblast cells), the tertiary object will be the cell body minus the nucleus (i.e., the cytoplasmic signal only).

a. Insert the “IdentifyTertiaryObjects” module.

*Note:* The larger object will serve as the parent object and will surround the smaller (child) object. In this case, the parent object will be the output generated from the secondary object module and the child object will be the output generated from the expand or shrink module for the primary objects.

b. Assign a name for the objects identified and select “No” for the option to shrink objects prior to subtraction, as the previous shrink object module has already reduced the primary object’s area (Figure 6).

*Note:* CellProfiler™ is supposed to correctly associate objects when necessary, but the following steps serve as a means to manually check that the correct nuclei and cell bodies
are being counted. This helps avoid issues where the nucleus that is in a frame is counted, while the body (secondary object) is excluded due to previously set up parameters.

c. Open the module search tool and load the “DisplayDataOnImage” module into the pipeline.

*Note:* This module will display the assigned count numbers of each object, allowing you to check that the correct nuclei are assigned to the correct cytoplasm for downstream measurements (*Figure 7*).

d. Select objects as the measurement and select the input objects you would like to view.

*Note:* The category will be number, and the measurement will be object number. Select “Yes” for displaying the background image and select the image you would like as the background.

13. This step describes the settings for measuring object intensities and output data.
   a. Insert the module titled “MeasureObjectIntensity”.
   b. Select the images you would like to measure.

*Note:* It is a good idea to choose a variety of images to compare in order to make sure the proper regions/objects are being analyzed.

c. Select the object(s) you would like to measure. For the FISH assay, the oligo-dT signal density in the nucleus and the cytoplasm will be measured (*Figure 8*).

d. Insert the “ExportToSpreadsheet” module.

*Note:* Depending on experiment requirements, you can choose to include or omit certain measurements such as the area and locations objects occupy. This is achieved by selecting “No” for the option to export all data measurements (*Figure 9*).
Based on oligo-dT signal density in the nucleus (Primary Object) and the cytoplasm (Tertiary Object), calculate the ratio of dT(Nuc/Cyt). The higher ratio of dT(Nuc/Cyt) indicates compromised nuclear mRNA export.

**Note:** Take special note of the names of both images and objects, as the same object may be identified on more than one image.

### EXPECTED OUTCOMES

**Measure the nuclear mRNA export by FISH assay**

We have examined the mRNA distribution in patient-derived motor neurons (MNs) from DYT1 dystonia (a movement disorder) and the healthy controls (Ding et al., 2021). The nuclear mRNA export was measured by the ratio of nuclear to cytoplasmic oligo-dT signals (dT(Nuc)/dT(Cyt)). The nuclear dye HST was used to define the nucleus, and the generic neuronal marker MAP2 was used to identify the soma (Figure 10). In healthy control neurons, most mRNAs were localized in the cytoplasm. In contrast, substantial amounts of mRNAs were accumulated inside the nucleus of DYT1 neurons. The specificity of FISH signals was confirmed by the lack of signals when samples were treated with RNase A or when oligo-dA was used as a probe (Figures 10A and 10B). Image analysis and quantification data showed that the ratio dT(Nuc)/dT(Cyt) was significantly higher in DYT1 neurons than in control ones (Figure 10D), suggesting that the nuclear mRNA export was impaired in DYT1 neurons.

**Figure 7. Assigned count numbers of each object**

(A and B) Count verification for primary objects (A) and tertiary objects (B). Ensuring that each nuclear signal (primary object) is associated with the correct cytoplasmic signal (tertiary object) for correct measurements using grayscaled confocal micrographs. Scale bar: 20 μm.

**Figure 8. Select image and image objects to be analyzed**

Options for associating specific images with a specifically identified object that allows measurement of the signal intensity.
Using this protocol, we have also measured the influence of sample sex and neuronal maturation on nuclear mRNA export (Ding et al., 2020). We found that the developmental stages, but not sample sex, affect the mRNA subcellular distribution (Figure 11). These studies demonstrated that the measurement of nuclear mRNA export by FISH assay is reliable and reproducible.

Measure the protein NCT by the dual reporter

The dual reporter system consists of a fused GFP with a NES (GFP-NES) and an RFP fused with NLS (RFP-NLS). In cells with normal NCT activity, GFP and RFP are localized in the cytoplasm and nucleus, respectively; in cells with compromised NCT, this subcellular distribution will be disrupted (Figure 12A). An increased ratio of nuclear to cytoplasmic GFP (GFP$_{\text{nuc}}$/GFP$_{\text{cyt}}$) represents compromised protein export, while a higher ratio of cytoplasmic to nuclear RFP (RFP$_{\text{cyt}}$/RFP$_{\text{nuc}}$) indicates impaired protein nuclear import. HEK cells were used to validate the expression and the distribution of reporters. As expected, both GFP-NES and RFP-NLS are robustly expressed and almost exclusively distributed in the cytoplasm and the nucleus, respectively (Figure 12B). The exposure to Lepromycin B, a specific nuclear export inhibitor (Jang et al., 2003), caused the signals of GFP-NES gradually accumulated inside the nucleus, but the distribution of RFP-NLS was not affected (Figures 12B and 12C). Using lentiviral delivery of this dual reporter to the reprogrammed human neurons, both GFP-NES and RFP-NLS are highly expressed. Similarly, in neurons that were derived from health fibroblast cells (Sepehrimanesh et al., 2021), GFP and RFP signals are exclusively distributed in the cytoplasm and the nucleus, respectively (Figure 12D).

Using this reporter system, we have examined the protein NCT in reprogrammed DYT1 neurons and healthy controls (Ding et al., 2021). In healthy control neurons, both GFP and RFP were mainly localized in the cytoplasm and nucleus, respectively. In contrast, a substantial amount of GFP accumulated inside the nucleus, and obvious RFP can be noticed in the cytoplasm in DYT1 neurons (Figure 13A). Image analysis and quantification data showed that the ratios of GFP$_{\text{nuc}}$/GFP$_{\text{cyt}}$ and RFP$_{\text{cyt}}$/RFP$_{\text{nuc}}$ were significantly higher for DYT1 neurons than for control neurons (Figures 13B and 13C), suggesting that the protein NCT activities are impaired in DYT1 neurons.
LIMITATIONS

FISH assay

In this protocol, we combined the FISH assay using DIG-dT probes together with ICC to measure the distribution of total mRNAs. Compared with other methods, in which dT probes are directly conjugated with fluorescent dye (e.g., Quasar 670 etc.) (Aksenova et al., 2020), this protocol used DIG-dT probes. The advantage of DIG-dT probes is that anti-DIG antibody and corresponding secondary antibodies could be employed to amplify the signal. At the same time, different combinations of antibodies could be used for detecting transcripts and other protein markers by ICC, practically important if you need to distinguish different cell types that are co-cultured on a single coverslip. One limitation of the FISH assay in this protocol is that antibody staining could give rise to non-specific signals. We usually set up two negative controls to verify the specificity of FISH results (Figures 10B and 10C). If new antibodies will be used in the assay, you also need to set antibody controls, including both primary and secondary antibodies. This protocol only described the
measurement of mRNAs using dT probes. If you need to measure some gene-specific mRNAs or other types of RNAs, sequence-specific probes need to be designed and synthesized.

**Dual report assay**

Protein nuclear transport has been used more frequently to evaluate the NCT activities. There are several strategies to evaluate the protein movement across the nuclear membrane. Based on immunostaining, the subcellular localization of specific endogenous proteins could be examined, such as the NFAT (nuclear factor of activated T cells) transcription factors that require the changes of the subcellular localization to fulfill their transcriptional regulations (Ding et al., 2018; Muramatsu et al., 2021). In this protocol, the protein transport activities will be measured by monitoring the kinetics of ectopically expressed reporters, GFP-NES and RFP-NLS. Compared with other alternative methods, there are several advantages of this protocol. First, the signals of GFP-NES and RFP-NLS could be simultaneously examined to evaluate both export and import activities in single cells. Second, GFP and RFP signals can be visualized directly under a fluorescent microscope and samples do not need to be stained with antibodies. Third, cultured cells expressing reporters can be visualized in living condition, and do not need to be fixed and/or permeabilized, making it possible to reveal the protein transport activities under a more physiological condition.

One limitation of the protein transport assay using this reporter system is that lentivirus needs to be prepared for the transduction of postmitotic cells such as primary cells or reprogrammed neurons. The quality of lentivirus will significantly influence the transduction efficiency and the expression levels of reporters (Ding and Kilpatrick, 2013). The proper signal density of reporters is important to give rise to reliable results in the following quantification assay. The growth condition of different cell lines may also affect the transduction efficiency. Another limitation is that the protein nuclear transport activities measured by this dual report only reveal the overall transport activities for proteins contain typical NLS or NES signal peptide. For certain protein cargos that use different
signaling peptides or different signaling pathways, their transport and subcellular distribution need to be measured specifically, such as by antibody-based ICC assay.

Image analysis
Confocal images of FISH assay or reporter assay could be analyzed using software such as ImageJ or CellProfiler™ either manually or automatically with specific settings based on the purpose. Usually, if you have low number of samples, quantify images manually will work. If you need to quantify a large number of cells, you may need to set up a method using some software for automagical image analysis. In this protocol, we used CellProfiler to quantify signal density and subcellular distribution. CellProfiler is a free, open-source software that allows to automatically quantify images. Compared with other software, CellProfiler is easy to use and requires little programming knowledge. It offers pipeline saving option including modules and settings. This feature enables CellProfiler to generate reproducible data. At a single cell level, the subcellular distributions of dT signal or fluorescent proteins are measured, and the ratios of cargos’ subcellular distributions are calculated to evaluate the NCT activities. For each cell, as large an area as possible to be measured within the nucleus or cytoplasm. To avoid potential bias, we recommend an unbiased approach for data collection. For example, the person who will analyze the images will be completely blinded to the sample information.

TROUBLESHOOTING
Problem 1
Weak oligo-dT signal in the FISH assay.

The oligo-dT signal is weak in the FISH assay (steps 1–3).

Potential solution
The weak FISH signal could be caused by several reasons. 1) The degradation of mRNAs in samples. To prevent potential RNA degradation, it is important to prepare all related solutions using
nuclease-free water. If samples cannot be processed immediately after fixation, you may store them at 
−20°C in 70% ethanol for a few weeks without an obvious decrease of oligo-dT signals, but the signal 
quality of some protein markers could be diminished. 2) Low concentration or poor quality of oligo-dT probes. Make fresh probes with proper concentration. 3) Antibodies. Make sure the anti-
bodies used are of good quality.

**Problem 2**
High background of FISH signal in negative controls.

Substantial oligo-dT signals can be noticed in negative control samples in the FISH assay (steps 1–3).

**Potential solution**
To validate the specificity of oligo-dT signals, two negative controls are recommended. One nega-
tive control is the sample control, which is treated with RNase A to degraded RNAs. The second negative control is the probe control, in which samples are hybridized with oligo-dA probes that

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**Figure 13. Protein nucleocytoplasmic transport assay in reprogrammed human neurons**
(A) Representative confocal images of the reporter distribution in healthy and DYT1 diMNs at 6 wpi. HST stained nuclei. Scale bar: 20 μm.
(B) Subcellular distribution of GFP-NES from four replicates; ns, not significant; ****p<0.0001.
(C) Subcellular distribution of RFP-NLS from four replicates; ns, not significant; ****p<0.0001. Adapted from Ding et al., 2021.
will not bind to mRNAs. If the RNase A treated samples show high background, the RNase A reagent and the digestion conditions need to be validated. The high background of oligo-dA probe control could be caused by the cross contamination with oligo-dT probes during hybridization. To systematically decrease the background signals, more stringent wash conditions need to be considered by adding more times of wash and/or washing longer each time. If necessary, antibody controls could be added to validate the specificity of new antibodies. These negative controls are processed simultaneously with other samples. The specificity of oligo-dT signals could be verified if negative controls show no or very little signals under the same confocal settings (Figures 10B and 10C).

**Problem 3**
Low expression of the dual reporter in transduced cells.

In transduced cells, the expression level of the dual reporter is low (step 7).

**Potential solution**
The low expression of the dual reporter in transduced cells is usually caused by the poor quality of lentivirus. To prepare high-quality lentivirus and validate the titer, please refer to our previous reports (Akter et al., 2022; Ding and Kilpatrick, 2013; Sepehrimanesh et al., 2021).

**Problem 4**
More cells died after transduction.

Large quantities of transduced cells died (more frequently occurs in neurons) (steps 6 and 7).

**Potential solution**
More cells dying is usually caused by the transduction of a very high dosage of the virus. To achieve a reasonable infection efficiency, the dosage of the virus should be optimized to transduce different cell types. Too much virus may cause more cell death. The transduction efficiency and the cell survival should be well balanced. Generally, about 60% transduction efficiency is good enough to examine the protein transport in most samples. We usually employ 5 MOI (multiplicity of infection) to infect fibroblast cells, and around 2 MOI to transduce primary or iPSC-derived neural progenitor cells. For transduction of post-mitotic and differentiated neurons, we suggest using 3 or 4 MOIs.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Baojin Ding (baojin.ding@lsuhs.edu).

**Materials availability**
The majority of materials required in this protocol are commercially available.

**Data and code availability**
This study did not generate/analyze any datasets or code that were submitted to any repositories.

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Conceptualization, B.D.; Methodology, B.D., H.C., and C.C.; Investigation, B.D., H.C., C.C., M.S., M.A., and M.A.H.; Writing, B.D., M.S., H.C., and C.C.; Funding Acquisition and Supervision, B.D.
DECLARATION OF INTERESTS
The authors declare no competing interests.

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