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mRNA transport & local translation in neurons

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Neurons are amongst the most structurally complex cells and exhibit a high degree of spatial compartmentalization. Also, neurons exhibit rapid and dynamic signaling by processing information in a precise and, sometimes, spatially-restricted manner. The signaling that occurs in axons and dendrites necessitates the maintenance and modification of their local proteomes. Local translation of mRNAs into protein is one solution that neurons use to meet synaptic demand and activity. Here we review some of the key findings and recent discoveries that have shaped our understanding of local translation in neuronal function and highlight important new techniques that might pave the way for new insights.

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RNA localization—the local transcriptome

In the early 1990s, the first studies demonstrating mRNA localization in neurons [1,2] using in situ hybridization techniques were published. These were followed by studies using advanced RNA labeling methods (eg, RNA fluorescence in situ hybridization (RNA-FISH)) demonstrating that the localization patterns of specific mRNA species varied significantly between brain regions and neuronal subtypes [3–5]. For example, the Allen-Brain-Atlas project [6] used a high-throughput procedure for in situ hybridization and data acquisition to describe gene expression patterns with a cellular level resolution. This approach revealed, without optimizing for the detection of dendritic mRNAs, about 65 mRNAs in dendrites. More recently, however, microarray studies and RNA deep sequencing have identified a much larger population of localized mRNAs. Microarray analysis of RNA isolated from mechanically separated processes of hippocampal cultures grown on porous membranes or from the dissected stratum radiatum of the hippocampal CA1 region identified □100 and 178 localized mRNAs, respectively [7,8]. Six years later, the combination of RNA deep sequencing, NanoString analysis and high-resolution in situ hybridization expanded this pool of localized mRNAs to an astonishing number of 2550 mRNA species in the hippocampal CA1 neuropil (the local transcriptome) (Figure 3a) [9]. The collective efforts of these studies changed our notion of the capacity for mRNA localization and local translation but also presented new challenges to the field. For example, how many mRNA molecules actually populate a single dendritic arbor and how does this number relate to the amount of mRNA within the neuronal cell body? It also remains unknown how heterogeneous individual dendritic transcriptomes are and how they change in response to synaptic activity.

In the past few years, there has been extraordinary progress in the development of RNA sequencing (RNA-seq), enabling the sequencing of single cells [10–12] and revolutionizing our understanding of the diversity underlying complex cell populations. In the nervous system single-cell gene expression profiling has also been used to classify neural cell types by their RNA composition (reviewed in detail: Ref. [13]). Optimistically speaking, further refinements may enable the analysis of transcriptomes from even smaller units such as subcellular compartments. Only recently, two studies added morphological information and electrophysiological properties to the gene expression profiles of single neurons. In these studies, the cellular content of the soma was aspirated by a patch pipette to acquire RNA for RNA-seq. The multi-facted nature of the information obtained by this technique can facilitate the identification of neural subgroups that are indistinguishable by RNA-seq alone [14,15□]. In principal, a similar approach could be used to capture neuronal compartments such as dendrites or axonal segments to analyze their RNA content [16].

In addition to RNA-seq, exciting new approaches in single-molecule imaging have enabled the discovery of copy numbers and localization patterns of thousands of genes, and thus, could be a major advance in describing the spatial transcriptome of neurons. Prior to this, the constraints of spectral overlap had limited RNA-FISH techniques (reviewed in: Ref. [17]) to the simultaneous detection of a few mRNA candidates. These constraints have now been obviated by techniques that are based on sequential rounds of hybridization and imaging. For example, seqFISH [18,19] removes previously bound probes using DNAse digestion to free and reuse previously assigned color channels. This allows the generation of in situ mRNA barcodes and hence, exponentially

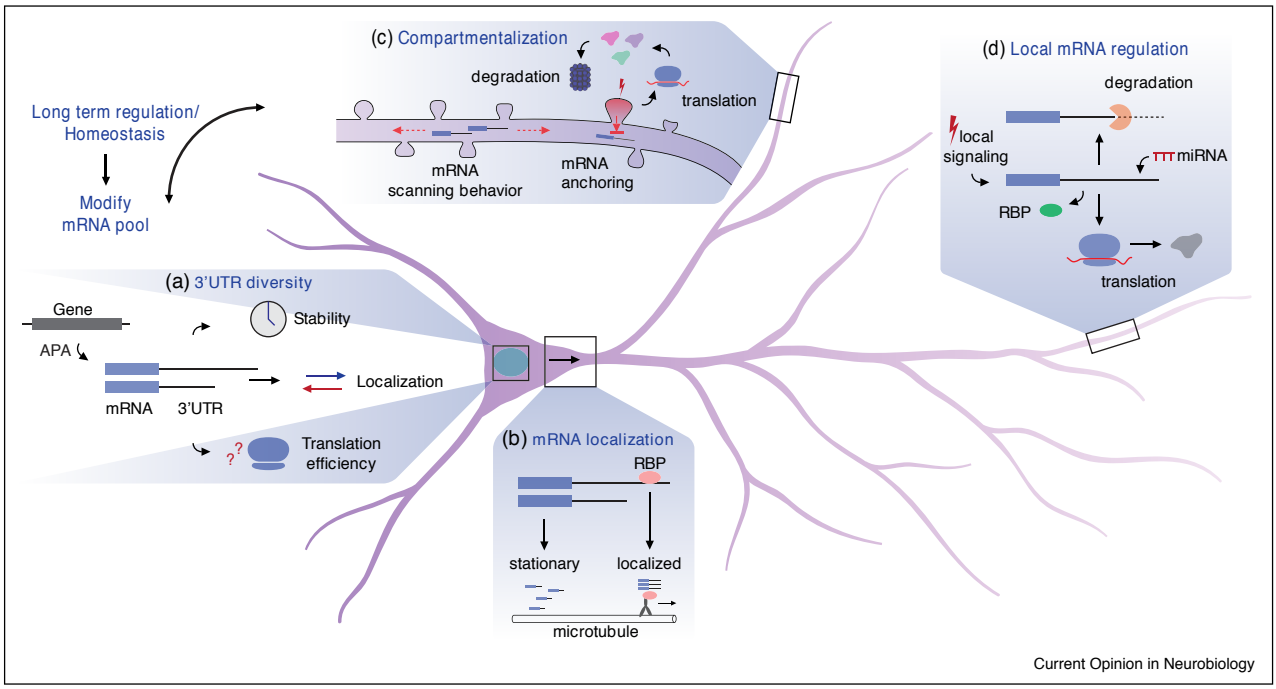
increases the number of mRNAs that can be studied in parallel. In addition, fluorescent in situ sequencing (FISSEQ) was developed [20,21]. This technique allows the simultaneous detection of up to thousands of genes by directly reading the bases of cross-linked complementary DNA amplicons within a biological sample. Another technique that relies on sequential hybridization is multiplexed error-robust FISH (MERFISH) [22,23▫]. Here, sequential hybridization is combined with an error-robust encoding scheme to enhance the sensitivity and accuracy of RNA identification. Taken together, these newly emerging in situ RNA imaging techniques promise to describe the unique localization patterns of genes in neuronal processes. However, their direct applicability in neurons remains to be tested. Also, it is still unclear how RNA characteristics such as structure, length and quality affect the sensitivity of these approaches.

RNA transport

As indicated above, recent microarray and RNA sequencing studies have dramatically expanded the list of localized mRNAs, spanning many functional gene classes, in dendrites and axons, [7–9]. These findings not only suggested enormous potential for local translation, but also raised the question of the specificity of mRNA transport. For many genes the specificity for localizing mRNAs to distinct regions of a cell is likely accomplished by RNA-binding proteins (RBPs), which bind cis-acting elements within the mRNA and attach them either to cytoskeletal motor proteins or adaptors for transport (reviewed in: Ref. [24]) or in some cases to anchors, which will tether the mRNA to a specific location (Figure 1b) [25,26]. These cis-acting elements or 'zipcodes' are mostly found in the 3^0 untranslated region (3^0UTR) of an mRNA (Figure 1a) [27]. It is now known that genes can give rise to transcripts with different 3^0UTR isoforms that differ in length and thus in the capacity to host regulatory platforms [28,29]. This diversity of 3^0UTRs is caused by the process of alternative polyadenylation (APA, reviewed in: Ref. [30]). By adding or removing sequences that harbor microRNA (miRNA) or RBP binding sites, for example, APA has the potential to potently affect the regulatory potential and hence the fate of an mRNA with respect to its localization, longevity and translation efficiency [31]. In some cases, 3^0UTRs can even drive the localization of the actual protein [32▫].

In comparison to other tissues, the brain predominantly favors extended 3^0UTR isoforms, suggesting an elevated need for posttranscriptional regulation in neuronal cells

Figure 1



Regulation of mRNA in neurons.
Alternative polyadenylation of mRNA precursors leads to the generation of an mRNA with different 3^0UTRs. The 3^0UTR serves as binding platform for RNA-Binding-Proteins (RBP) and other factors, which provides an opportunity for differential regulation of the same mRNA species (a). Utilizing this mechanism, the cell can regulate mRNA translocation to distal dendritic/axonal parts (b). mRNAs sorted into dendrites can exhibit a 'scanning' behavior and tend to be anchored close to previously activated synapses to undergo lcoal translation (c). mRNAs may not only be regulated by anchoring them at sites of translation but also regulated by local translational efficiency or mRNA stability (d).

[33]. However, the functional consequences of differential 3′UTR isoform choice and usage in neuronal compartments has not yet been studied. In future studies, sequence analysis of localized 3′UTR isoforms may be used to determine motifs that can predict mRNA localization. To analyze protein binding to RNA, methods such as HITS-CLIP [34,35] (see also PAR-CLIP [36], iCLIP [37], eCLIP [38], hiCLIP for secondary structure binding sites [39]) can then be used to confirm computationally predicted binding sites or define new binding sites. These methods make use of ultraviolet cross-linking of RBPs to their cognate RNA molecule followed by immunoprecipitation against a protein of interest. When coupled to high-throughput sequencing, these methods can provide a comprehensive genome-wide identification of protein–RNA interaction sites. CLIP data, however, only provides snapshots of interactions and do not describe the dynamics of the protein–RNA interaction. In a recent study, dynamics were examined by combining heterospecies partition analysis with single-molecule mRNA detection to measure the association between zipcode binding protein 1 (ZBP1) and b-actin mRNA in hippocampal neurons. This approach showed that ZBP1 predominately binds b-actin mRNA perinuclearly in the soma, then the complex moves into dendrites before ZBP1 is released prior translation [40].

It is still unclear whether mRNAs form static outposts in dendrites to fuel local protein synthesis or if they are dynamic and shared between compartments. In single-molecule imaging experiments both dynamic and stationary mRNAs had been detected [41,42]. However, a more recent study reported that most of the particles of b-actin mRNA are indeed dynamic when monitored over a longer time. In this study mRNAs exhibited a scanning behavior by alternating between mobile and stationary phases (Figure 1c) [43]. These data indicate that at least some mRNAs not only function as anchored outposts but also can be shared between compartments along the length of a dendrite. In addition, this study described a scenario in which b-actin mRNAs are specifically transported to sites of neurotransmitter stimulation, where they are directly translated into actin protein. Interestingly, synaptic activity did not change the total net count of b-actin mRNAs in the dendrites, (*eg*, due to an increased transport from the soma) but rather promoted the redistribution of b-actin mRNAs from local pools [43].

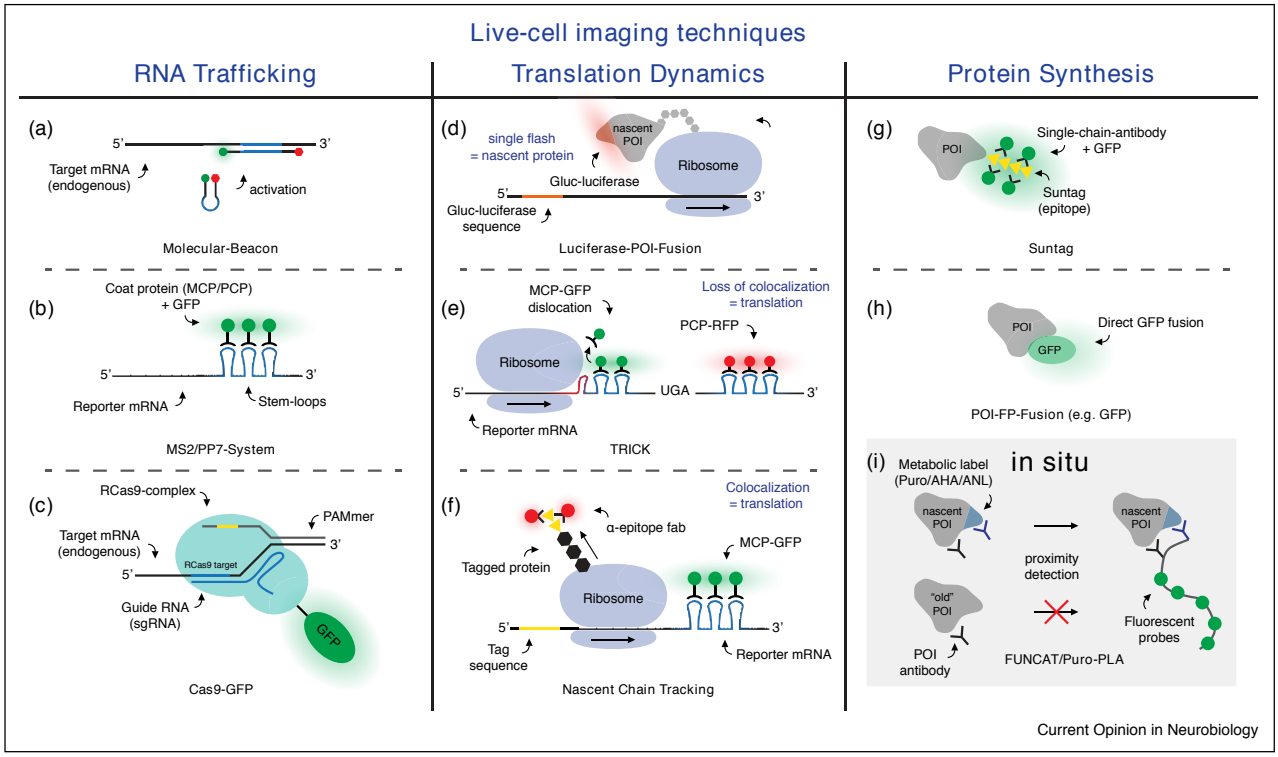
To date, the majority of single-molecule mRNA tracking experiments have relied on exogenous reporters to describe mRNA dynamics. For example, the MS2 bacteriophage system makes use of the binding between the MS2 coat protein (MCP) and a unique RNA hairpin sequence (stem-loops) that is cloned into the 3′UTR of the mRNA of interest. The fusion of a fluorescent protein to the MCP enables the live-cell imaging of mRNA dynamics (Figure 2b) [44]. More recently, similar systems from other bacteriophages such as l and PP7 have been discovered and can be used for the simultaneous monitoring of two mRNA species (reviewed in: Ref. [45]). It is not clear, however, the extent to which these reporters actually reflect the dynamics of endogenous mRNAs. So far this has only been shown for b-actin mRNA using a transgenic mouse line that expresses an MS2-cassette in the 3′UTR of the essential b-actin gene [41,42]. In future studies, tagging endogenous loci by genome editing may be used to extend current methods and illuminate mRNA dynamics in a more physiological context [46]. In addition to the MS2 system, a Cas9-GFP fusion protein was successfully used to tag endogenous, unmodified mRNAs (Figure 2c) [47□].

Local translation

Now knowing the enormous extent of mRNA localization in neurons, the question arises: which mRNAs are used to produce protein locally? Also, little is known about how local protein expression is regulated as a function of mRNA abundance, protein production and degradation [48] (reviewed in: Ref. [49]). For example, how efficiently do local mRNAs have to be translated to fuel the local proteome? Recent studies have described substantial discrepancies between changes in mRNA abundance and protein production [50,51]. It is conceivable that a higher translation efficiency might be useful in distal neuronal compartments, where fewer copy numbers of individual mRNAs have been detected by *in situ* hybridization. On the other hand, protein life cycle measurements, using stable isotope labeling with amino acids in cell culture (SILAC) and mass spectrometry (MS), of cortical neuronal cultures revealed that nearly all synaptic proteins exhibited low turnover rates with half-lives in the range of 2–5 days [52]. This raises the question as to whether there exists only a very exclusive demand for the local production of highly localized proteins, which could potentially be even accomplished by translation from a monosome (a single ribosome loaded onto an mRNA). Recently monosomes have been shown to be translationally active in yeast [53□]. In addition, it remains largely unknown which mRNA species are constitutively translated in neuronal processes and which are translated on demand, for example following particular patterns of synaptic activity (*eg*, Ref. [54]).

A strategy that has been proven to be successful in addressing the above questions is the visualization and identification of newly synthesized proteins. For example, the antibiotic puromycin can be used to label sites of protein synthesis [55,56]. Puromycin integrates itself in growing peptide chains thus ‘labeling’ nascent proteins and can be subsequently detected by an antibody. Only recently, a photolabile-protecting group was added to puromycin to render it inactive until it is exposed to UV light [57,58]. By using a 2-photon laser, this puromycin variant can be ‘activated’ with high spatial and

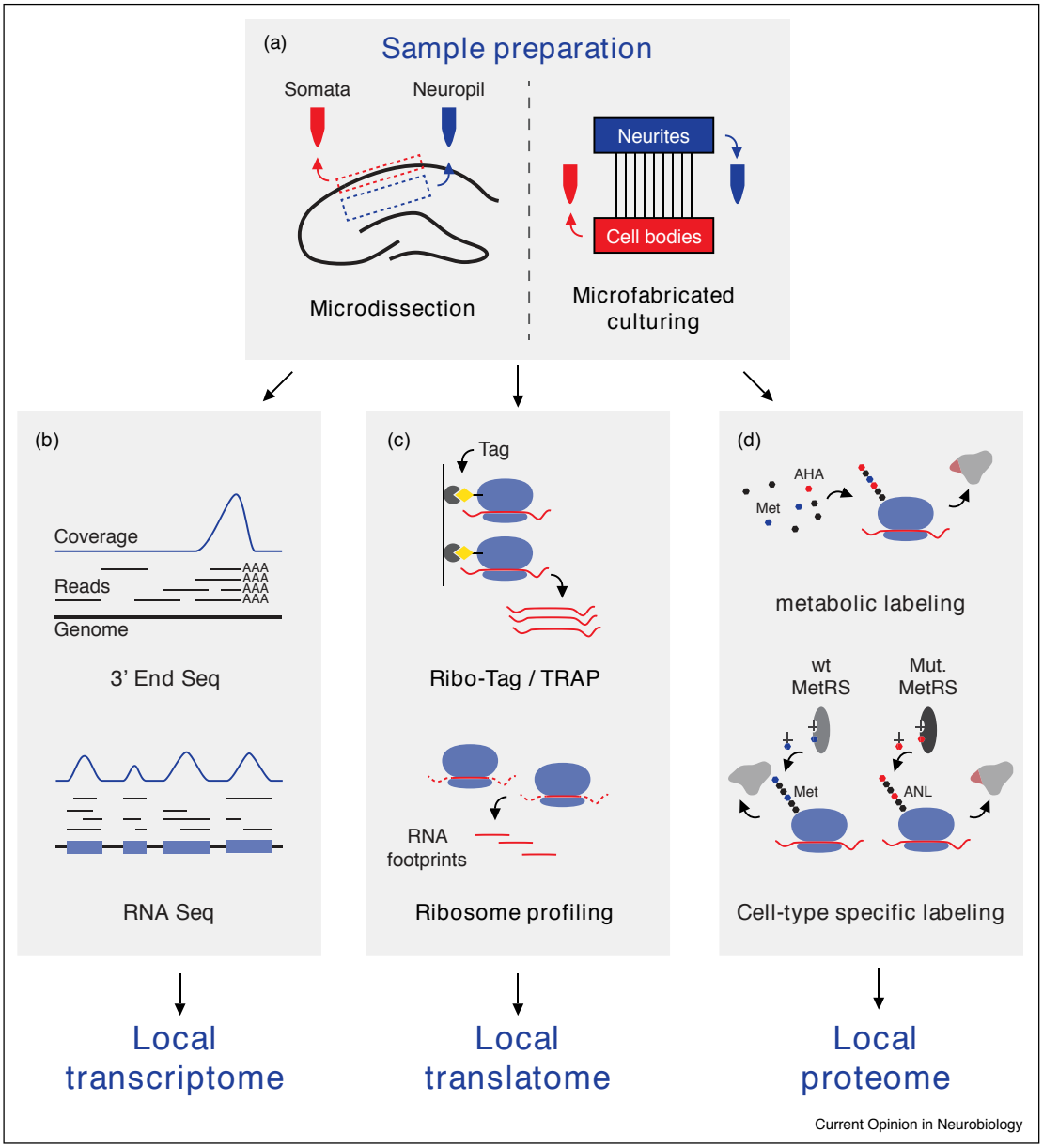
Figure 2



Technical overview on optical methods to follow different cell biological processes in living cells.
(a) Molecular-beacons are small hairpin shaped oligonucleotides with an internally quenched fluorophore in the unbound state. Fluorescence is restored upon binding the target mRNA sequence and the mRNA's position and dynamics can be monitored [85].
(b) The MS2/PP7-system utilizes the MS2/PP7 bacteriophage coat-protein fused to a GFP/RFP to recognize a mRNA reporter containing stem-loop structures from the phage genome [44,86].
(c) The RCas9–GFP fusion-complex can be specifically targeted to endogenous mRNA using a guide-RNA (sgRNA). No exogenous tags are required [47▫].
(d) The rapid maturation of the Gaussia luciferase (Gluc) can be exploited as a translation reporter. Following translation of a Gluc containing reporter mRNA, the luciferase within the nascent peptide consumes a substrate and emits a single flash [87].
(e) Translating RNA imaging by coat protein knock-off (TRICK) utilizes the MS2-stem-loops and PP7-stem-loops to label a reporter mRNA within the CDS and 3^{0}UTR with two different colors. The coat-proteins recognizing the stem-loops in the CDS are displaced during translation whereas the coat-proteins binding in the 3^{0}UTR are not. The subsequent loss of spectral colocalization serves as a read-out for translation [88▫].
(f) Nascent chain tracking (NCT) can sense translation by encoding a tag sequence within a reporter mRNA that, once translated, is recognized by antibody-based fluorescent probes. Another RNA-tag (MS2-system) within the reporter allows for simultaneous mRNA-detection [40,79,80,82].
(g) The Suntag-methodology utilizes a repeating peptide array fused to the protein of interest (POI), which can recruit multiple antibody-gfp-proteins for long-term imaging of single proteins [89].
(h) The classic approach consisting of a direct fusion between POI and a fluorescent protein (e.g., GFP). Limited single molecule imaging capabilities and photostability.
(i) Fluorescent non-canonical amino acid tagging (FUNCAT) in combination with a proximity-assay (PLA) is an in situ technique to detect specific nascent endogenous proteins in a spatiotemporal manner. Metabolic labeling with non-canoncial amino acids (e.g., AHA/ANL) or certain antibiotics (e.g., puromycin) tag nascent proteins. Subsequent antibody detection of the metabolic modifications and a specific POI lead to a signal amplification only if both antibodies are in close proximity. Thus only nascent POI are fluorescently labeled [65].

temporal resolution and thus can be potentially used to examine local protein production. The same strategy had already successfully been used to apply anisomycin, a translation inhibitor, locally [59]. In addition, new methods have been developed that make use of noncanonical amino acids to selectively label the pool of newly synthesized proteins. These amino acids freely cross cell membranes, get charged onto tRNAs by the cell's own tRNA synthetase, and then get incorporated into nascent polypeptides. These techniques, bio-orthogonal noncanonical amino acid tagging (BONCAT; [60]) and fluorescent noncanonical amino acid tagging (FUNCAT; [61,62]) can be used to purify or visualize newly synthesized proteins, respectively. The combination of these metabolic labeling approaches with a proximity ligation assay (PLA) [63,64] enables visualization of newly synthesized proteins of interest (Figure 2i) [65]. This has been used, for example, to detect the local suppression of

Figure 3



System approaches to acquire locally restricted information on transcripts and proteomes.
To get comprehensive insights into the RNA/protein composition of subcellular regions, pure samples with high yield of the targeted structures are required. This can be achieved by mechanically dissecting highly structured regions of the brain (e.g., the hippocampus) or by utilizing microfabricated culture techniques (e.g., microfluidic chambers or porous membranes) in which growth-barriers lead to a separation of subcellular structures (a). These compartment-enriched samples are then subjected to different down-stream applications depending on the desired information. Isolated RNA can be sequenced to obtain the local transcriptome and depending on the sequencing technique, further insights on specific mRNA populations can be obtained (e.g., 3'UTR diversity) [90–92] (b). By isolating ribosomes first and subsequently eluting bound mRNA the local translatome can be gained, the subpopulation of the local transcriptome that is currently translated [70,71] (c). Mass spectromentry on isolated proteins yields information on the protein population. Combining this approach with metabolic labeling enables the detection of the nascent or cell-type-specific proteome (d).

CamK2a synthesis within dendrites following maturation of a miRNA (Figure 1d) [66□□]. Recent work has increased the specificity of bio-orthogonal labeling techniques by restricting the labeling of nascent polypeptides to specific cell types. This has been achieved by using the noncanonical amino acid, azidonorleucine (ANL), which requires the expression of a mutated tRNA synthetase to be charged onto its tRNA (Figure 3d) [67]. This

approach has been used in *Caenorhabditis elegans* and *Drosophila melanogaster*, to purify cell type-specific proteomes, including neurons [68,69].

As an alternative to proteomic approaches, protein production can also be determined by examining the mRNAs that are actively translated (translatome). Methods such as TRAP (translating ribosome affinity purification) [70] and Ribotag [71] allow for the cell type-specific measurement of translating mRNAs in complex tissues, including the brain (Figure 3c) [72,73]. Both techniques utilize the expression of a tagged ribosomal subunit (RPL22-HA; RPL10-GFP) that is used to immunoprecipitate the ribosomal complex along with its associated mRNA. The mRNA can then either be partially digested by RNase, and the ribosome protected fragments can be sequenced (ribosome profiling, see also Refs. [74–77]), or the entire mRNA can be sequenced, for example, to gain information about the translation of individual mRNA variants. Only recently, this approach was used to describe dynamic changes in the translatome of retinal axons during development in the mouse brain [78▪▪]. While ribosome profiling displays a genome-wide snapshot of the translatome, it represents the 'average' of the tissue subjected to the analysis and hence does not describe heterogeneity in translation dynamics between cells or subcellular compartments. Recently, several groups developed tools for live imaging of single mRNA translation *in vivo* that allow the direct monitoring of the location and the dynamics of protein synthesis (Figure 2f) [79,80,81▪▪,82] (reviewed in: Ref. [83]). All of these studies combine fluorescent tagging of individual mRNAs using the MS2 system with the rapid binding of fluorescent antibodies to tandem repeats of an epitope in the nascent peptide. These approaches provided the first direct observations of translation kinetics in cells (elongation rate: 3–10 codons per second), which roughly agree with previous measurements by ribosome profiling (elongation rate: 5.6 codons per second [93]. In addition, these studies have been the first to measure translation initiation rates, occurring every 30–40 s on actively translating mRNAs. The data obtained also highlighted a previously unrecognized heterogeneity in translation dynamics among individual mRNA molecules. In neurons, this approach revealed that the majority of the reporter mRNA was actively translated in proximal dendrites, whereas in distal dendrites the reporter mRNA tended to be translationally repressed. Interestingly, dendritically localized transcripts showed 'bursts' of translation compared to a constitutive translation behavior in the soma. Additionally, it was found that mRNAs can, in contrast to previous assumptions [84], actively translate while scanning the dendrite [81▪▪]. Together these studies have been the first to demonstrate mRNA-to-mRNA variability of translation within different subcellular compartments. In the future these methods might be used to describe translation in response to changes in neuronal activity with a high spatiotemporal resolution.

Summary

In recent years our view of RNA localization and translation in dendrites and axons has changed dramatically. There is now mounting evidence that the directed transport of mRNA followed by local translation provides a mechanism that likely compartmentalizes neurons with specific functions and facilitates the local processing of extrinsic signals, such as different synaptic inputs. Here we have highlighted some of the key findings in the field and sought to raise questions about mechanisms of local control that remain unanswered. Technical advances in both, systems and imaging approaches show great promise to tackle these questions and further decode the regulation of RNA function in neurons.

Conflicts of interest statement

Nothing declared.

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- ▪▪ of outstanding interest

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