Review

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Single-molecule insights into mRNA dynamics in neurons

Adina R. Buxbaum[1,2,3], Young J. Yoon[1], Robert H. Singer[1,2,3], and Hye Yoon Park[3,4,5]

[1] Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA
[2] Gruss Lipper Biophotonics Center, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA
[3] Janelia Farm Research Campus, Howard Hughes Medical Institute, 19700 Helix Drive, Ashburn, VA 20147, USA
[4] Department of Physics and Astronomy, Seoul National University, Seoul 151-747, Korea
[5] Institute of Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Korea

Targeting of mRNAs to neuronal dendrites and axons plays an integral role in intracellular signaling, development, and synaptic plasticity. Single-molecule imaging of mRNAs in neurons and brain tissue has led to enhanced understanding of mRNA dynamics. Here we discuss aspects of mRNA regulation as revealed by single-molecule detection, which has led to quantitative analyses of mRNA diversity, localization, transport, and translation. These exciting new discoveries propel our understanding of the life of an mRNA in a neuron and how its activity is regulated at the single-molecule level.

RNA localization in neurons

Neuron morphology, a complex arborization of hundreds of branches up to millimeters in length, raises the biological enigma of how proteins can be targeted to their appropriate destinations. Neural activity-dependent modifications in the molecular composition of neurons and synapses underlie normal brain function and behavioral changes [1]. One mechanism to precisely target proteins is through the transport of mRNAs to distal regions in neuronal branches combined with local protein synthesis [2]. There is much evidence to suggest that mRNA localization and local translation in neurites is important for proper neuronal physiology [3–5]. For example, synthesis of new proteins is necessary for the induction of synaptic plasticity [6–9] (reviewed in [10]). Considerable effort has been made to understand how mRNAs are targeted to distal regions in neurons, which RNAs are present and under which conditions, and how translation is regulated (reviewed in [2,11]). As mRNA labeling methods and optical sensitivity have improved, so has our understanding of these questions. Techniques to visualize single mRNAs in fixed and live cells deliver single molecule intracellular resolution of endogenous mRNAs in non-neuronal cells (reviewed in [12]) and can now be applied to the field of neurobiology. The advantage of single-molecule methods is that they provide absolute quantitative measurements of RNA expression. Single-molecule imaging allows the measurement of discrete states rather than ensemble averages; therefore, it reveals subpopulations of molecules or transient states of dynamic processes. These properties of single-molecule detection have provided important advantages for studying regulation of RNAs within all cells. The ability to identify single RNAs increases the confidence in expression abundance, whereas lower-resolution techniques have a higher probability of false positives because the threshold of detection of a specific signal is undefined. In live neurons, single-molecule approaches have expanded our ability to explore the precise mechanisms of RNA motility and localization [13,14]. Recent work highlighted here, utilizing single-molecule fluorescence *in situ* hybridization (FISH) to endogenous mRNAs and live imaging of single mRNAs in neurons has resulted in novel and at times even reformed insights into how mRNAs are localized in the brain [15–17]. For simplicity, technologies that are capable of yielding single-molecule resolution are discussed as single-molecule imaging without focusing on whether this sensitivity was achieved in each study. We further discuss insights into neuronal mRNA movements, targeting and translational regulation provided by single-mRNA imaging, and what the future hopefully holds for the field.

Neuronal mRNA distribution is highly diverse

The elongated morphology of neurites implies that active transport plays a role in the delivery of RNAs to distal regions. Due to the high energy demands of active transport, RNA localization must be selective for RNAs with critical local roles. The hierarchal and segregated structure of the hippocampus facilitates the differentiation of somatic and neurite layers, offering a platform to address this hypothesis via the quantification of RNA in different regions. Early demonstration of *in situ* hybridization in hippocampal slices and cultured neurons distinguished mRNAs that were present in dendrites from those that were selectively excluded [18–20]. Moreover, contrasting patterns of mRNA localization in different hippocampal subfields strongly suggested that not only are neurons capable of sorting mRNAs, but localization of a single species of mRNA could vary between neuronal subtypes and brain regions [18,21–24]. How the differential subcellular localization of a single mRNA species is accomplished remains unknown. Potentially, high-resolution

Corresponding author: Park, H.Y. (hyeyoon.park@snu.ac.kr).
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RNA-imaging methods can shed light on this important question. Using deep sequencing, more than 2500 different mRNAs have been identified in the synaptic neuropil of the hippocampus [25]. Moreover, *in vitro* single-molecule imaging of the neuropil transcriptome using nanostring technology revealed that relative mRNA abundances varied in excess of 1000-fold [25]. The sensitivity of the assay demonstrated that many species of mRNA are present in widely varying amounts in the neuropil. To complement these and other unbiased techniques, single-molecule detection of endogenous mRNAs has enabled researchers to probe the precise quantity and the exact location of each transcript within single neurons in culture (Figure 1). Further, single-molecule labeling of endogenous RNA in brain tissue is beginning to put *in vitro* measurements into their biological context [14,16,25–29] and can even quantify the coexpression of distinct mRNA species in a single neuron *in vivo* [28]. The presence of such a diverse population of mRNAs in neurites underscores the significance of compartmentalized gene expression in neurons and indicates that a large repertoire of proteins may be necessary to achieve neuronal function within specific compartments.

Mechanisms of mRNA transport in neurons

Much attention has been dedicated to understanding how mRNAs and RNA-binding proteins (RBPs) interact to achieve motor-based mRNA transport in neurons. Directed RNA transport requires binding of *cis*-acting RNA elements and RBPs, which mediate interactions with molecular motors that transport along microtubules (reviewed in [30]). Actively transported mRNAs typically contain specific *cis*-acting sequences or targeting elements that confer subcellular localization [31–35] (reviewed in [36]). These elements, typically located within the 3′ untranslated region (UTR) of the mRNA, interact with RBPs and other proteins to form messenger ribonucleoprotein (mRNP) complexes that are transported along microtubules by kinesin or dynein (reviewed in [37]) (Figure 2). Significant effort has been dedicated to characterizing localization elements and minimal functional elements and to understanding how they contribute to neuronal mRNA transport and localization [31–35]. Single-molecule FISH can be used to measure the relative contributions of targeting elements or RBPs to localization. For instance, quantification of the localization of reporter mRNAs with alternative Spinophillin dendritic targeting elements (DTEs) directly demonstrated how the presence of the DTE sequence in the mRNA affects absolute mRNA counts in dendrites [38]. In lieu of direct visualization of mRNAs, fluorescently tagged RBPs can function as proxies to reveal the dynamics of mRNA transport in neurites [39–42]. Collectively, RBP-imaging studies have revealed that while most mRNPs were stationary, a subpopulation exhibited persistent and oscillatory bidirectional transport in neurites punctuated by pauses in motion. Loss or mutation of RBPs, combined with live tracking, has also been informative regarding the contribution of individual RBPs to active transport. Amyotrophic lateral sclerosis (ALS)-associated mutations in TDP-43 altered the transport

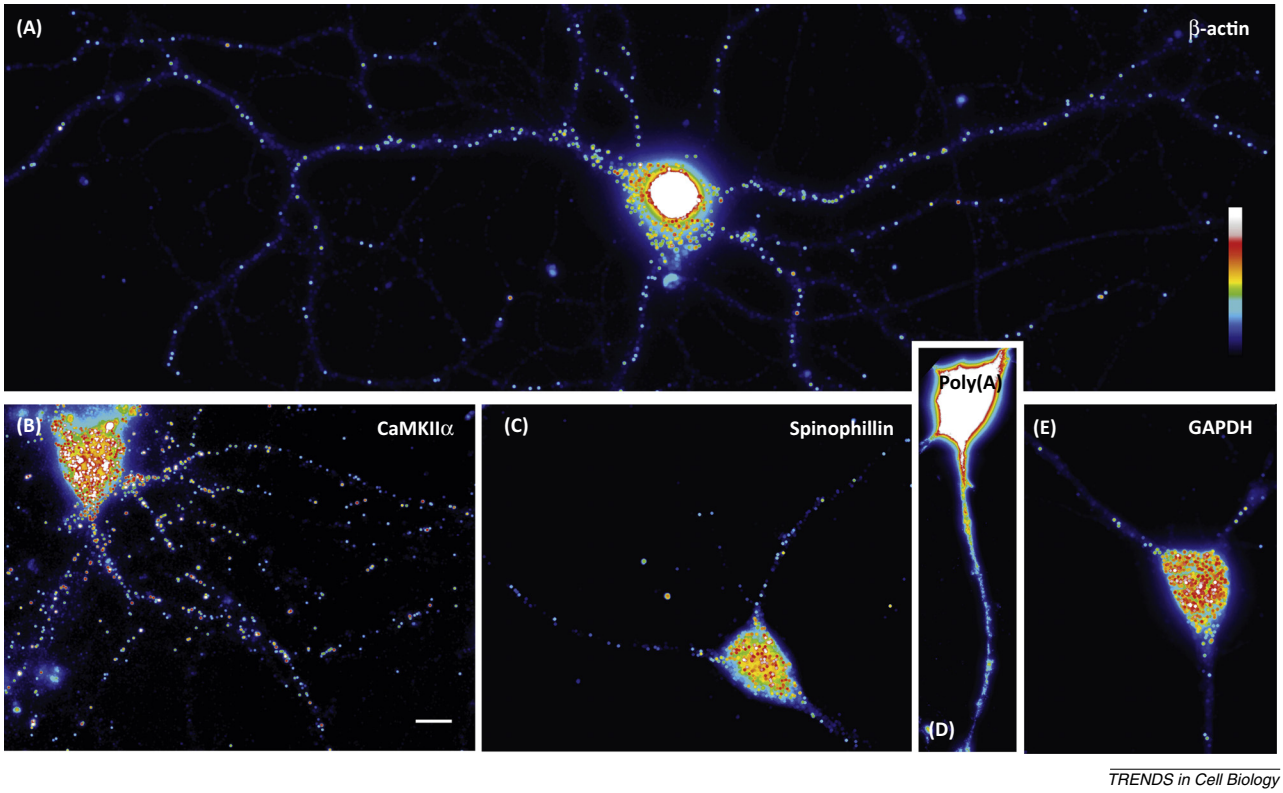

Figure 1. mRNA distribution in neurons varies widely. **(A)** Single-molecule fluorescence *in situ* hybridization (smFISH) of endogenous β-actin mRNA in cultured primary hippocampal neurons demonstrates that the mRNA localizes along neurites. **(B)** smFISH of CaMKIIα mRNA in cultured neurons. **(C)** smFISH of Spinophillin mRNA localized in neurites. **(D)** FISH using dT sequences complementary to poly(A) demonstrates the total amount of polyadenylated mRNA in dendrites. **(E)** Visualization of endogenous GAPDH mRNA with smFISH demonstrates that the mRNA is largely restricted to the soma. In all images, the mRNA signal was dilated by two pixels to aid viewing. The heat map represents the relative fluorescence intensity. Bar, 10 μm. All images presented at the same scale.

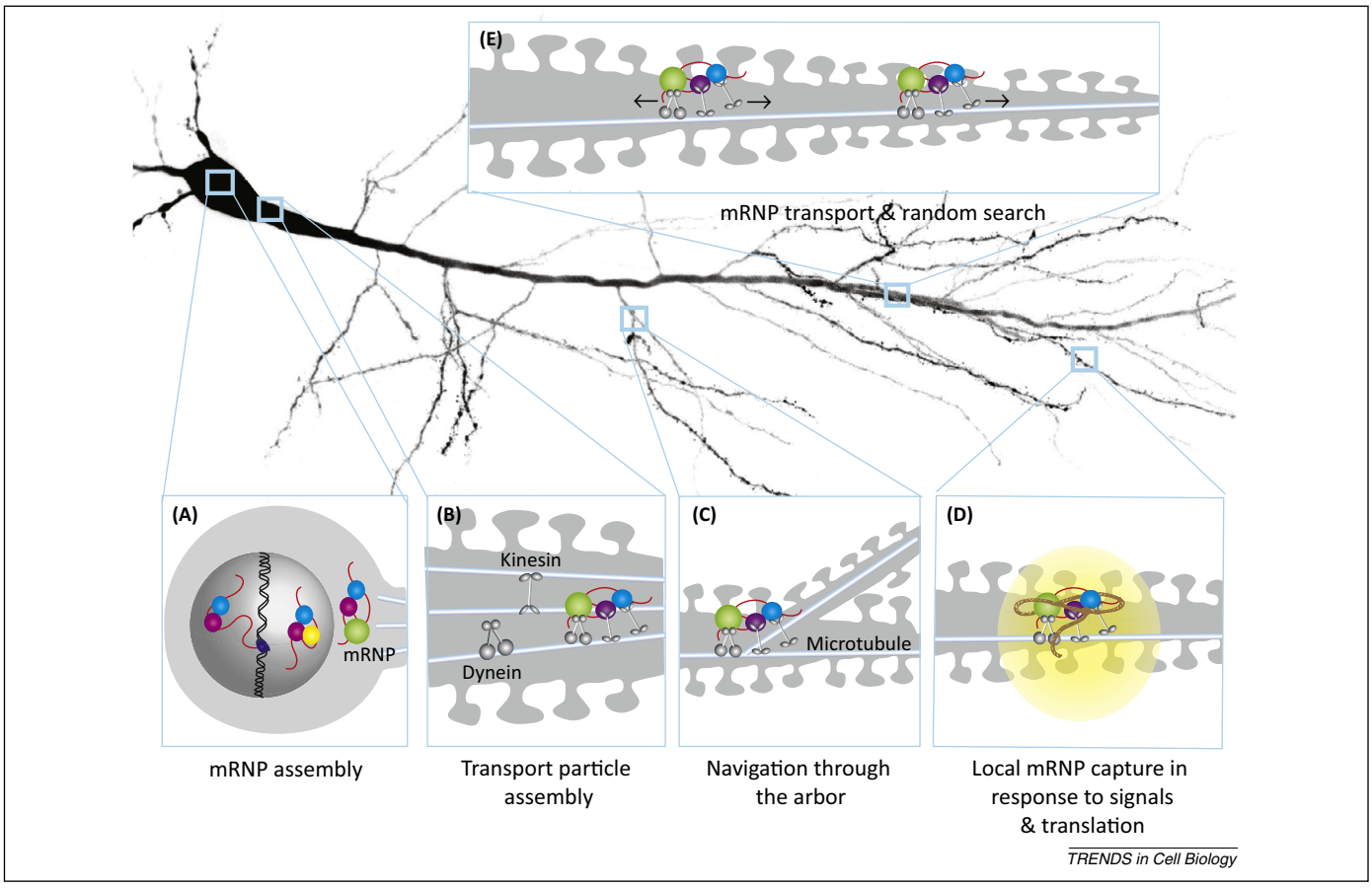

Figure 2. The life of a localized mRNA in a neuron. **(A)** During or shortly following synthesis, mRNAs are recognized and bound to RNA-binding proteins (RBPs), which play a role in all aspects of the mRNAs' travels and life. **(B)** In dendrites, a large proportion of mRNAs are actively transported. Although it remains unclear where precisely the mRNA binds motors, it is possible that assembly of the transport machinery occurs at or near the soma since many mRNAs can be seen to be actively transported in regions near the soma. **(C)** mRNA transport must account for the highly branched morphology of a neuron. Navigation of mRNAs through the arbor is a poorly understood process. Whether transport is random or biased toward specific branches or regions is unknown. **(D)** mRNAs can be captured or stalled at specific regions in response to local activity. This may allow the specific targeting of proteins to particular regions or synapses through local translation. **(E)** mRNA imaging highlights the oscillatory behavior of actively transported mRNAs, reminiscent of random searching. This could possibly be due to multiple opposing motors associated with the mRNP, the bipolar orientation of microtubules, or alternating transport on microtubules and actin filaments. Future work will reveal more on how this behavior is regulated and how random it is.

capability of the mRNP and reduced the net displacement of the mRNP in motor axons [42]. Similarly, reduced mRNP motility was observed in *Drosophila* neurons lacking fragile X mental retardation protein (FMRP) compared with wild type [43]. Consistent with the importance of properly regulated intracellular RBP–mRNA stoichiometry [44], overexpressing *Drosophila* FMRP also resulted in decreased mRNP movement, presumably due to excessive recruitment of motors to mRNPs, which can inhibit active transport [43]. These results suggest the importance of unimpaired mRNA transport in proper neuronal function.

Single-mRNA movement in neurites

Direct visualization of individual mRNAs in live neurons has yielded an enhanced understanding of mRNA transport dynamics. By tracking the movement of single mRNAs, changes in velocity or direction during motor-based transport can indicate regulation of motor binding and activity [45,46]. Measurement of mRNA diffusion rates can correlate mRNA movement with the cellular milieu. In neurons, high-resolution imaging of active transport and localization of mRNAs can be accomplished by microinjecting labeled mRNAs [31,47,48] or by using the MS2-GFP system [13,49–57] (Box 1). Observing the movements of mRNA in neurons lacking specific RBPs has yielded information on how RBPs regulate mRNA transport behavior. Live imaging of MS2 (Box 1) reporter mRNA demonstrated that huntingtin (Htt), Htt-associated protein 1 (HAP1), KIF5A kinesin, and dynein colocalized with β-actin mRNA during dendritic transport, and knockdown of these proteins reduced the transport of β-actin mRNA [57]. This study directly demonstrated that active mRNA trafficking in neurons was mediated by members of the kinesin and dynein motor families. Tracking reporter RNAs using the MS2 labeling system in neurons lacking FMRP revealed that FMRP can function as an adaptor for the kinesin light chain [50] and deliver CaMKIIα mRNA to dendritic spines in response to synaptic stimulation [52].

The aforementioned mRNA-tracking studies have relied on transfection or injection of exogenous RNA. However, an exogenous reporter RNA may not recapitulate the full repertoire of behavior of endogenous RNA for multiple reasons. Reporter RNAs are usually overexpressed and may lack some of the *cis*-regulatory elements and *trans*-acting RBPs because they do not undergo proper splicing and processing in the nucleus. To label endogenous mRNA, a recent study used knock-in and transgenic techniques and extended the utility of the MS2-GFP system in live

Box 1. Imaging single mRNAs in neurons

Single-molecule FISH

Since the first demonstration of single-molecule FISH [86], variations of FISH techniques have been used to detect individual transcripts with high accuracy (reviewed in [87]). Originally, five 50-nucleotide amino-modified oligodeoxynucleotides (ODNs), each of which directly labeled with five fluorescent dyes, were used for FISH in neurons [50]. This method enables highly sensitive detection of single probes bearing five dyes as well as single mRNAs labeled with up to 25 dyes [86]. The ability to count single fluorescent probes confirmed single-molecule detection. An analogous single-molecule FISH method was developed using 48 or more 20-nucleotide ODNs each labeled with a single fluorophore [88]. This method enabled cost-effective, high-throughput synthesis of FISH probes, which contributed to the wide spread use of the technique. In addition, amplification of fluorescent signal on a shorter target can be achieved using dendrimers [25] or sequential tethering of ODNs [89], where the complementary oligo is further bound by non-targeting fluorescent sequences. The use of shorter target sequences may increase false-positive or -negative signal; however, the fluorescence is significantly brighter due to the serial amplification of fluorescent dyes at the RNA.

Microinjection of labeled mRNA

Localization of mRNA in live neurons can be visualized using microinjection of fluorescently labeled mRNAs [31,47]. Fluorescent RNAs are transcribed *in vitro* in the presence of UTPs labeled with a fluorescent dye. Labeled RNAs are introduced into live neurons using an injection needle with a pressure-injection system. Although it is tedious and less efficient, microinjection has been an effective method to track individual mRNP particles.

Tagging RNA with GFP

Genetically encoded RNA reporters utilize highly specific binding between a RBP and its target RNA sequence. The MS2-GFP system, in which MS2 bacteriophage capsid protein fused with GFP (MCP-GFP) is coexpressed with an RNA tagged with the MS2 binding site (MBS), has been widely used to track single mRNAs (reviewed in [46]). To increase the signal-to-background ratio, a reporter RNA is tagged with 24 repeats of MBS stem–loops, each of which binds a dimer of MCP [90]. More recently, RNA–protein pairs derived from other bacteriophages such as λ and PP7 [91–93] have been developed for multicolor imaging of mRNAs in live cells (reviewed in [12]).

mice [14,26]. Tracking labeled, endogenous single β-actin mRNA revealed that RNA movements in neuronal dendrites are distinct from those in fibroblasts. Diffusion of β-actin mRNA in neurons ($D \approx 0.004\ \mu m^2$) was ~24 times slower than in fibroblasts, suggesting a more confined environment. While mRNA movements in fibroblasts were dominated by diffusive motion, most of the endogenous β-actin mRNA was stationary in neurons, consistent with the behavior of exogenous reporter RNA [13,49,52]. If travel in one direction for more than 1.5 μm is classified as directed motion, about 10% of mRNPs showed bidirectional motion, with a mean speed of 1.3 μm/s. The frequency ratio of anterograde to retrograde movement was approximately 1.3, suggesting that a bias to the anterograde direction mediates the constitutive delivery of β-actin mRNAs to distal dendrites. The ability to measure the dynamics of single mRNAs revealed the precise kinetics for subpopulations of mRNAs.

Finding the destination

Observation of the oscillatory behavior of a fraction of actively transported mRNAs in neurites suggests a scanning behavior near areas that may require local translation such as synapses. Given that synaptic transmission and modification of the efficacy of transmission strength can be specific to single dendritic spines [58], it is likely that mRNA localization may also be specific for individual spines. An attractive model for mRNA targeting to stimulated synapses is that mRNAs transport stochastically along neurites and are captured or stalled at sites of stimulation [32,59] (Figure 2). This model conveniently circumvents a mechanism of predetermining a destination for a traveling mRNA. Stimulated synapses may express a synaptic tag (reviewed in [60]), which leads to mRNA capture, thus targeting protein synthesis to locations requiring modifications [32]. Single Arc and β-actin mRNAs have been shown to aggregate or cluster in certain regions, suggesting that specific areas in dendrites may be more prone to inducing mRNA pausing or stalling [13,14,61]. Live observations of transfected Arc reporter mRNA revealed that actively transported mRNAs often reduce velocity before coming to a stop [13] and single Arc mRNAs are stably positioned at the base of spines [51]. Furthermore, in some cases, actively transported mRNAs may 'overshoot' a spine, reverse direction, and return before arresting motility [13]. Observations of these behaviors may contribute insight into how a mRNP is instructed to stop or travel to a synapse. *In silico* measurements show that local cues such as metabolic gradients that cause motor detachment from a randomly searching mRNA cargo can efficiently deliver multiple cargos to their targets [62,63]. These studies suggest that by using a motile fraction of randomly searching mRNAs and by altering their motility in response to local cues, docking at specific synapses is likely to be quickly and efficiently achieved.

Exploring molecular candidates responsible for mRNA docking at synapses is starting to illuminate the mechanisms of targeting mRNAs. For example, FMRP, an RBP with roles in regulating mRNA transport and translation, has been shown to play an important role in translocating CaMKIIα mRNA to dendritic spines after stimulation of metabotropic glutamate receptors (mGluRs) [52,64]. The RBP Fused in Sarcoma/Translocated in Liposarcoma (FUS/TLS) also moves to dendritic spines in response to mGluR stimulation [65]. In addition, molecular motors themselves may participate in delivering or docking mRNAs to synapses. The actin-based motor myosin Va has a role in mRNP accumulation at actin-rich dendritic spines [66]. Several RBPs can associate with actin filaments by interacting with myosin Va, leading to a mechanism of mRNA and RBP docking at dendritic spines [65,67]. Nevertheless, many questions remain: how mRNAs are induced to pause at specified locations and how transport is regulated to achieve precise positioning. By combining single-mRNA imaging with perturbation of RBPs or other molecules that play a role in mRNA docking, mechanisms of specific mRNA targeting to synapses can be revealed.

Seeing the tree from the forest: changed perceptions

Before single-mRNA detection, visualization of mRNAs lacked sufficient sensitivity and spatial resolution, making the quantification of mRNAs and their exact positions difficult. With high-resolution techniques some

discrepancies have emerged regarding the abundance of certain localized mRNAs in neurons. Due to the highly diverse and abundant nature of localized neuronal mRNA, early reports proposed that numerous localized mRNAs traveled together as transport granules. This conclusion addressed how multiple mRNAs could be induced to translate in unison and contribute a pool of synaptic or plasticity-related proteins as well as increase the efficiency of mRNA transport. Microinjected CaMKIIα and Septin7 RNAs, which share similar DTEs, may colocalize around 10% of the time in the same particles in dendrites [31]. Single-molecule imaging of endogenous β-actin mRNA in neurons revealed continuous assembly and disassembly of large mRNP complexes containing multiple copies of β-actin mRNAs [13].

However, some single-molecule studies in cultured neurons demonstrated that different mRNA species do not colocalize to a great extent [15,68]. In brain tissue, Arc and CaMKIIα mRNAs, which share A2 DTEs, were present in dentate gyrus dendrites following stimulation, but in distinct populations of particles [16]. Whether mRNAs are single or clustered may depend on the transient masking effect of mRNAs in granules where they are not accessible by FISH probes, particularly in unstimulated neurons [61]. In addition, there is an inherently low probability of finding granules that contain two specific mRNA species chosen for each FISH analysis while there can be many other mRNA species that are not labeled. Therefore, the aforementioned studies may not necessarily be in opposition as they all observe a low amount of coalescing mRNAs ranging from 2% to 10%.

Since high-resolution detection of mRNA can determine the distribution of compartmentalized mRNAs, it can also determine that certain transcripts are absent. Recent studies utilizing FISH to detect endogenous mRNA have led to some alternative interpretations of dendritically localized mRNA. For example, the nascent transcripts encoding brain-derived neurotrophic factor (BDNF) undergo alternative splicing, which results in two isoforms with different 3′ UTR lengths (short and long). Previously, it had been shown that the long 3′ UTR isoform of BDNF mRNA was capable of trafficking to distal dendrites [69]. However, high-resolution FISH revealed only sparse amounts of BDNF mRNA in the soma relative to other neuronal transcripts and none of the long isoform in distal dendrites [70]. In addition, FISH of neurotransmitter receptor mRNA provided new insight into the localization and regulation of the post-synaptic response to the neurotransmitter glutamate. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, a type of glutamate receptor, may comprise various combinations of subunits (GluA1–4) and inclusion of the GluA2 subunit selectively diminishes the receptor's permeability to extracellular calcium. Branched-DNA labeling of endogenous mRNA showed that the GluA2 subunit mRNA of the AMPA receptor was not present in dendrites but instead was largely restricted to the soma [17]. By contrast, earlier studies reported dendritic localization of AMPA receptor subunit mRNAs (GluA1–4) and that subsequent synthesis of AMPA receptors could be modulated locally in an activity-dependent manner [71–75]. These results demonstrated that the enhanced sensitivity of single-molecule assays may alter our assumptions concerning mRNA abundance and location. Due to the central role glutamate receptors play in synaptic transmission, it is likely that neurons have developed multiple and redundant ways to modulate expression during periods of plasticity. Moreover, differential neuronal signaling properties could lead to different AMPA receptor mRNA expression and localization profiles in various neuronal subtypes and brain regions [24]. While improvements in the detection of RNA in neurons will inevitably introduce some discrepancies with previously published reports, the findings will eventually enhance our understanding of local protein synthesis in neurons.

Localization is only half the battle: regulating local translation of mRNAs in processes

While the localization of mRNAs into dendrites implies the occurrence of local translation, visual measurements of active local translation have been scarcer and have yielded lower-resolution measurements than mRNA imaging in neurons [75–80]. It would be illuminating to complement the visualization of mRNA localization with measurements of the translational status and quantitative translational output of mRNAs.

Because certain mRNAs are thought to translate locally in response to a synaptic cue it is likely that constitutively localized mRNAs are largely in a translationally repressed state in dendrites [61,81]. By regulating both the location and the translational status of mRNAs, neurons can orchestrate local gene expression with high spatial fidelity.

Many RBPs prevent translation while transporting mRNAs. Moreover, higher-order mRNP complexes in neurons such as RNA granules, which comprise many RBPs and ribosomes, are integral in maintaining translational repression of the mRNAs they contain. The ability to detect β-actin mRNA with single-molecule FISH was used to report on the presence of single mRNAs in repressive granules thereby indicating their translational status [61]. Single-molecule detection quantitatively demonstrated that β-actin mRNAs in repressive structures were released during synaptic stimulation. This also showed that mRNA detection in neurons may be subject to obscuring structures, necessitating more aggressive detection methods [61,82].

Other single-molecule assays for translation have utilized imaging of a fluorescent protein reporter to assess rates of translation in neurons. Use of the fast-folding, bright, and easily bleached fluorescent protein Venus revealed that stimulation of metabotropic glutamate receptors or inhibition of action potentials increased and decreased, respectively, the translation of Venus-ARC and Venus-FMRP reporters within minutes [48]. Translation of Venus-PSD95 synaptic protein also increased in response to metabotropic glutamate stimulation [83]. Neurons lacking the translational regulator FMRP lost the ability to regulate translation at steady state as well as in response to the stimulation [83]. In contrast to other methods that require minutes to hours to detect an appreciable amount or change in protein synthesis [76,77,79,80,84,85], the ability to count single events with this method greatly

Box 2. Imaging transcription dynamics

Advances in single-molecule mRNA imaging have led to revelations of how mRNAs move and translate in neuronal processes; however, the technology also lends itself to single-molecule resolution of mRNA synthesis dynamics. FISH or live imaging of endogenous mRNAs combined with normalizing the total intensity of mRNAs present at sites of transcription to the intensity of single mRNAs can result in valuable information on transcription kinetics [92,94,95]. For example, depolarization of hippocampal slices to stimulate neurons increases the number of nascent β-actin mRNAs [14]. Arc mRNA levels are low in steady-state conditions; however, environmental or electrical stimulation robustly activates Arc transcription and dendritic localization [16,59]. To quantify mRNA synthesis in relation to a stimulus, FISH labeling of Arc transcription sites in brain slices is used as a measure of gene expression activation during behavior [96,97]. Quantitative assessment of the transcriptional dynamics of different mRNAs in brain tissue will illuminate how the transcriptional response to a stimulus varies among mRNAs and neuronal subtypes. These measurements will be critical for our understanding of how constitutively expressed and modular plasticity-related genes differ in their regulatory mechanisms of expression during plasticity paradigms related to learning.

enhanced the time resolution of monitoring changes in translation.

Beyond the messenger

While it is now possible to detect and image messenger RNA at the single-molecule level, the advent of imaging technologies in conjunction with RNA-labeling techniques also allows high-resolution detection of noncoding RNAs. Quantitative amplification of fluorescent signal using branched-oligo FISH labeling can facilitate the detection of sequences as short as 20 nucleotides in length. For example, single transcripts of miR-124 were observed along dendrites, offering novel *in situ* insight into the regulation of local translation via miRNAs [17]. Similarly, Ube3a mRNA, which produces a seemingly nonfunctional protein, localizes to dendrites, and plays a role in regulating the availability of miRNA-134 [82]. Branched-DNA FISH also allowed the detection of single circular forms of RNAs, which are highly enriched in neural tissue, by targeting probes to the head-to-tail junction [27]. This facilitated the observation that the expression of circular forms of certain mRNAs can be modified during development and by activity. The use of high-resolution FISH techniques to address the precise abundance and localization of mRNAs, noncoding RNAs, and even mRNA transcription dynamics (Box 2) will describe the multifaceted regulatory mechanisms of localized gene expression.

Concluding remarks

mRNA transport and local translation have been increasingly recognized as important mechanisms of regulating neural plasticity and development. Recent studies using single-molecule imaging of mRNA have shed light on detailed aspects of mRNA dynamics with unprecedented spatiotemporal resolution. Digital detection of individual molecules has enabled quantitative measurements of even trace amounts of RNA inside single neurons. Recent advances in RNA labeling *in situ* has allowed the visualization of miRNAs, noncoding RNAs, and circular RNAs, expanding the repertoire of visualization beyond messages.

Observations of highly heterogeneous distribution and movement of mRNPs have revealed complex interactions of mRNAs, RBPs, and motor proteins in the regulation of local gene expression. However, the mechanisms of targeting mRNAs to specific sites and regulation of local translation remain largely unknown (Box 3). It is important to continue to advance single-molecule imaging technologies for visualizing RNA–protein interactions as well as local

Box 3. Outstanding questions

- How is the differential expression of an mRNA species regulated in neuronal subtypes and brain regions? While our understanding of localization elements and their interaction with RBPs and motors describes how distinct mRNAs have contrasting localization, it is unexplained how the same mRNA exhibits divergent localization patterns depending on its context.
- What is the minimal *cis*-acting RNA sequence that determines RNA transport into dendrites and/or axons? Methodical identification of the targeting and minimal targeting elements of neuronal genes will certainly reveal conserved sequences and axioms that target mRNAs into dendrites and axons.
- Many questions remain regarding the molecular composition of mRNPs and which proteins play roles in regulating the unique localization of individual mRNAs. These include: What are the adaptor proteins that recruit motor proteins to mRNP complexes in neurons? How specific are RBPs to one type of mRNA? Are there neuron-specific RBPs or proteins that generate unique mRNA behavior in neurons such as docking at synapses or long-distance transport? Enhancing our understanding of mRNP identity and function will lead to understanding how localization is determined.
- How are mRNPs recognized and captured at the activated dendritic regions or synapses? Identification of synaptic tags that lead to the capture of mRNAs and proteins following synaptic activity and to consolidation and memory formation will be transformative for our understanding of how local translation is directed to stimulated synapses or regions.
- While dendritic mRNAs may dock at spines, how do mRNAs behave in axons? Although mRNAs have long been established to localize to dendrites as well as the axons of certain types of mature neurons, information regarding the latter compartment has been subject to scrutiny due to claims of non-axonal mRNA contamination obfuscating legitimate axonally localized mRNAs [98]. Notwithstanding this, there remains a great deal of opportunity to observe how mRNAs behave in axons using single-molecule technologies. Optical techniques such as single-molecule FISH and live tracking may reveal that mRNAs that have been previously characterized in dendrites behave in a different manner in axons. The unipolar microtubule orientation in axons may inhibit retrograde mRNA movements, which will be evident on live observations of the same mRNA species in both types of neuronal process.
- How do the transport kinetics of different mRNAs with different functions compare with those of well-characterized mRNAs such as β-actin and Arc? Although thousands of mRNAs are known to be transported into neuronal dendrites, very little is known about how and why different mRNAs respond differently to the same stimuli and why different mRNAs are regulated either at the level of their transcriptional or translational activity or in their positioning in the neuron. Live imaging and tracking of diverse mRNAs will demonstrate the unique and convergent transport mechanisms of mRNAs and perhaps expose regulatory mechanisms of transport that conform to the unique function of the mRNA being observed.

translation processes in real time. In addition, further work is needed to investigate RNA dynamics in the intact neural network in live animals to understand the physiological consequences of normal and disrupted RNA regulation.

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