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# Mechanical Fractionation of Cultured Neuronal Cells into Cell Body and Neurite Fractions

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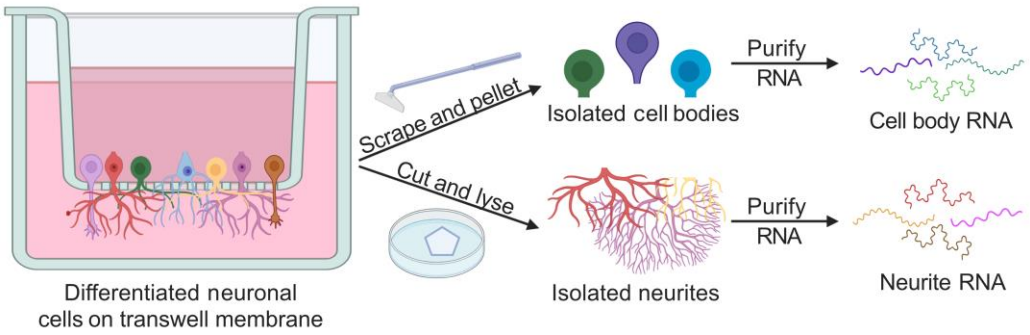
**[Abstract]** Many cells contain spatially defined subcellular regions that perform specialized tasks enabled by localized proteins. The subcellular distribution of these localized proteins is often facilitated by the subcellular localization of the RNA molecules that encode them. A key question in the study of this process of RNA localization is the characterization of the transcripts present at a given subcellular location. Historically, experiments aimed at answering this question have centered upon microscopy-based techniques that target one or a few transcripts at a time. However, more recently, the advent of high-throughput RNA sequencing has allowed the transcriptome-wide profiling of the RNA content of subcellular fractions. Here, we present a protocol for the isolation of cell body and neurite fractions from neuronal cells using mechanical fractionation and characterization of their RNA content.

**Graphic abstract:**




**Fractionation of neuronal cells and analysis of subcellular RNA contents**

**Keywords:** RNA localization, RNA transport, Post-transcriptional regulation, RNA trafficking, Subcellular transcriptomics

**[Background]** In eukaryotic cells, proteins are asymmetrically distributed to define spatially specialized regions. In cells that have complex morphologies and/or large sizes，like neurons, this asymmetry is often more extreme. These extended morphologies require a regulated and efficient sorting process to ensure that proteins are correctly localized. For many proteins, this process is facilitated through the

transport of RNA molecules to the site of protein function (Engel *et al*., 2020). On-site translation of these RNAs produces a protein that is immediately correctly localized. In neurons, this is a widespread process as up to 1000 different RNA species are enriched in the projections of these cells relative to their cell bodies (Cajigas *et al*., 2012; Taliaferro *et al*., 2016).

RNA localization is widely used as a gene expression regulatory strategy and contributes to a diverse set of biological processes, including mating-type switching in yeast (Bertrand *et al*., 1998), developmental patterning in *Drosophila* (Ephrussi *et al*., 1991; Lécuyer *et al*., 2007), and nutrient response in intestinal epithelial cells (Moor *et al*., 2017). The misregulation of RNA localization is associated with a range of neurological diseases (Wang *et al*., 2016), including spinal muscular atrophy (Fallini *et al*., 2011), amyotrophic lateral sclerosis (Chu *et al*., 2019; Briese *et al*., 2020), and Fragile X Syndrome (Dictenberg *et al*., 2008; Goering *et al*., 2020).

Despite the increasing recognition of the role of RNA localization in promoting a range of cellular functions, several questions remain unanswered. Among them is perhaps one of the simplest questions that exists regarding RNA localization: what RNAs exist at a given subcellular location and what are their relative abundances? To answer this question, at least in the context of neuronal cells, a variety of techniques have been developed and applied, including laser capture microdissection (Zivraj *et al*., 2010), compartmentalized culture chambers (Gumy *et al*., 2011), growth on microfluidic devices (Nijssen *et al*., 2018), and microdissection of rodent brains (Cajigas *et al*., 2012).

In this protocol, we describe a similar approach to separate neuronal cells into neurite and cell body fractions. This technique relies on microporous culture membranes. Cells are cultured on top of these membranes, which have pores (usually 1-3 µm in diameter) large enough to allow neurites to pass through them to the underside but small enough to keep the cell bodies on the top of the membrane. After growth on the membranes, the cells can be mechanically fractionated by scraping the top of the membrane with a cell scraper and removing the dislodged cell bodies. Neurites remain attached to the underside of the membrane and can be lysed for RNA extraction. Following RNA isolation, the RNA content of the fractions can be analyzed by reverse transcription-quantitative PCR (RT-qPCR) or by high-throughput RNA sequencing.

Although the focus of this manuscript is the fractionation of neuronal cells, this method can also be applied to cellular protrusions from a variety of cell types, including fibroblasts (Mili *et al*., 2008) and migrating cancer cells (Mardakheh *et al*., 2015; Dermit *et al*., 2020). In the context of neuronal cells, this procedure has been successfully used with neuronal cell lines (Taliaferro *et al*., 2016; Goering *et al*., 2020), primary mouse cortical neurons (Taliaferro *et al*., 2016), and iPS-derived neurons (Goering *et al*., 2020; Hudish *et al*., 2020). It is likely compatible with most neuronal cell types.

## Materials and Reagents

1. Deep well 6-well cell culture plates (Corning, catalog number: 353502, store at room temperature)
2. Microporous transwell membranes (transwell cell culture inserts), 1 µm pore diameter (Corning, catalog number: 353102, store at room temperature)
3. Matrigel (VWR, catalog number: 47743-706, store at -20°C)
4. RNase-free pipetting equipment (*e.g.*, aerosol-resistant filter tips)
5. Cell lifter/scraper (Fisher, catalog number: 07-200-364, store at room temperature)
6. Quick RNA Microprep kit (Zymo, catalog number: R1051, store at room temperature)
7. Cell culture media (dependent on the needs of the specific cells being grown)
8. Mouse anti-beta actin antibody (Sigma, catalog number: A5441)
9. Mouse anti-histone H3 antibody (Abcam, catalog number: 10799)
10. iScript reverse transcription supermix (BioRad, catalog number: 1708841)
11. Taqman probes and master mix (ThermoFisher, catalog number: 4444556)
12. MOPS running buffer (Invitrogen, catalog number: NP0001)
13. Protein sample buffer (Invitrogen, catalog number: NP0008, store at room temperature)

## Equipment

1. Cell culture tabletop centrifuge (for example, Eppendorf, model: 5702R)
2. Benchtop microcentrifuge (for example, Eppendorf, model: 5424R)
3. SDS page and western blotting materials
4. Thermocycler
5. qPCR-enabled thermocycler

## Procedure

A. Prepare transwell membranes
   1. Dilute Matrigel to 0.2% in cell culture media (see Note 1).
   2. Place the transwell membranes upside down in a 15 cm cell culture plate. Add 1 ml of diluted matrigel on the top, coating the bottom (underside) of each transwell membrane.
   3. Incubate at 37°C for 1 h.

B. Prepare cells
   1. In the meantime, wash cells with 1× PBS and trypsinize them.
   2. Pellet by centrifuging at 500 × *g* for 5 min.
   3. Resuspend in cell culture media to a concentration of 500,000 cells per ml. Two milliliters of this cell suspension is needed for each transwell membrane (see Note 2).

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C. Plate cells

1. Remove Matrigel solution from the transwell membranes.
2. In each well of a deep well 6-well plate, place 4 ml of cell culture media.
3. Put one transwell filter in each well.
4. Place 2 ml of cell solution (Step B3) onto each filter.
5. If a media change following plating is necessary (*e.g.*, a change into differentiation-inducing media), allow cells to attach for 1 h, then replace the media above and below the membrane. Change pipettes in between dealing with the solutions above and below the membrane to avoid introducing cells into the lower chamber.
6. Allow cells to incubate at 37°C for 48 h.

D. Fractionate cells

*Note: For a description of this procedure, see* ***Video 1****.*


**Video 1. Mechanical fractionation of neuronal cells for subcellular RNA analysis.** This video details the procedures for mechanical fractionation of neuronal cells using microporous membranes and highlights key points of the protocol.

1. Gently remove media above and below the membrane by aspiration.
2. Replace the media with PBS, using 2 ml below the membrane and 1 ml above the membrane.
3. Remove the cell bodies on the top of each membrane.
   a. Gently but thoroughly scrape the top of each membrane with a cell lifter, making sure to get the edges of the membrane where it joins the plastic housing (Figure 1A) (see Note 3).

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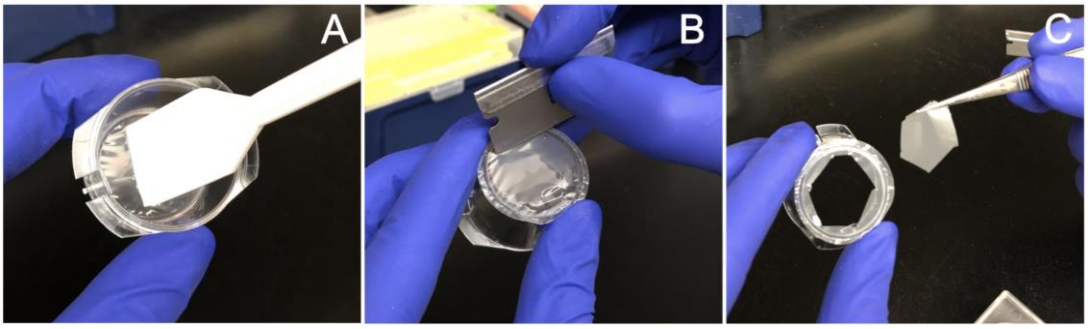


**Figure 1. Mechanical fractionation of cells.** A. The top of the membrane is scraped to remove cell bodies. B. The membrane is then removed from the plastic housing using a razor blade. C. The cut membrane is placed in a dish of lysis buffer using tweezers.

b. Transfer 700 µl of the cell body suspension into a 15 ml conical tube on ice.
c. Tilting the membrane, scrape any remaining cell bodies into the remaining 300 µl of PBS.
d. Thoroughly transfer the PBS into the 15 ml conical tube.
e. Place the membrane upside down on a clean surface (for example, the lid of the 6-well plate).
f. Repeat Steps D3a through D3e for the remaining membranes.

4. After scraping and removing the cell bodies from all six membranes, place 550 µl of RNA lysis buffer from the Zymo RNA Microprep kit into a 6 cm dish.
5. Remove each membrane from its plastic housing.
   a. Using a fresh razor blade, cut each membrane to remove it from the housing, leaving approximately 3 mm around the edge (Figure 1B) (see Note 4).
   b. Using tweezers, carefully put the released membrane into the RNA lysis buffer with the neurite-containing side facing down (Figure 1C).
   c. Repeat for all six membranes, placing all in the same 6 cm dish.
6. Incubate the dish at room temperature with rocking for 15 min to lyse neurites.
7. In the meantime, centrifuge cell bodies at 2,000 × *g* at 4°C for 7 min. Resuspend in 600 µl of PBS (*i.e.*, 100 µl per membrane).
8. Reserve samples for western blotting.
   a. Take 10 µl of the 600 µl cell body suspension. Add 90 µl of Protein Sample Buffer.
   b. Take 50 µl of the 550 µl neurite lysate. Add to 50 µl of Protein Sample Buffer. Guanidine in the RNA lysis buffer will precipitate from solution upon addition to Protein Sample Buffer, but that is expected.
   c. Store these samples at -20°C until analysis of fractionation efficiency by western blotting.
9. Isolate RNA
   a. Take 100 µl of cell body suspension and isolate RNA according to the instructions of the Zymo Quick RNA Microprep kit, beginning with the addition of 350 µl of RNA lysis buffer.
   b. Take the remaining 500 µl of neurite lysate and isolate RNA according to the kit's instructions, beginning with the addition of 500 µl of 95-100% ethanol.

10. Elute RNA

    Elute RNA in 15 µl RNase free water (see Note 5).

E. Analysis of fractionation efficiency using western blotting (see Note 6)
   1. Heat the samples that were reserved for western blotting (Step D8) for 5 min at 98°C.
   2. Load 5 µl of cell body sample per lane.
   3. Load 15 µl of neurite sample per lane while it is still hot. This helps any precipitated guanidine to dissolve and facilitates loading.
   4. Run a SDS-PAGE gel and transfer proteins to a nitrocellulose or PVDF membrane.
   5. Blot with primary antibodies.
      a. Use the β-actin antibody at 1:5,000 dilution.
      b. Use the histone H3 antibody at 1:10,000 dilution.
   6. Probe with an appropriate mouse secondary antibody.
   7. Image blot. See **Figure 2** for a blot depicting an efficient fractionation.

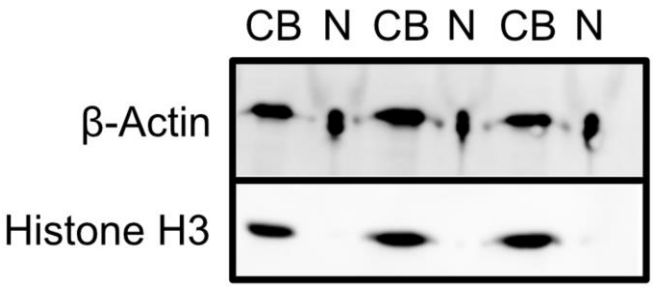


**Figure 2. Assessment of fractionation efficiency by western blotting.** Cell body samples are indicated by CB, and neurite samples are indicated by N.

F. Analysis of fractionation efficiency using RT-qPCR (see Note 7)
   1. Reverse transcribe 100 ng of RNA from each sample (see Note 8)
      a. Combine 2 µl of 5× iScript RT master mix, RNA, and water into a 10 µl reaction.
      b. Incubate in a thermocycler for 5 min at 25°C, 20 min at 46°C, and 1 min at 95°C.
      c. Dilute reaction with water to a 20 µl final volume (see Note 9).
   2. Perform qPCR

      Using a qPCR master mix of your choice, perform qPCR to quantify the desired marker RNA molecules in each fraction. Use 2 µl of cDNA from Step F1c per reaction. It is usually best to quantify the ratio of two RNA molecules in each sample, with one of the two known to be neurite enriched. The accuracy of the ratio in each sample can be improved using TaqMan qPCR probes to allow simultaneous quantification of the two species in the same reaction.
   3. Assess qPCR results

      From the qPCR results, calculate the relative abundance of the neurite-enriched and control RNAs in the cell body and neurite fractions. If the fractionation was successful, the ratio of the neurite-enriched RNA to control RNA will be higher in the neurite fraction than in the cell body

fraction. See Figure 3 for qPCR results from a successful fractionation. The increased variation in neurite quantification relative to cell body quantification is typical and likely reflects the technical variation inherent to differences in fractionation efficiency between replicates.

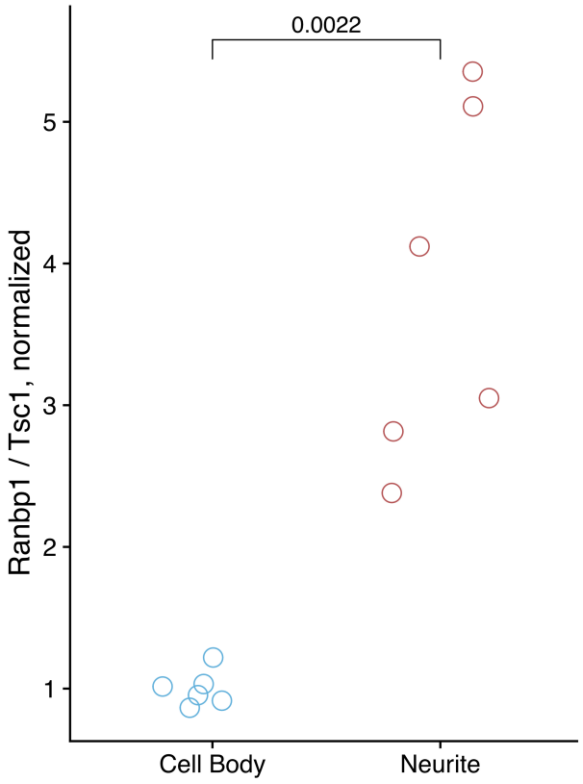


**Figure 3. qPCR results from a successful fractionation.** *Ranbp1* RNA is known from previous experiments to be enriched in neurites, while *Tsc1* is known from previous experiments to be enriched in cell bodies. The relative amounts of these RNA species were quantified in cell body and neurite samples using Taqman qPCR.

G. Construction of high-throughput sequencing libraries

Use the purified RNA to make high-throughput RNA sequencing libraries. Several commercial kits are available for this purpose, although we have had consistently good results using the mRNA Hyperprep kit from KAPA (Kapa KK8580). Importantly, provide the same amount of input RNA (*e.g.*, 100 ng) for all samples in the kit, even though significantly more RNA can be isolated from cell bodies than from neurites.

## Data analysis

1. Analysis of western blot results can be done by visual inspection. Generally, there should be very little to no signal from histone H3 in the neurite samples. Significant histone H3 signal in the neurite samples is indicative of a poor fractionation. The level of β-actin signal may or may not be similar between the cell body and neurite fractions. What is important to consider is the

relative ratio of β-actin to histone H3 signal in the two fractions.

2. Identification of RNAs that are differentially localized between the two fractions can be done using standard differential gene expression techniques with high-throughput sequencing data.

## Notes

1. We recommend thawing the Matrigel stock overnight in a 2°C to 8°C refrigerator. Additionally, dilute matrigel in cold cell culture media as it starts to form a gel at 10°C. As matrigel is very viscous, aspirate and dispense the stock slowly.
2. This concentration of cells ensures that they are essentially confluent when plated on the membrane. This can be desirable because it can result in neurite outgrowth being forced down through the pores of the membrane rather than laterally across the surface. If this is not desirable, adjust the cell concentration accordingly.
3. Using too much pressure when scraping can result in the membrane being torn away from the plastic housing. If this happens, discard the torn membrane and move to the next one.
4. It is often difficult to completely remove cell bodies from the corner formed by the membrane and the plastic housing. For this reason, it is often best to avoid this area when cutting the membrane out of the housing. Do not worry about removing all of the membrane when cutting.
5. When this procedure is performed with N2A or CAD mouse neuronal cell lines, expect 5-10 µg of RNA from the cell body sample and 500-1,000 ng of RNA from the neurite sample. This is the expected amount when all wells of a 6-well plate are combined.
6. The fractionation efficiency can be assessed by probing the cell body and neurite fractions for specific proteins. β-Actin should be present in both fractions, whereas histone H3, being nuclear, should be restricted to the cell body fraction. The detection of significant histone H3 signal in the neurite fraction indicates poor fractionation efficiency. The high amount of salt in the neurite fractions may cause them to appear compressed during imaging or while running the gel. This is a purely cosmetic defect, and the ability to detect protein bands within these samples is not hindered.
7. The efficiency of fractionation can also be assessed using RT-qPCR. This requires knowledge of RNA species enriched in each fraction. We have observed that mRNAs encoding ribosomal proteins are reproducibly neurite-enriched across several neuronal cell types and species. In this example, we use *Ranbp1* and *Tsc1* RNAs as markers, which we have previously observed to be neurite-enriched and cell body-enriched, respectively.
8. It should be noted that qPCR requires ~100 ng RNA from both cell body and neurite fractions. Cells from two wells are enough to serve as one replicate for qPCR. Thus, a full 6-well plate can be split into three replicates for qPCR. We highly recommend performing a minus RT control to ensure that there is no genomic DNA contamination in the samples.
9. The dilution factor for the RT reaction depends on the expression of the genes that need to be tested. For highly expressed genes, the RT reaction can be diluted to 50 µl final volume.

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## Competing interests

The authors declare no financial or non-financial competing interests.

## Ethics

No animal or human subjects were used during this study.

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