In highly polarized cells such as neurons, compartmentalization of mRNA and of local protein synthesis enables remarkably fast, precise, and local responses to external stimuli. These responses are highly important for neuron growth cone guidance, synapse formation, and regeneration following injury. Because an altered spatial distribution of mRNA can result in mental retardation or neurodegenerative diseases, subcellular transcriptome analysis of neurons could be a useful tool for studying these conditions, but current techniques, such as transcriptome analysis of neurons could be a useful tool for studying these conditions, but current techniques, such as in situ hybridization, bulk microarray, and RNA-Seq, impose tradeoffs between spatial resolution and multiplexing. To obtain a comprehensive analysis of the cell body versus neurite transcriptome from the same neuron, we have recently developed a label-free, single-cell nanobiopsy platform based on scanning ion conductance microscopy that uses electrowetting within a quartz nanopipette to extract cellular material from living cells with minimal disruption of the cellular membrane and milieu. In this study, we used this platform to collect samples from the cell bodies and neurites of human neurons and analyzed the mRNA pool with multiplex RNA sequencing. The minute volume of a nanobiopsy sample allowed us to extract samples from several locations in the same cell and to map the various mRNA species to specific subcellular locations. In addition to previously identified transcripts, we discovered new sets of mRNAs localizing to neurites, including nuclear genes such as Eomes and Hmgb3. In summary, our single-neuron nanobiopsy analysis provides opportunities to improve our understanding of intracellular mRNA transport and local protein composition in neuronal growth, connectivity, and function.

In highly polarized cells, such as neurons, compartmentalization of mRNA and local protein synthesis is implemented in rapid, remarkably precise, local responses to external stimuli, allowing tight and accurate regulation of subcellular composition and content (1). This implies that at each subcellular compartment, the amount of specific proteins depends on the local rate of translation rather than the absolute amount of mRNA (i.e. transcription) (2). So far, analysis of mRNA species in dendrites and axons has revealed thousands of transcripts that are differentially localized (3–7). Some sequence motifs at the 3′-UTR, 5′-UTR, and retained intron regions of the mRNA have been found to regulate the localization of transcripts to neuronal processes (8, 9) in the translationally repressed state during mRNA trafficking (1). In addition, mRNA transport and local translation are involved in different aspects of neuronal homeostasis, such as growth cone guidance (10, 11), axon maintenance (12), injury response (13), and synapse and memory formation (14). Altered mRNA transport and translation can result in devastating consequences, including mental retardation or neurodegenerative disease, such as amyotrophic lateral sclerosis (15).

Comparative subcellular transcriptome analysis of neurons has faced many technical limitations. To detect genes specific for the axons or dendrites, the neurites must be separated from the soma. This can be achieved either by culturing neurons in compartmentalized chambers (3, 4); microdissection of specific brain areas where the cells have highly ordered, uniform arrangement, e.g. the CA1 region of the hippocampus (5); or laser microdissection and glass micropipette aspiration of neurites of cultured neurons (16–19). Currently available techniques (such as in situ hybridization, bulk microarray, or RNA sequencing) impose a tradeoff between spatial resolution and multiplexing; in situ hybridization can visualize only a few kinds of transcripts at a time, whereas when tissue, cells, or whole neurites are harvested for multiplexed microarray or RNA sequencing, all spatial information is lost. In addition, previous studies used different cell types for axonal and dendritic transcriptome analysis, making data comparison very difficult. There was no available method for multiplexed, neurite transcriptome analysis at the single-cell level. Our group recently developed a label-free, single-cell nanobiopsy platform based
mRNA localization in neurons revealed by nanobiopsy sampling

A. approach

penetration

nanobiopsy

analysis

B

Figure 1. Schematic of single cell nanobiopsy. A, illustration of automated approach to cell surface, penetration in the cell cytosol, followed by controlled aspiration of cytoplasmic material by electrowetting. B, optical micrographs of a neuronal cell projection nanobiopsy procedure illustrating the cell membrane penetration and aspiration via electrowetting. Two nanobiopsy samples were collected from the same neuronal projection. Scale bars, 25 μm.

Results

Nanobiopsy sampling of neuronal cells

To study the spatial pattern of mRNA compartmentalization within neuronal cells, we extracted samples from the cell bodies and neurites of neurons using our nanobiopsy platform. The SICM-based setup maps the cell surface by measuring the ion current flowing through a quartz nanopipette. Our custom-coded control software enables automatic cell surface finding, penetration, and aspiration of cellular material through electrowetting (Fig. 1). The aspiration volume is ~50 fl, corresponding to 1% of the cell’s total volume. Our method of sampling causes minimal damage to the cells such that cell viability is maintained and thus several samples can be collected repeatedly from the same cell (20). We collected 43 nanobiopsy samples from 33 cells in total, of which 13 samples were derived from cell bodies and 30 from neurites (Table S1). The cDNA was prepared from each individual sample for library preparation. Altogether, we generated 43 single-nanobiopsy RNA-sequencing libraries. Because of the minute amount of input RNA used, the sequencing depth ranged between 10 and 2900 mapped reads per nanobiopsy sample.

The timing of nanobiopsy can be controlled by adjusting the voltage applied to the electrode, targeting a very small subcellular region, with practically no contamination from other sources. Thus, even genes displaying a low number of reads mapped uniquely to an annotated transcript may be scored positive for expression. To confirm this, we prepared negative control sequencing libraries from External RNA Controls Consortium (ERCC) RNA Spike-In Mix RNAs (Ambion) without adding any RNA derived from nanobiopsy samples. The controls consist of a set of unlabeled, polyadenylated transcripts designed to be added to an RNA analysis experiment after sample isolation, to infer library quality (21). Contrary to our nanobiopsy sequencing libraries, we had very few reads mapped to human genome (Table S2, Sample_NC). As for the false negatives, we must consider the following two things. ERCC RNA Spike-In Mix was added to each nanobiopsy sample for quality control and sensitivity check (Fig. S1A). After RNA sequencing read mapping and analysis of the ERCC reads, we found that the lower limit of reliable detection is ~50–100 mRNA copies (Fig. S1B). Thus, if a nanobiopsy sample contains less than 50–100 copies of a transcript, it is likely that it will not be detected and will be a false negative.

We considered genes to have higher expression level if they had at least four reads mapped in a nanobiopsy sample (approximately the top 20% of genes). Mapping of the sequencing reads to the human genome identified 10–1000 genes expressed in each sample (Fig. 2, A and B, and Table S2). This very wide range indicates that the concentration and/or homogeneity of mRNA transcripts vary greatly depending on the precise subcellular location. The total number of genes found in all 43

The abbreviations used are: SICM, scanning ion conductance microscopy; ER, endoplasmic reticulum; SOM, self-organizing map; GO, Gene Ontology Enrichment Analysis; RNA-seq, RNA sequencing; SSC, saline-sodium citrate.

don scanning ion conductance microscopy (SICM),2 which uses electrowetting within a quartz nanopipette to extract cellular material from living cells with minimal disruption of the membrane and cellular milieu. Using electron microscopic measurements and geometrical calculations, this volume was estimated to be ~50 fl, which corresponds to ~1% of the volume of a cell (20).

In this study, we used our nanobiopsy platform to extract samples from the soma and neurites of human induced pluripotent cell-derived iCell® neurons and analyzed the mRNA pool by multiplex RNA sequencing. Because of the minute volume of a nanobiopsy sample, it was possible to extract cytoplasm from multiple locations in one cell. We found that the subcellular mRNA pools showed great mosaicism and that cell regions are fundamentally different from each other in terms of their mRNA composition. Neuronal cell bodies showed enrichment for transcripts encoding proteins involved in transcriptional regulation and protein transport, whereas neurites were enriched in genes related to protein synthesis, protein targeting to endoplasmic reticulum (ER), and mRNA metabolism. In addition to the previously identified transcripts, we report a new set of mRNAs that specifically localize to neurites, including mRNAs encoding proteins that were previously believed to localize exclusively to the nucleus. Here we provide evidence that single-neuron nanobiopsy studies can deepen our understanding of mRNA compartmentalization and open the possibility to study the molecular mechanism for specific neuronal functions, cellular circuitry, neuronal growth, and network formation.

2 The abbreviations used are: SICM, scanning ion conductance microscopy; ER, endoplasmic reticulum; SOM, self-organizing map; GO, Gene Ontology Enrichment Analysis; RNA-seq, RNA sequencing; SSC, saline-sodium citrate.
nanobiopsy samples was 2011, of which approximately ~1300 mRNA transcripts were identified in cell bodies and ~930 in neuronal processes (Fig. 2C and Table S3). The list of the most abundant transcripts based on the number of nanobiopsies in which they were detected can be seen in Table S4. Previously, other groups have used laser microdissection or aspiration by glass pipette to collect individual neurites from cultured neurons for transcriptome analysis. Zivraj et al. (16) used laser-capture microdissection to collect axon segments. After pooling 250 axon segments and microarray analysis, they identified...
~5000 transcripts that localize to axon. Francis et al. (18) used glass micropipette to collect a pool of 100–400 dendrites for microarray analysis and found ~11,000 genes in dendrites. The advantage of neurite microdissection compared with bulk sampling was that contamination from cell body could be reduced very significantly. The most important difference between microdissection and nanobiopsy sampling is the amount of sample being collected. When hundreds of whole neurites are dissected and pooled, a large amount of input material is present; therefore a high number of transcripts can be identified. A single nanobiopsy, however, collects ~1% of the volume of a cell. This enables us to collect samples from very small, targeted area. The number of transcripts sequenced is smaller compared with microdissection sampling, but we can get a much higher spatial resolution. In addition, the cell viability does not decrease after the procedure, allowing us to collect multiple small samples from the same cell.

We analyzed this high dimensional data set using the self-organizing map (SOM) described by Kim et al. (23). The SOM provides an intuitive way to visualize and examine the repertoire of genes in each nanobiopsy sample. We mapped 2011 genes onto a SOM, where each hexagon represents a set of genes whose expression patterns are most similar to one another. These units are clustered and located in the same positions across all nanobiopsy components of the SOM. Thus, in this 2D heat map, spatial proximity corresponds to expression pattern similarity. Each nanobiopsy transcriptome was visualized as a component of SOM (Fig. 2D; four representative nanobiopsy components shown for each cellular compartment type).

**Nanobiopsy is an unbiased and sensitive way of neurite transcriptome sampling**

Nanobiopsy can sample repeatedly from cell bodies and neurites of the same cell, and because we can collect sample from a very small subcellular area, the transcript sets do not need to undergo any data filtering. To examine the sensitivity of nanobiopsy sampling, we compared the mRNA repertoire in our nanobiopsy samples to all genes detected in axon or dendrite by bulk microarray analysis, RNA sequencing, and in situ hybridization in previous studies (3–7, 16, 18, 19, 24–41) (Fig. 3A and Table S5). Sampling from 30 neurites enabled the identification of 929 transcripts that localize to neuronal processes. Although the total number of genes was smaller compared with bulk sampling, we found 178 transcripts in neurites that were not detected in previous studies (Fig. 3A). When performing bulk sampling, the relative number of rare transcripts is very low, partly because the fragments can be lost during purification steps, and their sequencing read signal intensities may be regarded as noise. Our nanobiopsy platform can collect a miniscule amount of cytosolic material, and a rare transcript could be present in a relatively higher amount in a smaller sample. Nanobiopsy samples are directly reverse transcribed, and the cDNA is amplified, without cell lysis or RNA purification steps, thus further reducing the loss of rare transcripts.

The new discovered transcripts localizing to neurites belong to various functional categories, such as neuron development, immune system, cell signaling, and even proteins annotated as nuclear. Some of the new genes we found encode proteins involved in immune response. Our highly sensitive nanobiopsy sampling of neuronal cells has shown for the first time that the mRNAs encoding the Toll-like receptor 5 (Tlr5) are part of the mRNA repertoire of neurites. We were surprised to see that many of the new transcripts we sampled from neuronal processes encode proteins that function in the nucleus. This includes transcription factors, which are important in nervous system development, such as EOMES. Nanobiopsy sampling has also shown that mRNAs encoding many histone-remodeling enzymes are transported to neurites as well, such as the lysine demethylase Kdm5c and general constituents of the DNA replication or the transcription machinery such as the double-stranded DNA unwinding protein HMGB3, the mini-chromosome maintenance binding protein MCMBP, and the transcription elongation factor TCEAL7.

To verify the localization of these transcripts in neurites, we performed fluorescent in situ hybridization on a chosen set of genes (Fig. 3C). We selected a diverse set of genes for the validation experiment, including genes encoding nuclear proteins, a component of the translation machinery, a protein that has differential expression in brain, and a component of mitochondrial inner membrane. We put more emphasis on nuclear genes because their localization to neurites is less understood. All of the mRNAs for which we developed probes were detected in the neurites. Control experiments lacking the initial probe showed no fluorescent signal. The experiment has shown that these mRNAs are localized to neurites in only ~10–15% of the neurons and that they are present in neurites in a very low amount as shown by the low number of fluorescent spots. Some RNAs with low copy numbers in neurites included genes that encode nuclear genes such as the double-stranded DNA unwinding protein HMGB3, the mini-chromosome maintenance binding protein MCMBP, and the transcription elongation factor TCEAL7 (Fig. 3C). Some of the non-nuclear genes we detected were brain-expressed X-linked 5, eukaryotic translation initiation factor 2, subunit 3, and apolipoprotein A (Fig. 3C). Thus, fluorescent in situ hybridization confirmed that nanobiopsy is a sensitive and reliable method for the detection of low abundance transcripts in neuronal extensions.

**The cell body is a transport hub of neuronal mRNA and protein compartmentalization**

We next compared the list of genes we identified in our cell body and neurite nanobiopsy samples (Fig. 3B and Table S6). The two gene sets showed little overlap (~200 genes), demonstrating the functional difference between the two subcellular compartments.

To get a general overview of the repertoire of mRNAs that localize to neuronal cell bodies, we analyzed the pooled expression data from all cell body nanobiopsy samples (13 samples, 1292 genes) (Table 1, Table S7, and Fig. S2). This gene set was significantly enriched for the Gene Ontology (GO) terms “cel-
Table 1
Gene Ontology Enrichment analysis of all transcripts identified in all nanobiopsy samples taken from cell bodies and neurites of human induced pluripotent stem cell–derived neuronal cells

| Function                                      | Cell body p value | Neurite p value |
|-----------------------------------------------|-------------------|-----------------|
| Cellular localization                         | 2.23E-26          | 3.83E-15        |
| Intracellular transport                       | 9.66E-22          | 5.66E-14        |
| Cellular protein localization                 | 1.17E-20          | 7.52E-14        |
| Translational initiation                      | 3.97E-14          | 2.32E-11        |
| Cellular component biogenesis                 | 2.34E-13          | 2.34E-11        |
| Cytoskeleton organization                     | 2.64E-11          | mRNA metabolic process | 4.96E-11 |
| RNA processing                                | 1.76E-11          |                 |

Figure 3. Nanobiopsy sampling/RNA-seq is able to collect and identify rare transcripts that are not detected via bulk sampling methods. A, Venn diagram of the genes detected by neurite nanobiopsy, and the genes identified in axon and dendrite by previous bulk microarray, RNA-seq or in situ hybridization studies (3–7, 16, 18,19, 24–41). B, Venn diagram of the genes detected by cell body and neurite nanobiopsy of neuronal cells. All Venn diagrams are area-proportional; the labels indicate the number of genes. C, validation of nanobiopsy sampling and RNA-seq by high-resolution fluorescent in situ hybridization. The cells were first treated with a set of unlabeled predecoding oligonucleotides binding the target gene and subsequently hybridized with fluorescently labeled decoding oligonucleotides. Transcripts validated include Hmgb3, Mcmbp, Tceal7, Bex5, Eif2s3, and Apoo. White spots encircled with red indicate fluorescent spots corresponding to the target mRNA. Blue indicates 4',6'-diamino-2-phenylindole–stained nuclei.
Table 2  
Gene Ontology Enrichment analysis of all transcripts that had at least four reads mapped to the human genome in cell body nanobiopsy samples taken from human iPS-derived neuronal cells

| Category                        | p value   |
|---------------------------------|-----------|
| Neuron projection development   | 4.25E-7   |
| Nervous system development      | 1.78E-6   |
| Microtubule-based process       | 1.08E-5   |
| Neuron differentiation          | 1.58E-5   |

mRNA localization in neurons revealed by nanobiopsy sampling

To get a deeper insight into the spatial dynamics of mRNA compartmentalization and distribution in neuronal cells, we analyzed single-nanobiopsy components of the SOM (Fig. 4). Because the amount of cytosolic material collected during each biopsy sampling is only ~1% of the cell’s total volume, we could compare very small segments of the cellular space to each other. Nanobiopsy samples showed great variability in terms of the number (Fig. 2, A and B) and the identity of transcripts (Fig. 4) they contained. This indicated that the cellular mRNA pool cannot be considered well-mixed and homogeneous and that subcellular areas fundamentally differ from each other in terms of their mRNA composition. The two most commonly found gene clusters (Cluster 1 and Cluster 2) (Table S8) did not show any significant enrichment for any Gene Ontology term. These clusters included genes such as the large ribosomal subunit protein Rpl8, the DNA-unwinding protein Hmgb3, the mitochondrial import inner membrane translocase subunit Timm10, or the E3 ubiquitin ligase complex substrate adapter protein Keap1.

mRNAs encoding functionally related proteins often localize to the same subcellular place

To dissect the functional significance of the elaborate mRNA compartmentalization pattern in neuronal cells, we examined the gene clusters that showed similar expression patterns across samples (Fig. 4 and Table S8). Most gene clusters contained functionally unrelated transcripts. However, we were surprised to see that a subset of the gene clusters was enriched for a common GO term (Table S9). Cluster 4, found in one cell body nanobiopsy sample, was significantly enriched for the GO term “intracellular transport” (p value, 4.29E-11). 120 genes were annotated to this GO term, including the kinesin-like protein Kif13a or the protein transport gene Sec24d. Cluster 3, another cell body nanobiopsy sample, showed enrichment for the GO term “protein dephosphorylation” (p value, 1.31E-3). Seven protein phosphatases mapped to this GO term, including Ptpreb and Dusp1. Cluster 7, found in one of the neurite nanobiopsy samples, showed enrichment for the GO term “respiratory electron transport chain” (p value, 4.44E-7). Five mitochondrial genes were annotated to this term, including NADH-dehydrogenase subunits and cytochrome c-oxidase subunits. Cluster 14, found in another one of the neurite nanobiopsy samples, showed significant enrichment for the GO term “translational initiation” (p value, 7.60E-6). Nine genes were annotated to this GO term, including the ribosomal proteins Rpl39, Rpl10, Rpl11, and Rpl17. Overall, these results suggest that neuronal cells take advantage of sophisticated mRNA-transport mechanisms to establish defined mRNA compartmentalization patterns to fine-tune the molecular functions of the subcellular segments in response to extracellular stimuli.

Discussion

Neurons are highly polarized cells that take great advantage of compartmentalizing mRNA and locally translating it wherever and whenever it is needed (1). To examine the pattern of mRNA compartmentalization in neuronal cells, we extracted minuscule cytosolic samples from cell bodies and neurites using
Figure 4. Analysis of mRNA compartmentalization pattern in neuronal cells by nanobiopsy sampling and RNA-seq analysis using the self-organizing map. The self-organizing map consists of units representing a group of genes whose expression patterns across samples are most similar. The units are clustered and have fixed positions across all nanobiopsy components of the SOM. Individual nanobiopsy transcriptomes can be plotted by mapping the genes expressed in the sample onto the SOM. Gene clusters are defined as groups of units that are next to each other and are often co-expressed in cells. Above, representative single-nanobiopsy transcriptomes depicted as individual components of the SOM are shown. Boxes represent individual nanobiopsy samples taken from neuronal cell bodies (A) or neurites (B). Gene clusters are outlined in gray.
mRNA localization in neurons revealed by nanobiopsy sampling

our label-free, single-cell nanobiopsy platform, prepared the cDNA, and performed next generation RNA sequencing. Our easy-to-operate, flexible platform allowed us to sample from any subcellular compartment of neural cells with high spatial resolution and precision. We collected 43 nanobiopsy samples in total and identified more than 2000 transcripts.

Our analysis has shown that the repertoire of mRNAs that localized to cell bodies is highly enriched for genes related to intracellular transport including genes regulating cytoskeleton organization, protein and RNA-transport, vesicle trafficking, and transcription activation. This indicates the importance of distributing the mRNA and protein to the peripheral sites of the neuronal cell through highly regulated and sophisticated transport mechanisms.

The neurite transcriptome was highly enriched for genes related to protein synthesis, protein targeting to ER, ribosome biogenesis, and RNA metabolism. It has been reported before that neuronal growth cones respond to attractive (brain-derived neurotrophic factor, netrin) and repulsive (SEM3A, SLIT2B) guidance cues by regulating the local translation of the cytoskeletal protein β-actin and the actin cytoskeleton remodeling proteins coflin and RhoA (11, 45, 46). Newly synthesized β-actin has higher polymerization ability compared with old, covalently modified β-actin. Thus, it significantly affects growth cone dynamics (47). Neurotrophic factors also induce the axonal translation of Bcl2 that prevents axonal degeneration and promotes axon maintenance (12). Following neuron injury, local translation is required for neurite regeneration (13). Taken together, regulating local translation of mRNA and regulation of protein levels is one of the major ways that neurites respond to extracellular stimuli and maintain a physiologically accurate composition.

Our highly sensitive nanobiopsy sampling approach enabled the detection in neurites of ~200 very low expression level transcripts that could not be found by using previously employed bulk sampling methods. To our great surprise, many of these transcripts encode nuclear proteins, such as the transcription factor EOMES, the double-stranded DNA unwinding protein HMGB3, the mini-chromosome maintenance binding protein MCMBP, and the transcription elongation factor TCEAL7.

Although unexpected, there is a growing body of evidence indicating the presence of transcripts encoding nuclear proteins in axons and dendrites. Nuclear genes can have additional functions, unrelated to nucleus. The nuclear scaffolding protein Lamin B2 is translated in axons, where it binds mitochondria, and is required for the maintenance of axonal integrity (48). Some transcription factors (e.g. CREB and SMAD 1/5/8) are translated in axons following neurotrophic factor stimulation or injury, retrogradely transported to nucleus, and act as transcription factors to promote neuron survival (49, 50). Up to now there was only limited evidence showing the importance of the dendritic synthesis of transcription factors in the dendrites, soma signaling (51, 52). Ainsley et al. (6) have shown that H4 histone and Mediator complex member mRNAs are localized to dendrites. Thus, transcription factors synthesized in the periphery and interacting with the local signaling environment may carry some unique information or function, allow a local response, and contribute to the synapse–axon–nucleus signaling axis. In addition, the time delay required for these transcription factors to be translated in the axon and retrogradely transported to the nucleus may be an essential part of the temporal dynamics of neuronal cell physiology. Alternatively, the signal for transport to the nucleus may depend upon other environmental cues encountered at the periphery. Thus, our study can help establish the comprehensive list of nuclear genes that localize to neurites and help us shed light on the possible function of these genes.

We have also definitively shown that the neuronal mRNA pool cannot be considered well-mixed and homogeneous, in that subcellular areas fundamentally differ from each other in terms of their mRNA composition. This is in agreement with a previous study showing that in Drosophila embryos, >70% of the mRNA transcripts analyzed appeared to be regionally distributed (53). Nanobiopsy samples showed great variability in the number and the kind of transcripts they contained. Analyzing gene clusters that showed similar expression pattern across samples we found that some gene clusters showed functional enrichment. For example, mRNAs encoding genes related to cytoskeleton remodeling or protein translation localized to the same subcellular space. Recent reports have shown the presence of translationally active hot spots in cells (54–56) and that in primary neurons, mRNAs display “bursting” translation (57). Translation machinery components are often anchored to the plasma membrane and are being released upon activation of transmembrane receptors and initiation of local signaling, enabling local translation of mRNA (58). Furthermore, proteins synthesized at distal sites are more likely to be rich in assembly-promoting domains and are often regulated by post-translational modification sites (59). Thus, compartmentalizing mRNAs encoding proteins of related function to the same subcellular area can contribute to the coordinated, local synthesis of these proteins, and the formation of cellular multiprotein assemblies in response to extracellular stimuli. Some sequence motifs located in the 3’-UTR, 5’-UTR, intron, or coding sequence of an mRNA, as well as RNA-binding proteins regulating the transport of mRNAs to neuronal processes, have been identified (9, 60, 61). However, the mechanisms by which most of the several thousand mRNAs that localize to neuronal axons and dendrites are transported are still unknown.

Our nanobiopsy sampling and analysis revealed that neuronal cells take advantage of sophisticated mRNA-localization mechanisms to establish defined mRNA compartmentalization patterns. This allows neuronal cells to fine-tune the molecular functions of the subcellular segments according to an endogenous program, as well as in response to extracellular stimuli. This technique could be of use for further probing of the subcellular localization of mRNA in various polarized cells.

Experimental procedures

Neuronal cell culture

The neuronal cells cultured for this study were hiPSC-derived neurons (iCell neurons) obtained from Cellular Dynamics International (Madison, WI). The cryopreserved cells were a more than 95% pure population consisting of GABAergic and...
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glutamatergic neurons, with less than 1% of dopaminergic neurons (information provided by CDI, Madison, WI). For nanobiopsy experiments, the cells were plated at 20,000 cells/cm² density in 3.5-cm diameter, CELLview™ cell culture dishes (Greiner Bio-One GmbH, Rainbach im Mühlkreis, Austria) pretreated with 0.05% polyethyleneimine (Sigma–Aldrich) for 1 h and coated with 5.0 μg/ml laminin (Sigma–Aldrich) for 1 h. For fluorescent in situ hybridization experiments, iCell GABANeuron cells (new catalogue name for iCell Neurons) were plated at 10,000 cells/well on eight-well chambered coverglass (Thermo Scientific), which were precoated with 0.01% poly-l-ornithine (Sigma–Aldrich) for 1 h and 5.0 μg/ml laminin (Sigma–Aldrich) solution for 1 h.

The cells were grown in medium provided by Cellular Dynamics supplemented with 1% PenStrep (Lonza, Basel, Switzerland) at 37 °C, with 5% CO₂, 95% air. To better visualize cell morphology for nanobiopsy, the cells were transfected with pEGFP-c1-Tau (Kan resistance) plasmid using GeneJuice® transfection reagent (Merck Millipore, Billerica, MA), according to the manufacturer’s instructions. The plasmid was a generous gift of Steve Finkbeiner’s laboratory from the Gladstone Institute at University of California, San Francisco.

Electrowetting in nanopipettes

Nanopipettes were fabricated as previously described (20). Briefly, nanopipettes with a mean diameter of 106 ± 16 nm were made from quartz capillaries (Sutter Instrument, Novato, CA) using a P-2000 laser puller (Sutter Instrument). Quartz capillaries were filled with a 10 nm tetrahexylammonium tetraakis-(4-chlorophenyl)borate (THATPBCl) solution in 1,2-dichloroethane (DCE). An Ag/AgCl wire was then inserted into the barrel of the nanopipette, whereas an Ag/AgCl wire was submerged in the medium of the cell culture acting as a reference-counter electrode.

SICM setup

The scanning ion conductance microscope was set up as described previously (20). The SICM consisted of an Axopatch 200B low-noise amplifier (Molecular Devices, Sunnyvale, CA) for nanopipette bias and current measurement. The nanopipette was spatially manipulated with an MP-285 micromanipulator (Sutter Instrument) for coarse control and a Nano-piezo actuator (Physik Instrumente, Irvine, CA) for fine control. A PCIe-7851R field-programmable gate array (National Instruments) was used for hardware control of the system. A custom-coded software written in LabVIEW (National Instruments) was used to operate the system, enabling cell surface finding, penetration, and collection of cellular material automatically. The volume of aspirated cellular material was ~50 fl, which corresponded to ~1% of the total volume of an average cell.

RNA-seq, cDNA synthesis, and sequencing

cDNA was synthesized from aspirated RNA samples using the ultra low RNA input SMART-Seq system (Takara Bio USA, Mountain View, CA). The cDNA was prepared for each individual aspiration for library preparation. All 43 nanobiopsy samples and 1 negative control sample were supplemented with 0.5 μl of 1:5000 diluted ERCC Spike-In Mix 1 (Ambion) for quality control and sensitivity check. cDNA was amplified by a KAPA HiFi HotStart ReadyMix PCR kit (KAPA Biosystems, Wilmington, MA), using SMART-Seq PCR primers. cDNA was purified by AmPure XP beads (Beckman Coulter Genomics, Danvers, MA). The quality and quantity of single-cell cDNA was evaluated using the Agilent Bioanalyzer 2100 high sensitivity DNA assay (Agilent, Palo Alto, CA).

Tagmentation of cDNA for the preparation of RNA-seq libraries was performed by Nextera XT DNA library preparation kit (Illumina, San Diego, CA). Multiplexed sequencing adapters were ligated to tagmented cDNA by limited-cycle PCR (10 cycles), according to the manufacturer’s instructions. The final, amplified libraries were purified by AmPure XP beads (Beckman Coulter Genomics, Danvers, MA), and size-selected in the range of 200 – 600 bp using the Caliper LabChip XT system (PerkinElmer). The final RNA-seq libraries were quantified using the Agilent Bioanalyzer 2100 high sensitivity DNA assay and KAPA library quantification kit and pooled together in equal concentration for sequencing. The pooled multiplexed libraries were sequenced for 2 × 150 bp paired-end or 2 × 80 bp paired-end reads on Illumina MiSeq (Illumina, San Diego, CA).

RNA sequencing read mapping

RNA-seq reads were aligned to the hg38 UCSC human reference genome using STAR aligner (62). Genes were scored positive for expression if at least one read mapped uniquely to an annotated transcript. Gene expression was quantified using HTSeq (63). Venn diagrams were created by the BioVenn web application (64).

Gene Ontology Enrichment Analysis

Gene Ontology Enrichment Analysis was performed using the DAVID Functional Annotation tool (65). Relevant GO terms were visualized as tree maps using the REVIGO server (66).

Self-organizing maps

SOM were constructed as described (23). Before constructing the Self-Organizing Map, each gene expression value was normalized on a gene-by-gene basis by subtracting each gene expression mean and dividing by its standard deviation across libraries. A self-organizing map was trained with the 1,000 genes of the highest variance among the nanobiopsy samples, using the R package Kohonen (67). The total number of map units was set to the heuristic value 3*sqrt(N), where N corresponds to the number of genes used for the training. Initial values were assigned to the grid by multiplying the first two principal components of the data by a sinusoidal function and the training lasted 200 epochs. Clusters were seeded by the local minima of the u-matrix, with a value for each unit defined as the average of the vector difference between that unit’s prototype and its six neighbors on the hexagonal grid. All other unit prototypes were then assigned to clusters according to the minimum vector distance to a seed unit. All expressed genes were mapped onto the SOM, and visualized using a custom Python code. The lists of clustered genes were submitted to the DAVID Functional Annotation tool (65) to determine enriched terms.

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**Synthesis of decoding fluorescent oligonucleotides**

Quasar 570 labeled oligonucleotide probes were prepared according to the previously described method (22). Briefly, 1 nmol of 5’-amino-modified oligonucleotides (Integrated DNA Technologies, Coralville, IA) were dissolved in 3 μl of nuclease-free 1× PBS buffer (pH 7.4). To this solution, 3 μl of sodium tetraborate (1 m, pH 8.5) in anhydrous dimethylformamide were added. The reaction mixture was incubated at room temperature for at least 3 h. Subsequently, fluorophore-coupled oligonucleotides were purified by nucleotide removal kit (Qiagen) and then further purified via a HPLC equipped with C18 column (Agilent). The purified product was dried completely in SpeedVac, subsequently dissolved in 200 μl of nuclease-free 1× PBS (pH 7.4), and stored at 4 °C as a stock solution.

**In situ RNA labeling**

Cultured iCell GABANeurons were first washed with nuclease-free 1× PBS for 5 min at room temperature and then fixed with fixation solution (4% formaldehyde (Polysciences) in PBS) at room temperature for 20 min. Subsequently, the cells were washed twice with 1× PBS at room temperature, each for 5 min. Finally, fixed cells were permeabilized with 70% ethanol for overnight at 4 °C.

Unlabeled predecoding oligonucleotides belonging to one library (each with a 25 pmol) were dissolved in 100 μl of nuclease-free 1× PBS (pH 7.4) to obtain a stock solution. To 100 μl of predecoding hybridization buffer (100 mg/ml dextran sulfate, 20 μg/ml bovine serum albumin, 1 mg/ml Escherichia coli tRNA, 2 mM vanadyl ribonucleoside complex, and 10% formamide in 2× saline-sodium citrate (SSC)), 1 μl of stock unlabeled probe library was added to obtain predecoding hybridization solution. Fixed iCell GABANeurons were first washed with wash buffer (10% formamide in 2× SSC, 2 mM vanadyl ribonucleoside complex) for 5 min at room temperature. Then cells were incubated with the predecoding hybridization solution at 37 °C for overnight. Next day, the cells were washed with wash buffer twice at 37 °C for 30 min.

To 100 μl of decoding hybridization buffer (10% formamide in 2× SSC, 100 mg/ml of dextran sulfate, and 2 mM vanadyl ribonucleoside complex), 5 μl of Quasar 570 labeled stock probe solution was added to obtain decoding hybridization solution. Then cells were incubated with decoding hybridization solution at 37 °C for 30 min and subsequently washed with wash buffer at 37 °C for 30 min once. The stained cells were first equilibrated with freshly prepared GLOX buffer (0.4% glucose, 10 mM Tris-HCl in 2× SSC) and subsequently imaged in GLOX solution (1% catalase, 0.37 mg/ml glucose oxidase, 0.4% glucose, and 10 mM Tris-HCl in 2× SSC).

**Fluorescent in situ hybridization imaging and data processing**

The stained cells were imaged under a Nikon Ti-E epifluorescence microscope equipped with 100X objective, using a 5-μm z range and 0.3-μm z spacing. All images were captured by CoolSNAP HQ2 camera and analyzed with NIS-Elements imaging software. Chroma filter 49004 was used to image Quasar 570 channel. All z images were stacked and aligned using ImageJ software.

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