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Genome-wide analysis of RNA and protein localization and local translation in mESC-derived neurons

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ABSTRACT

The subcellular localization and translation of mRNAs are fundamental biological processes. In neurons, they underlie cell growth and synaptic plasticity, which serves as a foundation of learning and memory. Multiple approaches have been developed to separate neurons on subcellular compartments – cell bodies (soma) and cell extensions (axons and dendrites) – for further biochemical analyses. Here we describe neurite/soma separation approach in combination with RNA sequencing and proteomic analyses to identify localized and locally translated RNAs and proteins. This approach allows quantification of around 7000 of local proteins and the entire local transcriptome. It provides a powerful tool for investigation of the mechanisms underlying RNA localization and local translation in neurons.

1. Introduction

The neuron is a highly polarized cell, consisting of cell body (soma) and neurite extensions (dendrites and axons). Such polarity is crucial to neuronal function and relies largely on asymmetric subcellular translation and localization of RNAs and proteins (reviewed in [1]). A number of human pathologies, including neurodegenerative disorders such as spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS), are associated with failure to localize mRNAs to specific subcellular compartments. Given the importance of the topic, a number of approaches have been developed, that allow separation of neurons on subcellular compartments - soma and neurites - for further biochemical analysis. Here we review such methods and provide a detailed description the neurite/soma approach relying on the usage of microporous membrane in combination with omics analyses. (1) Laser capture microdissection is one of such techniques that allows isolation of different subcellular compartments [2]. Although this approach is time-consuming, it has been successfully applied in omics studies. Thus, Schuman and colleagues [3,4] used it to isolate the synaptic neuropil of the hippocampus and analyze its mRNA content. An advantage of this approach is that analyzes tissues isolated directly from mouse brain. At the same time, the collected material is heterogenous and contains not only multiple types of neurons, but also non-neuronal glial cells. (2) Centrifugation in Percoll gradient was also described as a method to isolate synaptosomes, structures composed of pre- and postsynaptic compartments [5]. In this method, brain tissues are homogenized to separate nerve terminals from the rest of the cell, forming synaptosomes. Synaptosomes are then isolated from the rest of the cell by centrifugation in discontinuous Percoll gradient. (3) Neuronal compartments can also be isolated by growing neurons in compartmentalized microfluidic chambers [6]. (4) Finally, neurons can be grown on microporous membranes, so that cell bodies stay on one side and neurites grow on the other side of the membrane [7–9]. Cell bodies and neurites are then collected from the different sides of the membrane. In this manuscript we describe in detail how to apply this approach to mESC-derived neurons. Isolated soma and neurites material can be used for RNA-seq, proteomic analysis and ribosome profiling [9,10].

We use neurons differentiated from mouse embryonic stem cells (mESCs) through doxycycline-inducible expression of Achaete-scute homolog 1 (ASCL1) neurogenic transcription factor [9–13] (Fig. 1). As ASCL1 is overexpressed in every cell, neuronal differentiation is highly efficient (> 90%). Moreover, resulting neurons display all basic neuronal properties: (1) express mature neuronal markers, i.e. TUJ1, NeuN, and Synapsin, (2) form axons and dendrites clearly distinguishable with Neurofilament and MAP2 immunostaining [9] (Fig. 1A), (3) form functional pre- and postsynaptic structures [14–17], and (4) have typical passive and active intrinsic membrane potentials [11]. A major advantage of this test system is that neurons can be generated in large quantities, and resulting population is highly homogenous, a feature critical for omics approaches. Moreover, this system reduces animal use

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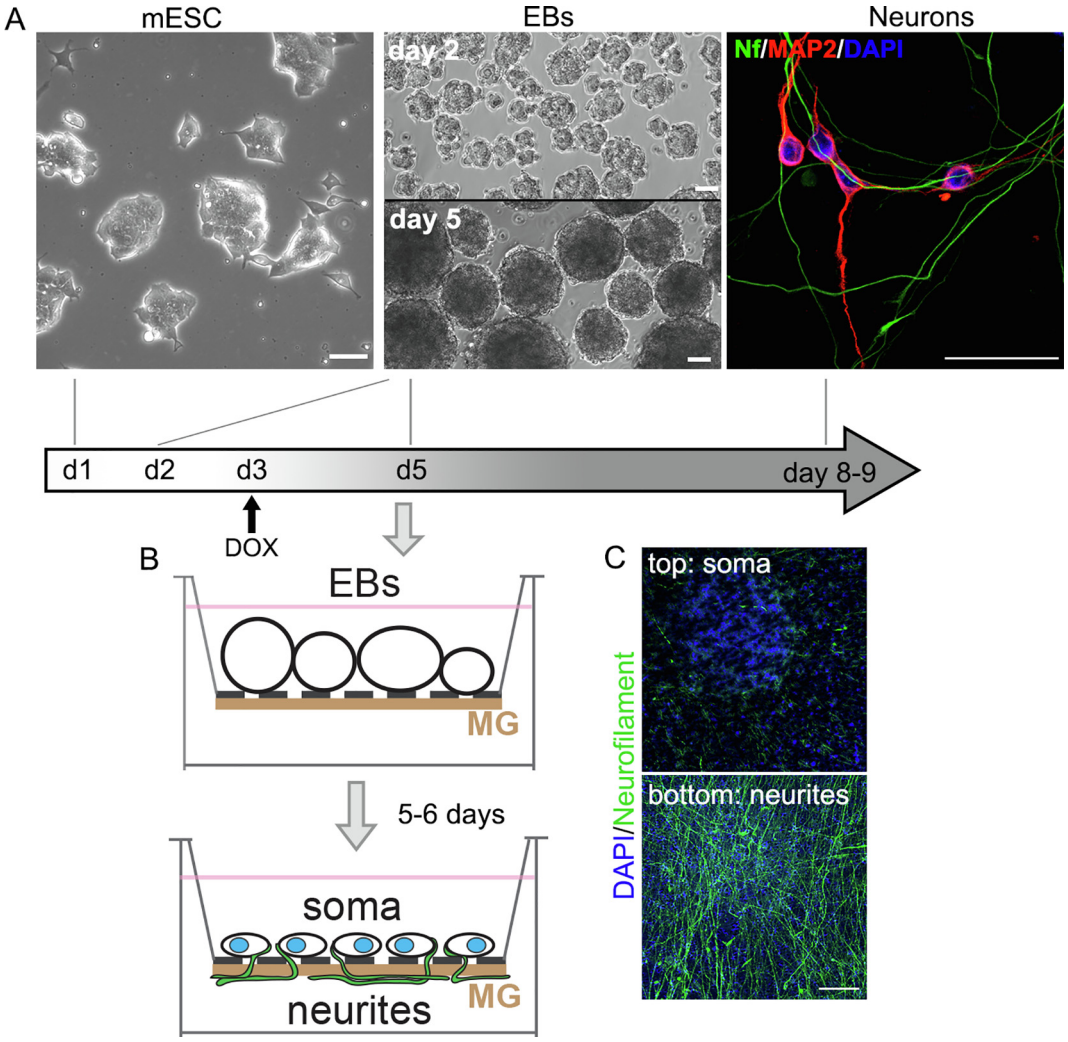

Fig. 1. ASCL1 mESC differentiation and plating schematic. (A) Example phase contrast (PH; mESC, EBs), and immunofluorescence (IF; Neurons, Nf: Neurofilament) images at indicated time point during differentiation. Scale bar = 50 μm (B) Schematic showing EB plating on the hanging filter inserts, and the separation of the compartments. MG: matrigel. (C) Example IF images of soma on the top of the filter and neurites on the bottom of the filter, stained with Neurofilament and DAPI. Scale bar = 50 μm.

following the good practice of 3R principle (Replacement, Reduction and Refinement).

In addition to being easily scalable, microporous membranes offer great experimental flexibility. For example, they can be used in co-culture with glia and other cell types, to examine their impact on local transcriptome and proteome in neurons. Furthermore, due to the wide range of available pore sizes, the method can be adapted to a variety of cell shapes and types, including primary neurons [8,10], dorsal root ganglion (DRG) explants [18,19], neuroblastoma cells [7], fibroblasts [20], and breast cancer cells [21]. Taken together, the diversity of cell systems that can be used with the microporous membranes, and the feasibility of soma/neurite separation approach makes this method easily accessible to most laboratories.

2. Materials

2.1. Cell culture and differentiation

1. Mouse embryonic stem cells (mESC) with doxycycline (dox) inducible expression of Achaete-scute homolog 1 (ASCL1) transcription factor [9,10].
2. Culture medium: 80–20 media (80% 2i medium and 20% mESC medium).
   a. 2i medium: 50% Advanced DMEM/F12 (12634028 Thermo) and 50% Neurobasal (21103049 Thermo) supplemented with 1 × N2 (17502048 Thermo), 1 × B27 (17504044 Thermo), 1 mM L-Glutamine (25030024 Thermo), 0.1 mM β-mercaptoethanol (βME) (31350-010 LifeTechnologies), 10^3 U ml^{-1} LIF, 3 μM CHIR99021 (MBS578900-50 Biozol), 1 μM PD03259901 (130–104–170 Milenyi Biotec).
   b. mESC medium: Knockout DMEM (10829018 Thermo) supplemented with 14% fetal bovine serum (FBS; 10439016 Thermo), 0.1 mM βME, 1 mM L-Glutamine, 1 × MEM non-essential amino acid (11140035 Thermo), 1 × nucleosides (ES008D Millipore), and 10^3 U ml^{-1} leukemia inhibitory factor (LIF, ESG1107 Millipore).
   c. Feeder medium: Knockout DMEM supplemented with 10% FBS, 1 mM L-Glutamine.
3. Gelatin for flask coating: dissolve 0.5 g porcine gelatin (G1890 Sigma) in 500 ml tissue culture (TC)-grade water (0.1% w/v). Sterilize by autoclaving (121 °C, 15 psi, 30 min) or filter sterilize (0.22 μm filter). Store at 4 °C; use within 2 months.
4. Differentiation medium:
   a. AK medium: 50% Advanced DMEM/F12 supplemented with 50% neurobasal, 10% knockout serum replacement (10828028 Thermo), 1 mM L-Glutamine, 0.1 mM βME, ± 3 μg ml^{-1}

doxycycline.
   b. Monolayer differentiation medium: Advanced DMEM/F12 supplemented with 1 × B27, 1 × N2, and 3 μg ml^{-1} doxycycline
5. Other cell culture reagents: PBS (P04-36500 PAN Biotech); TrypLE (12605028 Gibco)

2.2. Compartment separation

1. Matrigel (356237, Corning). Dilute the stock (9–12 mg/ml) to final concentration 0.3 mg/ml with ice-cold serum-free medium (Knockout™ DMEM) or PBS and prepare 0.5–1 ml aliquots. Since matrigel starts to form a gel above 10 °C, keep matrigel on ice, use pre-cooled tips and tubes, and freeze aliquots immediately.
2. Millicell hanging cell culture insert for 6-well plate (MCSP06H48 3 μm, Millipore)
3. Cotton swabs (large: 4–5.5 mm^2, 2–1019; small: 2 mm^2, 2–1022 neoLab)
4. Syringe needles
5. Forceps
6. Bench top centrifuge at 4 °C
7. Ice-cold PBS
8. Optionally: ice-cold 70% methanol (see *Note* in Section 3.3)

2.3. RNA isolation

1. Fume hood
2. peqGOLD TriFast™ (30–2010 VWR), or TRIzol (15596026 Thermo)
3. Chloroform
4. GlycoBlue Coprecipitant (15 mg/mL; AM9516 LifeTechnologies)
5. Isopropanol
6. 75% ethanol
7. RNAse free water. Add 0.1% (v/v) DEPC (D5758 Sigma) to double deionized water (for example: miliQ water). Shake on orbital shaker or mix with a magnetic stirrer 1hr to overnight (o/n). Remove DEPC by autoclaving: 15–45 min at 15psi on a liquid cycle. Traces of DEPC modify purine residues in RNA by carboxymethylation. Therefore, DEPC must always be removed from solutions or containers by autoclaving or heating at 100 °C for 15 min.
8. DNA Low-biding microfuge tubes (for example: DNA LoBind; 525–0130 VWR)

2.4. Protein isolation

1. Urea buffer [8 M Urea, 0.1 M Tris-HCl pH 7.5]
2. Bioruptor Plus (B01020001 Diagenode) or equivalent sonicator

2.5. Separation and quality controls

2.5.1. RNA

1. Qubit 2.0 or later
2. Qubit RNA HS Assay Kit (Q32852 Thermo)
3. Maxima First Strand cDNA Synthesis Kit for RT-qPCR (K1641 Thermo) or equivalent
4. SensiFast SYBR no-rox (BIO-98005 Bioline) or equivalent
5. qRT-PCR primers for neuritic and somatic enrichment quality control (see Table 1)

2.5.2. Protein

1. Protein concentrator columns (88512 Pierce, or analogous)
2. Qubit 2.0 or later and Qubit protein assay kit (Q33211 Thermo)
3. Standard western blotting equipment and reagents
4. Primary antibodies:
   a. mouse anti-Histone H3 (ab1791 Abcam)
   b. rabbit anti-β-Tubulin III (TUBB3, T2200 Sigma)

Table 1
List of qRT-PCR primers for quality control for genes enriched in neurites or soma.

Gene Primer	Sequence	Amplicon length
Neurite-enriched		
Tagln Fwd	TAGACCCCAGCGGCAACTAT	134 bp
Tagln Rev	GTTCCAGGTTCCAAGTCCACC	
Kif1c Fwd	GGAGCCTCCGTGAAAGTTG	178 bp
Kif1c Rev	CCGAAGTATGCGACCAGTAAGA	
Mapkapk2 Fwd	TTCCCCCAGTTCCACGTCA	122 bp
Mapkapk2 Rev	GCAGCACCTTCCCGTTGAT	
Col3a1 Fwd	CTGTAACATGGAAACTGGGGAAA	144 bp
Col3a1 Rev	CCATAGCTGAACTGAAAACCACC	
Soma-enriched		
Gng3 Fwd	GCACTATGAGTATTGGTCAAGCA	119 bp
Gng3 Rev	GTGGGCATCACAGTATGTCATC	
Tubb3 Fwd	CCAACAAGGGTCCATCCTACG	127 bp
Tubb3 Rev	ATCTGGGCGGCCTACATCA	
Equally distributed in Soma and Neurites		
Gapdh Fwd	TGACCTCAACTACATGGTCTACA	85 bp
Gapdh Rev	CTTCCCATTCTCGGCCTTG	
Thyn1 Fwd	CCCTAAATGGTCGATGGTGGA	94 bp
Thyn1 Rev	TTTGTGGGCTTGGTGATAGGT	
18S rRNA Fwd	AAACGGCTACCACATCCAAG	155 bp
18S rRNA Rev	CCTCCAATGGATCCTCGTTA	

   c. mouse anti-Neurofilament SMI312 (837904 Biolegend)
5. Secondary antibodies:
   a. rat anti-mouse IgG (L) HRP Conjugate (ab99632 Abcam)
   b. mouse anti-rabbit IgG (L) HRP Conjugate (ab99697 Abcam)

2.5.3. Immunofluorescent microscopy

1. Millicell hanging cell culture insert for 24-well plate (MCSP24H48 3 μm, Millipore)
2. PBS
3. 4% paraformaldehyde (PFA) in PBS, pH 7.4
4. 0.2% TritonX in PBS
5. 10% and 3% BSA in PBS (sterilized with 0.22 um filter)
6. Parafilm
7. PAP pen
8. Primary antibodies:
   a. chicken anti-Neurofilament Poly28226 (822601 Biolegend)
   b. guinea pig anti-Map2 (188 004 Sysy)
9. Secondary antibodies (fluorophore selection is given as an example, other combinations are also possible):
   a. Alexa Fluor 488 goat anti-chicken IgG (A-11039 Invitrogen)
   b. Alexa Fluor 647 goat anti-guinea pig IgG (A-21450 Invitrogen)
10. Prolong Gold Antifade Mounting with DAPI (P36931 Life Technologies)
11. Glass microscopy slides and cover glass
12. Forceps
13. Syringe needle

2.6. NGS library preparation

1. 1.5 ml LoBind tubes, Nuclease free (DNA LoBind, 525-0130 VWR)
2. PCR tubes, nuclease free
3. Bioanalyzer (Agilent)
4. Bioanalyzer Nano and/or Bioanalyzer Pico analysis kits (5067-1511; 5067-1513 Agilent), to check the quality of the RNA used for library preparation
5. Bioanalyzer DNA 1000 analysis kits (5067-1504 Agilent), to assess the libraries produced
6. TruSeq Stranded Total RNA Library Prep Gold (20020598 Illumina) with indexes (20020492). Other library preparation kits can be used.

7. ERCC spike-ins (4456740 Ambion)
8. Qubit DNA HS kit (Q32851 LifeTechnologies)
9. Agencourt AMPure XP beads (A63881 Beckman Coulter)
10. Agencourt RNAclean XP beads (A63987 Beckman Coulter)
11. Magnetic stand
12. Thermal cycler
13. NextSeq 500/550 High Output Kit v2 (FC-404-2004 Illumina). Other flow cells can be used.

2.7. SILAC, pSILAC and QuanCAT

1. Use dialyzed FBS for preparation of SILAC mESC, and Feeder media.
2. All SILAC media must be supplemented with 0.798 mM L-lysine and 0.398 mM L-arginine:
   a. Light: L-Lysine monohydrochloride (L5626 Sigma), and L-Arginine monohydrochloride (A5131 Sigma)
   b. Medium: 4,4,5,5,-d_4-L-lysine monohydrochloride (Lys-4; 616192 Sigma), and L-Arginine-$^{13}C_6$ monohydrochloride (Arg-6; 643440 Sigma)
   c. Heavy: L-lysine-$^{13}C_6$ $^{15}N_2$ monohydrochloride (Lys-8; 608041 Sigma), and L-arginine-$^{13}C_6$ $^{15}N_4$ monohydrochloride (Arg-10; 608033 Sigma)
3. Culture and differentiation media for SILAC are modified form Section 2.1 as following:
   a. 2i medium is prepared with: 50% SILAC Advanced DMEM/F12 Flex w/o D-glucose, w/o phenol red, w/o L-Arg, w/o L-Lys (A24943-01 Gibco) and 50% Neuropan Basal Medium with L-Gln, with 2.2 g/L NaHCO3, w/o L-Arg, w/o L-Lys (P04-00904 PAN Biotech) supplemented as indicated in Section 2.1 and with: 1.5 g/L D-glucose (A2494001, Thermo).
   b. mESC medium: Knockout DMEM with 4.5 g/L D-glucose, with sodium pyruvate, w/o L-Arg, w/o L-Gln, w/o L-Lys (ME16079L2, Gibco) supplemented as indicated in Section 2.1.
   c. Feeder medium: Knockout DMEM with 4.5 g/L D-glucose, with sodium pyruvate, w/o L-Arg, w/o L-Gln, w/o L-Lys (ME16079L2 Gibco) supplemented indicated in Section 2.1.
   d. AK medium: 50% SILAC Advanced DMEM/F12 Flex w/o D-glucose, w/o phenol red, w/o L-Arg, w/o L-Lys (A24943-01 Gibco) and 50% Neuropan Basal Medium with L-Gln, with 2.2 g/L NaHCO3, w/o L-Arg, w/o L-Lys (P04-00904 PAN Biotech) supplemented as indicated in Section 2.1, and with 1× D-glucose.
   e. Monolayer differentiation medium: SILAC Advanced DMEM/F12 Flex w/o D-glucose, w/o phenol red, w/o L-Arg, w/o L-Lys (A24943-01 Gibco) supplemented as indicated in Section 2.1, and with 1× D-glucose.
4. Click-iT protein enrichment kit (C10416 Thermo)
5. 10 mM DTT
6. Iodoacetamide (I6125 Sigma)
7. SDS buffer: 1% SDS, 100 mM Tris pH 8.0, 250 mM NaCl
8. Urea buffer: 8 M Urea, 100 mM Tris pH 8.0
9. 80% acetonitrile (271004 Sigma)
10. Lys-C (mass spec. grade; VA1170 Promega)
11. Trypsin (mas spec. grade; 90057 Thermo)

3. Methods

3.1. ASCL1-mESC culture

1. Grow ASCL1-mESC cells in 80–20 medium, in T75 gelatin-coated flasks without exceeding 75% confluency, at 37 °C and 5% CO_2. For gelatin coating, add 0.1% (w/v) gelatin solution to the cell culture flask, enough to cover the bottom, and incubate at room temperature (RT) for 20 min (min). Remove gelatin and rinse with PBS once. The gelatin-coated flask must not dry.
2. Passage cells 1:10–1:25 every 3–4 days. Between the passages change the media every other day. For passaging, rinse cells with PBS, and incubate with TrypLE for 1 min at 37 °C (1 ml/T75 flask). Dilute TrypLE with 5× excess Feeder media, resuspend cells by pipetting up and down, and collect the cell suspension into the 15 ml conical tube. Centrifuge the tube at 900 rpm for 4 min and remove supernatant. Resuspend the cell pellet in 80–20 medium and plate 1/10-1/25 part into a fresh gelatin-coated flask.

3.2. Neuronal differentiation of ASCL1-mESC into induced neurons

1. Estimate the number of cells to be differentiated based on the final amount of material needed. For example, 0.5×10^6 mESCs will be sufficient for 1 × 6-well filter, with the estimated yield of ~1 µg neuritic and ~10 µg somatic RNA or ~30 µg of neuritic and ~375 µg of somatic protein.
2. For differentiation, use cells at 75% confluency. On day 1, follow passaging instructions (Section 3.1, point 2), but resuspend the cell pellet in 1 ml of AK medium. Make sure to dissociate the pellet into single cells by trituration (P1000, 10–15 S). Add 5 ml of AK medium and count the cells. Plate 1 × 106 cells in 10 ml of AK medium per one 10 cm dish. Important: the dish used at this point must not be coated with gelatin, to allow cells grow in suspension and form embryoid bodies (EBs) (Fig. 1A).
3. On day 2 (24 h after plating), most of the cells form EBs: they should be of uniform size and round shape (Fig. 1A).
4. On day 3 (48 h after plating), split the EBs 1:2 in AK media supplemented with doxycycline to induce ASCL1 expression. For that, collect the EBs in a 50 ml conical tube and pellet them by centrifugation at 700 rpm for 3 min. Resuspend the EBs in 2 ml of AK media with 3µg/mL doxycycline (AK + dox). Add 1 ml of the EB suspension to a 10 cm plate containing 9 ml of AK + dox media and grow for 2 more days.
5. In the evening of day 4 or in the morning of day 5, thaw matrigel at 4 °C. For that, place required number of matrigel aliquots (~3 ml for one 6-well plate) in an ice bucket filled with ice and leave it a refrigerator or in cold room to thaw for ~3 h to o/n. Note that matrigel will polymerize rapidly if left at room temperature.
6. On day 5, coat the filters with matrigel. For that, place filter inlays in the 6-well plate, close the lid and turn the plate upside down. Lift the plate so that inlays remain standing on the plate lid. Pipet ~500 µl of matrigel on each filter and put the plate back on top of the lid with filters. Flip the plate and incubate for 3 h at 37 °C (cell culture incubator).
7. On the same day (48 h after ASCL1 induction), plate the EBs on matrigel-coated filters, using one 10 cm dish of EBs per one filter. Collect the EBs in a 50 ml conical tube and pellet them by centrifugation at 700 rpm for 3 min.
8. Directly before plating the EBs, rinse matrigel-coated filters with PBS and place them in a 6-well place with the monolayer medium supplemented with 3 µg/mL doxycycline (2 ml per well).
9. Resuspend the EBs deriving from one 10 cm dish in 2 ml of the monolayer medium supplemented with doxycycline and plate on top of the filter (Fig. 1B). Optional: the protocol can be shortened by 2 days, if EBs are generated in AK supplemented with doxycycline and plated on the filters next day (one 10 cm dish of the EBs per one filter) [9].
10. On day 8, exchange the medium with fresh monolayer medium supplemented with doxycycline.
11. On day 10, check the cells under the cell culture microscope: they should be differentiated, with axons and dendrites extending on the lower part on the membrane (Fig. 1C). At this point cells are ready for neurites and soma isolation.

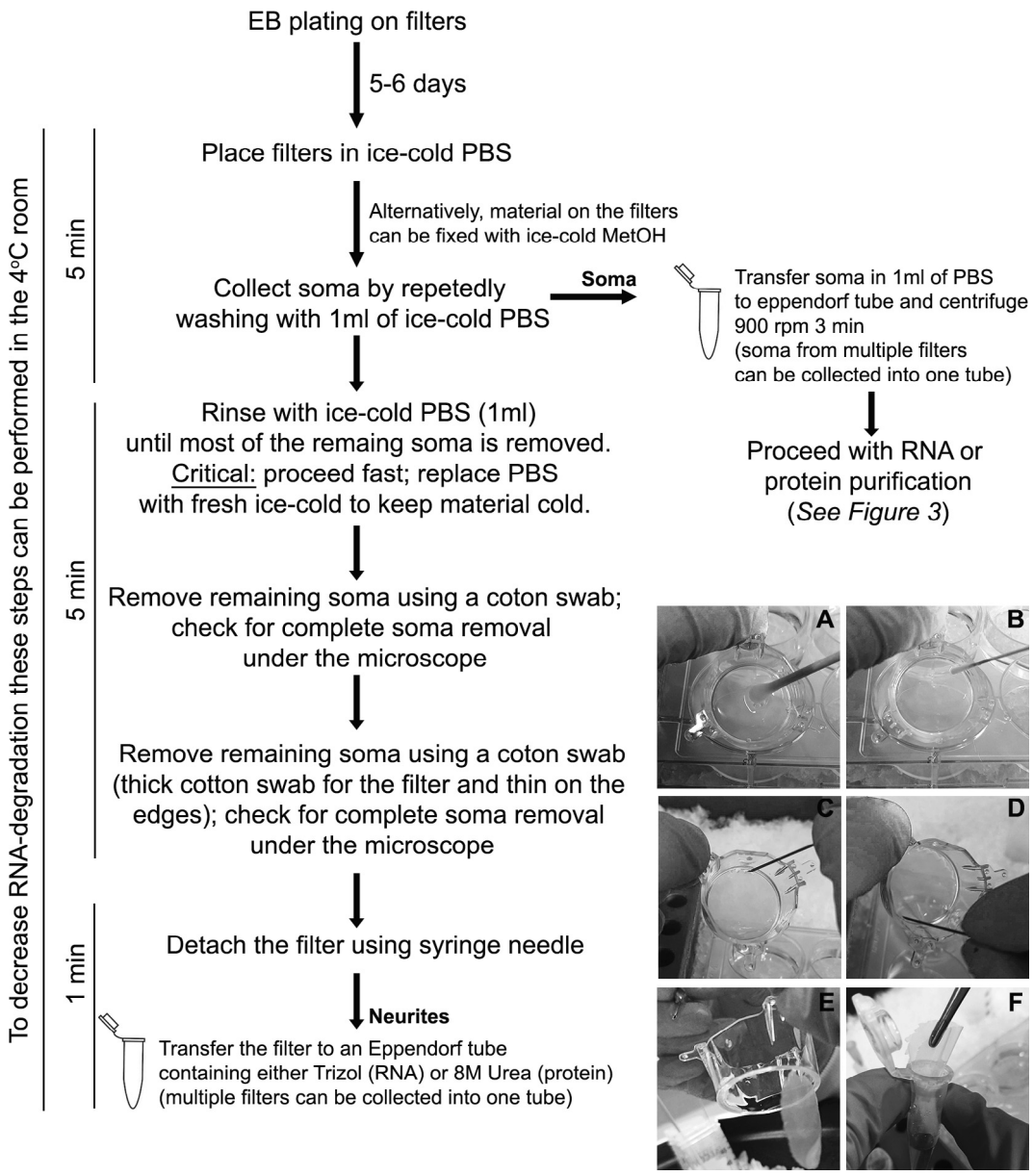

Fig. 2. Workflow of soma and neurites separation on the filters. (A–B) Removal of residual soma from the filters with cotton swabs. (C–E) Detachment of the filter from the insert. (F) Transfer of the filter to the eppendorf tube.

3.3. Separation of ASCL1-induced neurons on neurites and soma

1. On days 8–9, perform isolation of neurites and soma (Fig. 2). If the samples are to be used for RNA isolation, read the *Note* at the end of this section on working with RNA beforehand. Important: the following step must be done quickly (under 5 min) and at 4 °C to ensure the integrity of the isolated material.
2. Place a 6-well plate on ice and pour cold PBS in the wells. Transfer a filter inlay in a well of 6-well plates, using forceps, and rinse it in cold PBS. For soma isolation, detach it from the filter top by pipetting ~1 ml of PBS up and down the filter inlay with P1000 pipet. Transfer PBS to a 1.5 ml Eppendorf tube and pellet soma by centrifugation at 900 rpm for 3 min at 4 °C. Remove supernatant, immediately add 0.5 ml TriFast (for RNA isolation) or Urea buffer (for protein isolation) to the pellet and vortex. Safe stopping point: samples can be frozen and stored at −80 °C until further processing.
3. While soma is pelleted by centrifugation, isolate neurites. Use cotton swabs, dipped in cold PBS, to wipe the top of the filter inlay and remove the remnants of soma [22]. It is convenient to use large cotton swab to wipe most of the filer and small cotton swab at the perimeter where filter is glued to plastic ring (Fig. 2A–B). Confirm efficient soma removal using the cell culture microscope. Detach the membranes with a syringe needle (Fig. 2C–D) and place it with forceps into a tube (Fig. 2E–F) with 0.5 ml TriFast (for RNA isolation) or Urea buffer (for protein isolation), vortex.
4. Material isolated from multiple filters can be combined into one sample, if large amounts per replicate are needed. For RNA-seq or proteomics analysis, we usually combine material from 2 to 3 filters in one eppendorf tube with 0.5 ml TriFast (for RNA isolation) or Urea buffer (for protein isolation).

Note: Working with RNA. To minimize RNA degradation, use RNAse-free reagents and plastic, filter tips, wear gloves and keep RNA on ice, unless specified otherwise. Aliquot you RNA samples, to avoid multiple freezing-defreezing cycles. To obtain integral RNA during neurite/soma separation, work fast. If RNA degradation is still observed (Bioanalyzer profile), neurons can be fixed in 70% methanol prior to separation. For that, wash the filters with ice-cold PBS, and add pre-chilled 70% methanol. Keep on ice for

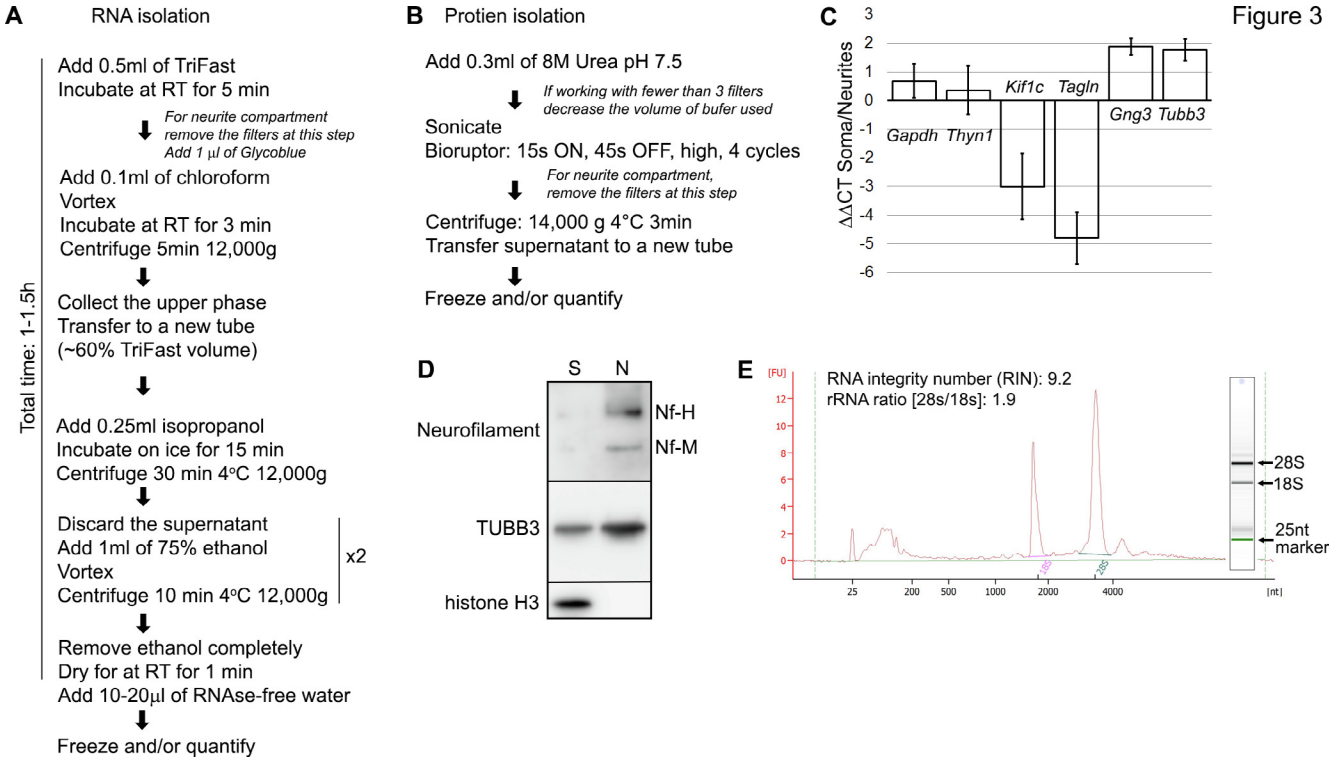

Fig. 3. RNA and protein isolation from neurites and soma after compartment separation. (A) Workflow of RNA isolation protocol. (B) Workflow of protein isolation protocol. (C) Representative qRT-PCR results showing equal distribution of *Thyn1*, and *Gapdh* between soma and neurites, enrichment of *Kif1c* and *Tgln* in neurites, and enrichment of *Gng3* and *Tubb3* in soma. Bar: mean; error bar: SD, n = 3 soma/neurite pairs, normalized to 18S rRNA. (D) Representative western blot showing enrichment of H3 in soma, equal distribution of TUBB3 between soma and neurites, and enrichment of Neurofilament in neurites (Nf-H: heavy isoform; Nf-M: medium isoform) (E) An example of expected Bioanalyzer profile.

15 min*. After fixation, remove methanol and again wash with cold PBS twice. Proceed with separation as described above.*

3.4. RNA isolation

1. Perform all the steps involving phenol and chloroform under the fume hood. Follow the instruction manual of TriFast or another phenol-based reagent you are using. For the scheme of the workflow, see Fig. 3A. After combining the material with TriFast reagent (3.3, Step 4), incubate the samples for 5 min at RT to ensure full dissociation of the RNP complexes. Add 1 µl of GlycoBlue to serve as a carrier during RNA precipitation. If working with neurites samples, before proceeding to the next step, transfer the sample to a fresh tube to remove the filters.
2. Add 0.1 ml of chloroform, vortex and keep for 3 min at RT (volume of chloroform is 1:5 of the initial TriFast volume: Section 3.3 Step 4). Centrifuge the tube at 5 min at 12,000*g* to separate the phases: lower red (phenol-chloroform phase), the interphase and the upper aqueous phase, which contains RNA.
3. Collect the upper phase into a fresh eppendorf tube. The volume of this phase is about 60% of the volume of the TriFast.
4. Precipitate the RNA with 0.25 ml of isopropanol (~1:2 of the initial TriFast volume). Keep samples on ice for 15 min and centrifuge for 30 min at 4 °C at 12,000*g*. The RNA pellet can be seen on the bottom of the tube as a light-blue precipitate.
5. Discard the supernatant and wash the RNA pellet twice with 75% ethanol. For that, add 1 ml of 75% ethanol, vortex and centrifuge for 10 min at 12,000*g* at 4 °C. Discard the supernatant.
6. After the last wash, quickly spin the tube again to collect the remaining ethanol on the bottom of the tube. Use P10 pipet to remove ethanol completely and leave the tube open for 1 min to dry the RNA pellet. Do not let the pellet to overdry as it will be difficult to dissolve the RNA.
7. Add 10–20 µl of RNAse-free water, incubate at RT for 1 min and pipette up and down to dissolve the RNA. Aliquot if needed. Safe stopping point: RNA can be stored at −80 °C.
8. To quantify the amount of RNA isolated, measure the concentration using Qubit analyzer and Qubit RNA HS Assay Kit following the manufacturer's instructions. Expected total RNA amount form one 6-well filter is 1 µg from neurites and 10 µg from soma.

3.5. Protein isolation

1. See Fig. 3B for the scheme of protein isolation. Sonicate the material resuspended in Urea buffer (3.3, Step 3). Use the following Bioruptor settings: 15 s ON, 45 s OFF, high, 4 cycles.
2. Remove cell debris by centrifugation at 14,000*g* for 3 min at 4 °C. Collect the supernatant into a fresh tube.
3. Use protein Qubit assay to measure protein concentration. Aliquot if needed. Safe stopping point: protein solution can be stored at −80 °C.

3.6. Quality control

1. To assess the efficiency of neurites and soma separation, perform RT-qPCR on RNA samples and western blotting on protein samples. Selected neuritic and somatic markers and loading controls are provided in Section 2.
2. For RT-qPCR, use 1–2 ng of total RNA per sample. We use Maxima first-strand cDNA synthesis kit to generate cDNA and sensiFAST SYBR No ROX qPCR kit for qPCR analysis, but other analogous reagents can be used too. Follow the instruction manual provided by the manufacturer.
3. Include technical duplicate and biological triplicates in the analysis.

See Table 1 for the list of suggested primers. *Kif1C*, *Tagln*, *Mapkapk2*, and *Col3a1* are enriched in neurites, *Gng3* and *Tubb3* are enriched in soma and *Gapdh*, *Thyn1* and *rRNA* are equally distributed between neurites and soma (Fig. 3C).

Note: To calculate ΔΔ C_T for soma and neurite comparison, first calculate the mean threshold cycle (CT) for each of the targets by averaging over technical replicates. Then, calculate Δ C_T for a given target in each of the compartments (Δ $C_T^{neurites}$; Δ C_T^{soma}) by normalizing to a reference transcript:

$$\Delta C_T^{neurites} = C_{target}^{neurites} - C_{reference}^{neurites}$$

$$\Delta C_T^{soma} = C_{target}^{soma} - C_{reference}^{soma}$$

We suggest to use rRNA, Gapdh or Thyn1 for normalization. Then calculate ΔΔ $C_T^{soma/neurites}$:

$$\Delta\Delta C_T^{soma/neurites} = \Delta C_T^{neurites} - \Delta C_T^{soma}$$

4. For western blot analysis, use 5–20 μg total protein per lane. If protein concentration is too low, column concentrators can be used. Protein solution in Urea buffer can be directly supplemented with 1/5 vol of 5× Laemmli loading buffer and processed as usual.
5. After protein transfer to the PVDF membrane and membrane blocking, cut the membrane between 15 kDa, 55 kDa and 130 kDa, using protein markers for orientation.
6. Incubate the lower part of the membrane with anti-Histone H3 antibody at 1:10,000 dilution; the middle part with anti-TUBB3 at 1:2000 dilution; and the upper part with anti-Neurofilament SMI312 antibody at 1:10,000 dilution. Incubate at 4 °C o/n.
7. Any conventional western blot protocol can be used for further steps. Histone H3 should be detected only or preferentially in soma, TUBB3 shows similar distribution between neurites and soma, and Neurofilament is enriched in neurites (Fig. 3D).

3.7. Additional quality controls: immunofluorescence on FILTERS

1. 1 Additional assessment of soma and neurites separation on filters can be performed using immunofluorescence antibody staining of the cells on the filters (Fig. 1C).
2. To decrease the amount of antibody used, use smaller filter size (for 12, or 24-well). Proceed with cell culture, differentiation, and plating as described for compartment separation. Plate 10–15 times fewer EBs on the 24-well filter as compared to 6-well filter. Cells should be fixed on the same day as compartment separation is performed.
3. All the washing steps should be performed carefully not to detach the cells from the filters. Pipet liquids slowly, use P1000 rather than an aspirator to remove washes and excess liquid.
4. To fix the cells, gently move the filter into a new well containing PBS and wash with PBS twice. Replace PBS with 4% PFA and incubate at RT for 15–20 min. Remove PFA and wash twice with PBS. Permeabilize the cells with 0.2% Triton X-100 in PBS at RT for 10 min. Safe stopping point: can keep up to a week at 4°C in PBS, or up to 1 month in 70% ethanol. To proceed with antibody staining, wash twice with PBS, and block in 10% BSA at RT for 1–3 h or at 4 °C o/n.
5. Prepare primary antibodies dilution in 3% BSA, the following antibodies can be combined: 1:5000 chicken anti-Neurofilament; 1:1000 guinea pig anti-Map2; 1:200 rabbit anti-TUBB3.
6. Cut a 5 by 5 cm parafilm piece and, using PAP pen, draw a circle (~1cm diameter) on the parafilm. Place a 50 μl drop of antibody dilution in the circle. Gently placed the Millicell filter on the drop of antibody solution. Add additional 20–30 μl of antibody solution on the top of the filter.
7. Incubate o/n at 4 °C. To avoid drying, place in a tightly closed container. The next day, place the filter in a well with 3% BSA and wash 3 times.
8. Prepare secondary antibodies dilution in 3% BSA; all the secondary antibodies are used at 1:1000. Repeat the procedure from Step 8 and incubate at RT for 1 h. Place the filter back in the well, and wash 3 times with 3% BSA.
9. Place a drop (~20 to 30 μl) of Prolong Gold Antifade Mounting with DAPI on a glass slide. Gently detach the filter from the plastic insert using syringe needle and forceps. Place the filter in the mounting media with soma side facing the slide. Carefully place a cover glass over the filters avoiding bubbles. Dry o/n at RT. An example image of soma and neurites stained with for Neurofilament are available in Fig. 1C.

Note: EBs are far larger than 3 μm *pores and do not migrate through on the other side of the membrane. However, in case cell bodies are detected on the bottom of membrane using DAPI, inlays with smaller pore size* (1 μm) *can be used.*

3.8. NGS library preparation from neurites and soma

1. If RNA is to be used for NGS library preparation, analyze the integrity using Agilent Bioanalyzer. Choose the Bioanalyzer chip based on the Qubit measurement from Section 3.4 Step 8. If using Nano Chip, RNA concentration should be within 25–500 ng/μL; for Pico Chip RNA concentration should be 50–250 pg/μl. Follow Bioanalyzer manufacturer's instructions to assess the quality of the RNA. Expected Bioanalyzer profile for integral RNA is provided in Fig. 3E. For library preparation, use only intact RNA with distinct 18 and 28S peaks (28S:18S rRNA ratios ~2), and RNA integrity number (RIN) above 7.
2. To prepare library for sequencing, follow the protocol provided by the manufacturer of the chosen library preparation kit. Custom library preparation protocols can also be used. When interested in mRNA expression levels, we use TruSeq Stranded mRNA Library Prep (RNA input 500–1000 ng). If other classes of RNAs (lncRNAs, circRNAs) are also of interest, we use TruSeq Stranded Total RNA Library Prep Gold (RNA input 100 ng–1 μg). If RNA amounts are limiting, custom protocols or kits compatible with low input can be used (for example: Ovation SoLo (NuGEN) for 10 pg-10 ng of total RNA or SMARTer Stranded Total RNA-Seq Kit v2 (TaKaRa) for 250 pg-10 ng of total RNA)).
3. It is important to prepare libraries in at least 3 biological replicates, i.e. 3 independent cell cultures, separation procedures and RNA preparations.
4. To be able to quantify absolute RNA levels, we also add to each RNA sample ERCC spike-in (1 μl of 1:100 dilution per 500 ng of total RNA as recommended by the manufacturer).
5. During library preparation, RNA is fragmented, reverse transcribed, ligated to adapters and PCR amplified. The resulting DNA library should be analyzed using DNA 1000 Bioanalyzer Chip to confirm narrow and uniform library size. Average library size ~300 bp is made up by ~150 bp fragments and ~150 bp of adapter sequences, therefore we sequence them with single-end 150 bp reads on an Illumina NextSeq 500 sequencer according to manufacturer instructions. Other sequencers (HiSeq, NovaSeq) can be used. We multiplex 6 to 12 libraries per NextSeq 500 High Output flow cell, yielding ~30 to 60 mln reads per sample (Table 2).

3.9. Data analysis and visualization

1. For RNA-seq data analysis we use PiGx pipeline [23]. See Fig. 4A for the data analysis workflow. For hands-on experience in shell and R, we recommend available online resources: The Unix Workbench (Coursera), Introduction to R (DataCamp). For introduction to statistical analysis see [24]

Table 2
Overview of sequencers and flow cell capacity.

Sequencer	Kit options	Read length	Max. # of single reads
NextSeq 500/550	Medium Output	150/300	130M
	High Output	75/150/300	400M
HiSeq 3000	SBS	50/150/300	2.5B
HiSeq 4000	SBS	50/150/300	5B
NovaSeq 6000	S1	100/200/300	1.3–1.6B
	S2	100/200/300	3.3–4.1B
	S4	100/200/300	8–10B

2. To obtain sequence (fastq) files for each library, sequencing data are demultiplexed. During this step sequences corresponding to multiple samples, which were sequenced as a pool, are separated based on the indexes present in their adaptors. For sequencing performed using Illumina sequencers, this step can be done using the bcl2fastq program, which is available from Illumina for linux-based systems.
3. Fastq files can be further processed by the PiGx RNA-seq pipeline [23]. The pipeline requires a tabular sample sheet, describing sample and file names as well as sample types (and covariates) used for differential expression analysis, and a YAML settings file to list the necessary genome assembly (fasta and gtf) and transcriptome sequence (fasta) files. Additionally the instructions for differential expression analysis between the neurites (case group) and soma (control group) samples need to be entered into this settings file as described in the pipeline manual. The pipeline will then automatically run quality trimming of the reads with trimgalore, followed by the genomic alignment and gene level counting with STAR. Next, the library and mapping statistic reports are

Figure 4

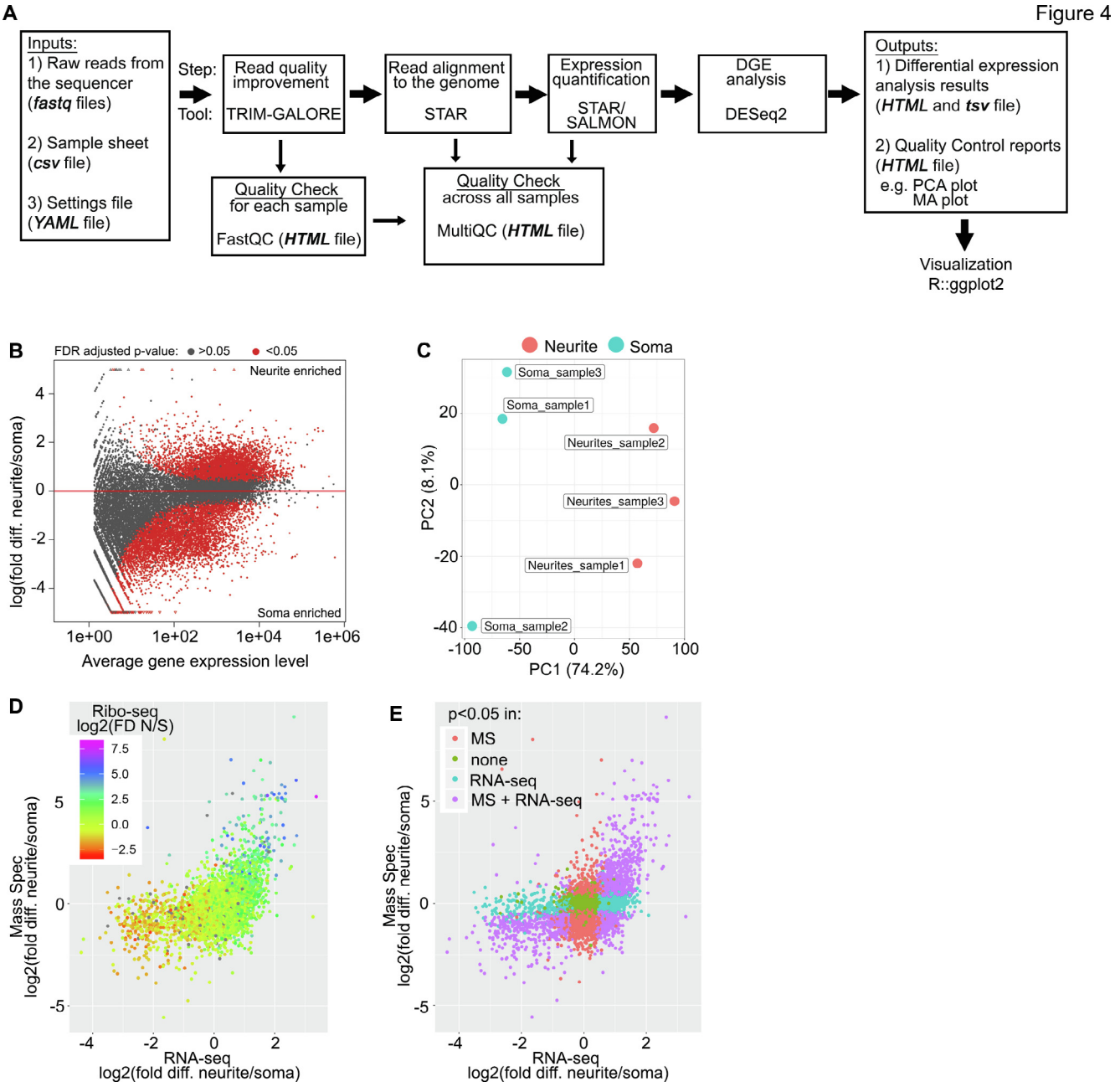

Fig. 4. Data analysis and visualization. (A) Overview of the PiGx pipeline. (B) Example of a typical MA plot. (C) Example of a typical PCA plot showing separation of somatic and neuritic samples along the first principal component (PC1). Examples of data visualization using third continuous (D, Ribo-seq log2 FC N/S) or discrete (E, p-value adjusted for multiple testing) variable created using R::ggplot2. Data are from [9].

summarized using multiQC. Then quasi mapping for gene and transcript level counts is carried out with salmon. Based on raw counts obtained from STAR and salmon, the final reports are generated using R/Bioconductor and DEseq2, including differential gene expression (DGE) analysis presented as log2 fold change between the samples.

4. One of the outputs of the PiGx pipeline is the multiQC html report. It contains a general quality check of the raw and processed sequence data. Every section is flagged as green, yellow or red, where red or yellow flags indicate potential problems that should be examined. Apart from the direct sequencing quality measured by fastQC (good phred scores are > 30) one important measure of the library quality is the total number of raw reads and the relative amount mapped reads per sample, which should ideally be above 60–70%. Lower than expected read numbers can indicate errors or contaminations (e.g. with ribosomal RNA) in library preparation, which could also result in higher amount of overrepresented sequences. The read number and mapping statistics, which are expected to show mainly uniquely mapped reads, should also be similar between the different samples.
5. One of the main outputs of the DGE analysis is a table with log2 fold changes between neurites and soma samples and associated adjusted p-values. From DGE, neuritically localized transcripts can be defined as those with a log2 fold change > 1 and adjusted p-values < 0.05, and somatically localized as those with a log2 fold change < −1 and adjusted p-values < 0.05. Another output is an html report that contains quality measures like the MA plot and principle component analysis (PCA). The MA plot (Fig. 4B) shows the log2 fold change between neurites and soma (log2 FC N/S) on the Y-axis and the mean expression values of all underlying samples (mean normalized counts) on the X-axis. Generally an MA plot should show a symmetrical distribution of log2 FC values. The PCA of the samples can be used to control for the quality of the data: the replicates should cluster together along the first principal component (Fig. 4C).
6. To visualize and explore the data, we recommend using ggplot2 (http://ggplot2.org). For hands-on experience in ggplot2, we recommend Data Visualization with ggplot2 (DataCamp). Generally genomic data can often be visualized with scatter plots with one data point for every gene or transcript, examples for this are MA plot (Fig. 4B), volcano plot or other customized plots (Fig. 4D–E). In addition to two numerical variables on the main axes it is useful to add a third variable, either discreet or continuous, by color-mapping the data. For example in the MA plot shown in Fig. 4B, statistically significant differentially localized transcripts are colored (adjusted p-value < 0.05). Other variable relationships or correlations can often be visualized this way as well, especially if the patterns are more complex than simple 2-way correlation. For example, differences in translation rate (Ribo-seq log2 FC N/S) can be presented as a color gradient on a plot showing relationship between protein (proteomics log2 FC N/S) and RNA (RNA-seq log2 FC N/S) enrichment in compartments (Fig. 4D). Discrete variables, like significance for the datasets plotted on the X and Y axes, can also be color-coded for easy visualization (Fig. 4E).

3.10. Proteomic analysis

1. Protein lysates prepared with Urea buffer (3.5 Protein isolation) can be analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). We use 20 µg of total protein per sample (an average yield of total protein obtained from one Milicell insert is ~30 µg for neurites isolation and ~375 µg for soma isolation).
2. Protein quantification can be performed using a label-free quantification (LFQ) method [25] or Stable Isotope Labeling by Amino acids in Cell culture (SILAC) [26]. SILAC detects differences in protein abundance between the samples using non-radioactive isotopic labeling and is advantageous for quantifying small differences.
3. For SILAC experiments, grow ASCL1- mESC in SILAC-customized 80% 2i/20% mESC medium: light (L) or heavy (H: Arg + 10 Da, Lys + 8 Da). See Section 2 for medium preparation. We observe complete proteome labeling (> 97%) within ~ seven cell passages.
4. Labelled ASCL1-mESC are differentiated into neurons as described in 3.2. (Neuronal differentiation), but using SILAC-customized differentiation media (L or H, see Section 2).
5. Neurites and soma lysates are then pooled (H neurites + L soma for forward and L soma + H neurites for reverse experiment) and subjected to LC-MS/MS. The forward and reverse experiments represent "label swap" replicates to eliminate biases introduced by the labeling procedure. The averages of H/L (forward) and L/H (reverse) ratios are used to measure relative protein abundance in neurites versus soma.
6. The protocol is also compatible with pulsed SILAC (pSILAC) [27] and QuaNCAT experiments [28], used to quantify de novo protein synthesis with pulse labeling.
7. For pSILAC, neurons are grown on the Millicell insert are pulse labelled for two hours before lysis, using pSILAC-customized monolayer differentiation medium: H (Arg + 10 Da, Lys + 8 Da (heavy pulse, H) or Arg + 6 Da, Lys + 4 Da (medium pulse, M). See Section 2 for media preparation. Samples are further processed as in case of SILAC. The average of H/M (forward) and M/H (reverse) ratios for each protein serve as a measurement of the relative amount of translation in neurites compared with soma.
8. QuaNCAT combines pSILAC and labeling of newly synthesized proteins with methionine analog azidohomoalanine (AHA). See Section 2, for formulations of QuanCAT-customized medium. AHA-containing proteins (M + H) are purified from the bulk of pre-existent proteins (L) by covalently linking them to alkyne bearing agarose beads using "click chemistry". This step reduces the background of pre-existing proteins (L) and enables shorter labeling pulse and measurement of larger number of proteins. For example, after 30 min AHA pulse, we could measure relative abundance of 380 newly synthesized proteins [9]. For comparison, with pSILAC we quantified 242 proteins after 2 h pulse.
9. For enrichment of newly synthesized AHA-containing proteins, protein lysates are mixed with alkyne-agarose-beads and linked to beads o/n using the Click-iT protein enrichment kit according to the manufacturer's instructions. To denature the proteins, the beads are incubated with 10 mM DTT at 65 °C and then alkylated by iodoacetamide. The beads are then washed with SDS buffer, followed by Urea buffer and finally with 80% acetonitrile. Then proteins are digested for 3 h with Lys-C and o/n with trypsin. As in pSILAC, the difference in proteins synthesized in the soma and neurites were quantified by the ratios H/M (forward experiment) and M/H (reverse experiment).

4. Results

Differentiation of ASCL1-mESCs into induced neurons takes 7–10 days. For the first 2–5 days the cells are allowed to form EBs in non-adherent culture. Some EB clumping might occur at days 3–5 of culture, but these aggregates should be easily dissociated with gentle pipetting with 10 ml pipettes. Excessive EB aggregation or stringing can lead to inefficient differentiation. Furthermore, there should be very few single cells remaining in the culture through these stages. Large numbers of single cells signal an issue with the originating culture or with differentiation. To monitor EB formation, we assessed their appearance throughout the process. At day 2 small round shape EBs formed and they were uniform in size with very few single cells present (Fig. 1A). As differentiation progressed, at day 5 the EBs grew in size but remained clearly separated and round with no aggregates forming and with only few single cells present (Fig. 1A).

In addition to assessing the EB formation, the quality of differentiated neurons can be easily tested by immunofluorescence. To do

this, at day 5 of differentiation (See Section 3.2), we dissociated the EBs with trypsin (1×, 5 min), and plated single cells in monolayer media with doxycycline on poly-L-lysine or poly-D-ornithine coated coverslips. At day 7, these cells were fixed and immunostained as described for filters (see Section 3.7). Induced neurons were positive for Neurofilament and MAP2, indicating that differentiation was successful (Fig. 1A). In addition, it allowed for clear distinction of axons (Neurofilament-positive neurites) and dendrites (MAP2-positive neurites) (Fig. 1A). This observation is consistent with induced neurons being mature.

Once EBs are plated in adherent environment (on filter), the neurites grow out. To test whether the neurites migrated through the pores and extended onto the lower part of the membrane, we performed immunostaining for Neurofilament (see Section 3.7). In addition, to ensure that the soma was present only on the top of the filter we stained the chromatin using DAPI. We observed clear Neurofilament positive outgrowths predominantly located on the bottom of the filter. The bottom surface was also clear of soma, which was abundantly present on the top of the membrane (Fig. 1C). These observations indicate that the neurite and soma compartments are successfully separated in culture.

Despite somatic and neuritic compartment being well separated during culture, cross contamination can occur during the mechanical separation. Soma is washed of the top of the filter and then the remnants of it are removed with cotton swabs (see Section 3.3). The complete removal of the soma from the filter before collection of the neuritic compartment is critical, as neurites generates smaller quantities of material (RNA and protein) than soma and any remaining cell bodies will influence compartment-specific enrichments. Therefore, in addition to visual inspection during the separation process, we performed qRT-PCR to assess distribution of soma (*Gng3*, *Tubb3*) and neurites (*Kif1c, Tagln*) specific mRNAs in the samples after collection (see Section 3.6). We also included uniformly distributed transcripts, *Gapdh* and *Thyn1*, as additional controls. ΔΔ CT values for soma/neurite comparison were −3 and −5 for *Kif1c* and *Tagln* respectively, and ~2 for both *Gng3* and *Tubb3*, indicating that somatic and neuritic fractions were strongly enriched for the appropriate compartments (Fig. 3C).

Similarly, to test if collected protein fractions were enriched for soma and neurites markers, we performed western blot analysis of the compartments. We observed that nuclear protein, histone H3, was detected exclusively in the somatic sample, indicating that soma was efficiently removed and did not contaminate neuritic fraction (Fig. 3D). TUBB3 showed similar distribution between the compartments, with slight enrichment in the neurites (Fig. 3D). Finally, Neurofilament was highly enriched in the neuritic sample, showing that majority of the neurites migrated through and extended on the bottom of the filter (Fig. 3D).

After successful quality control for compartment separation, but before RNA can be processed for NGS library preparation, we used Bioanalyzer to test whether isolated RNA was intact. The analysis profile showed distinct 18S and 28S peaks with ratio of 1.9. (Fig. 3E). The RNA integrity number (RIN) was 9.2 (Fig. 3E), confirming RNA integrity.

After NGS, the data was processed with PiGx pipeline (see Sections 3.7–8). The pipeline generates a comprehensive report that provides an insight into data quality. We used the MA plot to assess data distribution. Neurite-enriched transcripts were distributed in the positive range of the Y-axis and soma-localized RNAs in the negative range (Fig. 4C). The enrichment in the given compartments was not dependent on the expression levels as localized transcripts spread along the X-axis representing mean normalized counts (Fig. 4C). Further, we used PCA to evaluate the reproducibility of the biological samples used. The triplicates for soma clustered together and away from neurite samples along the first principal component (Fig. 4D). These results demonstrate reproducibility of the data.

At this point the data can be visualized to identify and explore patterns and correlations. To examine whether translation rate is dependent on the relationship between protein and RNA enrichment in compartments, we overlaid Ribo-seq log2 FC N/S [9] as a color gradient on the 2-way correlation of proteomics log2 FC N/S and RNA-seq log2 FC N/S. This visualization allows us to quickly identify genes for which high translation rate (dark blue) is correlated with neurite localized transcript and proteins (upper-right quadrant). In addition to visualizing the continuous variables, like Ribo-seq fold change, we also plotted discrete variable, significance, using color as a third dimension on the 2-way correlation between protein and RNA localization (Fig. 4E). This analysis allows us to quickly identify genes for which both transcript and protein were significantly enriched in any of the compartments or had opposite localization.

5. Discussion

Soma and neurite separation using microporous membranes, in combination with genome wide analysis of transcriptome, proteome and translatome, is a powerful tool to investigate mechanisms regulating local RNA and protein metabolism in neurons. Using this approach, we have previously identified hundreds of neuritically localized and translated transcripts, including polyadenylation and alternative splicing isoforms [9,10]. Moreover, we showed that mRNA localization is a key determinant of protein localization in the neurites [9] and that alternative 3′UTRs can direct localization of functionally distinct protein isoforms to different subcellular compartments [10].

Using microporous membrane for separation of subcellular compartments offers a number of advantages over other approaches. It allows for effortless scaling of the method by adjusting the size of membrane inserts used (24, 12, or 6-well). For example, one 6-well plate with neurons differentiated from 3×10^6 mESCs generates ~6 μg neuritic and ~60 μg of somatic RNA. This is comparable with the RNA amounts (~4 μg) obtainable from one rat synaptic neuropil, a region of hippocampus containing dendrites, axons, glia and a sparse population of interneurons [3]. Microporous membrane does not enable separation of axons from dendrites, a drawback that can be overcome with microfluidic chambers [6]. At the same time, microfluidic chambers produce rather low yields of neuritic material, and have been more frequently used for visualization and manipulation of synapses than for omics analyses. For example, to obtain 500 ng of message-derived RNA from axons of rat sympathetic neurons, Andreassi et al. [29] isolated material from 22 chambers and subjected isolated RNA to 2 rounds of linear amplification, based on cDNA synthesis and in vitro transcription [30]. Using similar microfluidic chambers, Taylor et al. [31] obtained 60 ng of axonal RNA per device from E17 mouse and E18 rat cortical and hippocampal neurons.

As axons and dendrites extend at large distances from the cell body, numerous mechanisms exist to allow for rapid and autonomous responses to the localized stimuli. Thus far identification of pathways involved in these processes was limited to investigation of specific candidates. The neurite/soma separation scheme in combination with omics analyses can be easily adapted to identify, genome-wide, novel pathways involved in regulation of local transcriptome and proteome. For instance, changes in localized events can be analyzed in response to neuronal depolarization with KCl or modulation of specific neuronal receptors. Furthermore, mESCs can be easily genetically modified prior to differentiation, using silencing or overexpression, enabling assessment of the role of potential players in RNA-localization process. Application of this method to compare local transcriptomes, proteomes, and translatomes across different neuronal types could shed light on pathways utilized by specific neurons.

Aside from deciphering local RNA metabolism in normal neuronal function, neurite/soma separation offers an untapped potential for investigation of disease processes. Defects in RNA metabolism underlay many neurodegenerative disorders, including ALS and SMA. As distant neuronal protrusions rely heavily on local transcriptome for their

function, it is not surprising that axonopathy is the first sign of neurodegeneration and that neuronal decay begins in neurites [32,33]. Despite growing clinical need to identify targetable pathways underlying neurodegeneration, molecular mechanisms affected by defects in RNA metabolism and contributing to neuronal decay remain largely undiscovered. Therefore, neurite/soma separation scheme combined with human stem cell derived neurons are an excellent tool to examine mechanisms of disease. For example, human induced pluripotent stem cells (hiPSC) or embryonic stem cells can be used to generate motor neurons either by induction with small molecules [34] or through overexpression of Ngn2, Isl2, and Lhx3 (NIL) transcription factors [35]. Using hiPSCs is especially compelling, as they can be generated from patient-derived skin fibroblasts, and therefore, harbor specific genetic milieu and disease-driving mutations. Combined with CRISPR-mediated [36] correction of mutations to generate isogenic control neurons, neurite/soma separation could enable identification of RNAs and proteins differentially localized and translated in diseased neurons as compared to healthy cells. This analysis could shed new light on the fundamental pathways underlying neurodegeneration and suggest new approaches for their treatment.

Taken together, we described in detail neurite/soma separation scheme combined with omics analyses to investigate local transcriptomes and proteomes in neuronal cells. The flexibility of the method allows for straightforward adaptation to different neuronal types and experimental conditions. Thanks to feasibility of the approach, the method can easily be adapted by other laboratories. Finally, we believe that this method has a great potential to generate insights into normal neuronal function and disease processes affecting neurons.

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