Visualizing mRNA Trafficking and Local Translation Within Individual Neurons

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Introduction

Neurons are remarkably polarized cells. They elaborate processes that extend great distances from the cell soma in order to form distinct subcellular compartments such as growth cones and synapses. Stimulus-induced changes in the structure and function of these compartments are essential to the formation and plasticity of neural circuits (Kandel, 2001). The persistence of such changes requires new transcription and translation, raising the question of how gene expression can be spatially restricted within neurons. Studies in many systems have indicated that mRNA localization and regulated translation provide one solution (Martin and Ephrussi, 2009). Translation of localized transcripts within distinct subcellular compartments in neurons has been shown to occur at several time points: in growth cones during axon guidance and synapse formation (Lin and Holt, 2008), at synapses during synaptic plasticity (Wang et al., 2010), and in axons during injury-induced regeneration (Willis and Twiss, 2006).

A challenge to the study of local translation in neurons is the need to study mRNA localization and protein synthesis within discrete compartments, which cannot always be easily separated from the rest of the cell. This challenge has necessitated the development of novel tools and approaches for isolating pure, or highly enriched, subcellular compartments and using these:

- To identify localized transcripts;
- To characterize and elucidate the mechanisms underlying their localization; and
- To characterize and elucidate the mechanisms whereby stimulation regulates their local translation.

In this chapter, we will describe techniques for identifying localized mRNAs and for visualizing their localization and translation within neurons.

Methods for Identifying Localized Transcripts Within Neurons

Determining the population of mRNAs present in axons or dendrites requires a means of isolating each of these compartments from the cell body. The purity of the neuronal-process preparation is critical because the amount of RNA that is present in processes is orders of magnitude smaller than the amount of RNA present in neuronal or glial cell bodies, such that even minimal somatic contamination can overwhelm detection of process-localized miRNAs. Investigators have devised a number of strategies to address this problem:

- Biochemical fractionation of CNS to enrich for synaptic terminals;
- Dissection of dendritic domains within brain tissue; and
- A variety of methods to mechanically separate neuronal processes from somata of cultured neurons.

J. Eberwine and colleagues pioneered a method in which individual dendrites of dissociated neurons in culture are transected and then aspirated into a micropipette containing buffer and reagents for T7-RNA amplification (Miyashiro et al., 1994). This method has been used to generate probes for microarray analysis and has led to the identification of approximately 400 dendritically localized transcripts (Eberwine et al., 2001a). T. Suzuki and colleagues (Tian et al., 1999) isolated RNA from rat forebrain postsynaptic density (PSD) fractions and used this approach to identify 130 transcripts present in this synaptic fraction. L. Bloch and colleagues (Zhong et al., 2006) dissected the stratum radiatum from rat hippocampal slices, purified its RNA, and used this sample as starting material to identify 154 “localized” transcripts by microarray analysis.

Our lab, in turn, has developed methods for culturing rodent hippocampal and cortical neurons on Millipore filters (Millipore, Billerica, MA) containing 1-μm or 3-μm pores through which neuronal and glial processes, but not cell bodies, can penetrate (Poon et al., 2006). Harvesting the bottom, process surface with a cell scraper provides a means of mechanically separating somata from axons and dendrites. The composition and separation of the two surfaces can be assessed by confocal optical sectioning of cultures stained as follows: using DAPI to detect nuclei, antibodies to NeuN to mark neuronal nuclei, GFAP to mark astrocytes and astrocytic processes, MAP2 to label dendrites, and tau to label axons. This technique revealed that with a 1-μm or 3-μm pore, the bottom surface was devoid of cell bodies and contained exclusively axons, dendrites, and glial processes; it further revealed that with 3-μm pores, 19% of dendrites penetrated to grow along the bottom surface.

To measure the purity of the soma/process RNA preparation, we performed quantitative (qPCR) for somatically restricted transcripts (e.g., the small nuclear ribonucleoprotein SM51) and for dendritically localized miRNAs (e.g., αCaMKII and MAP2). These analyses revealed that the process prep was enriched for known process-localized miRNAs and contained minimal somatically restricted transcripts. Microarray analysis of RNA purified from process and somatic/whole-cell compartments led to the identification of approximately 100 process-localized...
mRNAs; fluorescent in situ hybridization (FISH) of 19 of the identified localized transcripts revealed that all were present in dendrites. Similar methods have been used to isolate axonally localized mRNAs from dorsal root ganglion (DRG) neurons (Zheng et al., 2001) and sensory neurons (Cox et al., 2008).

Another method for mechanically separating neuronal processes from cell bodies (pioneered by N. Jeon and colleagues) involves growing neurons in compartmentalized microfluidic chambers (Park et al., 2006). In this method, neurons are plated into platforms in which processes and somata are separated by a physical partition containing embedded micrometer-sized grooves along which axons and dendrites, but not cell bodies, can traverse. By varying the distance of the partitions, one can obtain preparations containing pure axons, which extend greater distances than dendrites. This approach has been used by Taylor and colleagues to identify axonally localized transcripts in mature rodent cortical neurons (Taylor et al., 2009).

Neurites can be easily separated from the cell bodies of invertebrate neurons; in fact, neurites will remain viable in culture and continue to grow even in the absence of a cell body. We have used sharp electrodes to sever neuronal processes from Aplysia sensory

Figure 1. Rat hippocampal neurons were cultured on Millipore filters etched with 1-μm pores. Following fixation at 21 DIV, they were processed for immunocytochemistry with anti-MAP2, anti-tau, and anti-GFAP antibodies and counterstained with DAPI to visualize dendrites, axons, glial processes, and nuclei, respectively. Confocal sections of the top and bottom (separated by a 10-μm-thick filter) revealed that dendrites, axons, and glial processes grew through the pores and along the bottom surface but that cell bodies were confined to the top surface.
neurons, isolating RNA from the pure neurites and using this sample as the starting material for cDNA library synthesis (Moccia et al., 2003). The sequencing of clones from this library revealed approximately 250 localized mRNAs. As described below, the ability to obtain viable preparations of isolated processes is especially advantageous for studies of local translation at synapses.

Surprisingly little overlap is revealed by comparing the lists of dendritically localized mRNAs in rodent hippocampal neurons, identified using distinct approaches (Tian et al., 1999; Eberwine et al., 2001b; Poon et al., 2006; Zhong et al., 2006). The significance of these findings is not clear: They may indicate that a potentially very large population of mRNAs localizes to dendrites and/or that the population changes with development and with neuronal activity. Critically, however, they underscore the necessity of confirming localization by in situ hybridization (ISH), as described in the following section.

Detecting mRNA Localization Using in situ Hybridization

The methods described above for unbiased identification of localized transcripts within neurons are prone to problems with specificity. The major factor that contributes to the identification of false-positives is somatic contamination in the starting material. As a consequence, it becomes critical to confirm the localization of any identified transcripts using ISH.

Localization by ISH can be determined in dissociated cultured neurons and/or in tissue sections. The advantage of assessing localization in dissociated cultured neurons is that it is easier to resolve individual axons and dendrites than in tissue sections, where dense packing limits one's ability to discern individual axonal and dendrite processes. The major advantage of tissue sections is that they represent individual axonal and dendrite processes. The major advantage of tissue sections is that they represent an intact developing or adult brain in tissue sections.

Refining techniques for in situ hybridization

A consequence of the low amount of RNA in processes relative to cell bodies is that the signal in the cell bodies is often saturated before signal in the processes can be detected. In many published studies and publicly accessible databases, such as the Paul Allen Brain Atlas, the exposure time for ISH is stopped as soon as signal is observed in the cell body, limiting the usefulness of these resources for identifying localized transcripts. The requirement for long exposure times also underscores the importance of including negative controls in ISH studies. In addition to sense controls, ISH for transcripts that are known to be somatically restricted is essential. Additionally, the use of more than one riboprobe, covering distinct sequences in the RNA but showing the same pattern of localization, serves as a useful control. Finally, FISH is compatible with immunocytochemistry using some antibodies, such as anti-MAP2 antibodies, which allows for the colocalization of RNA signal with MAP2 immunoreactive dendrites (Poon et al., 2006).

Highly sensitive methods for FISH have greatly facilitated the identification of localized transcripts in neurons and other asymmetric cells. One study by H. Krause and colleagues (Lécuyer et al., 2007) used high-throughput FISH analyses to show that more than 70% of mRNAs showed very specific, hitherto unappreciated patterns of subcellular localization in Drosophila embryos. We have used FISH with digoxigenin or biotin-labeled riboprobes, from approximately 300 to 600 bp in length, coupled with tyramide-signal amplification, to detect localization of mRNAs in a variety of prepared samples: cultured hippocampal neurons, hippocampal sections, cultured Aplysia sensory-motor neurons, cryostat sections of Aplysia ganglia, and whole-mount Aplysia CNS (Lyles et al., 2006; Poon et al., 2006; Wang et al., 2009). In each of these preparations, we were able to optimize conditions in order to detect specific localization in distal neuronal processes with antisense (but not sense) probes. Important variables included the following: hybridization temperature, stringency of washes, and time of the tyramide signal amplification.

R. Singer and colleagues have developed methods for highly quantitative FISH by synthesizing several oligonucleotide probes, targeting adjacent sequences on an mRNA, and conjugating fluorochromes to specific sites on each oligonucleotide. This technique has made it possible to calibrate the signal to a known concentration of labeled oligonucleotide probes and, with appropriate imaging, to quantify the number of RNA molecules being detected (Femino et al., 2003). Although this method is more expensive than FISH using in vitro transcribed riboprobes, it is...
advantageous because it is quantitative and sensitive. Further, combining the labeling of oligonucleotides with a variety of distinct fluorophores allows investigators to detect multiple transcripts within a single sample.

The absence of an ISH signal does not necessarily mean that the transcript is not localized. Thus, transcripts may be present at low abundance (below the threshold for detection) or present in structures (e.g., RNA transport granules) that are inaccessible to hybridization by the riboprobe or oligonucleotides. Many ISH protocols include a protease step to digest proteins binding the RNA and thereby increase transcript accessibility during the hybridization step. As increasingly sensitive FISH protocols are developed and utilized to analyze mRNA localization in neurons, it is likely that highly distinct patterns of subcellular mRNA localization will emerge, analogous to what has been observed in *Drosophila* embryos (Lécuyer et al., 2007). Identifying these specific sites of localization may reveal subcellular compartments that had previously been unappreciated; thus, these findings may lead to a more detailed and nuanced understanding of neuronal architecture.

Dynamic Imaging of mRNA Localization in Neurons

ISH analysis provides only a snapshot of mRNA localization within neurons, and thus cannot provide much insight into mechanisms whereby the transcript is localized. Methods for dynamic imaging of mRNA transport in living neurons are needed to gain a better understanding, for example, of whether mRNAs are actively transported to particular sites, whether their localization results from anchoring within specific compartments, or whether their localization results from differential stability within the neuron.

Methods for directly visualizing RNA localization in neurons can be divided into two categories:

1. Methods using probes that hybridize to, and track the movement of, endogenous transcripts; and
2. Methods that track the movement of heterologously expressed mRNAs within neurons.

The advantage of the first approach is that the mRNAs are expressed at endogenous levels and undergo native processing within the nucleus and cytoplasm. It is nonetheless difficult to design probes that will hybridize and brightly label endogenous transcripts, and the possibility exists that any hybridizing reagent will interfere with the ability of the endogenous RNA to interact with its normal protein and RNA partners. The advantage of the second approach is that one can generate transcripts that are brightly labeled to facilitate live cell imaging. The disadvantages include saturation of trafficking mechanisms as a result of high levels of overexpression, as well as artifacts resulting from the fact that exogenously expressed cDNAs do not undergo the same processing in the nucleus as genomically transcribed RNAs. Studies of oskar mRNA localization in *Drosophila* have clearly illustrated that the “nuclear history” of the mRNA is critical to its subsequent localization within the cytoplasm (Hachet and Ephrussi, 2004).

Molecular beacons

One method for visualizing endogenous mRNAs is through the use of molecular beacons (Santangelo et al., 2006). Molecular beacons are small hairpin-loop nucleic acids with a fluorochrome attached to one of the free ends and a molecule that quenches the emission of that fluorophore attached to the other end. When the beacon hybridizes to its target mRNA sequence, the hairpin-loop structure is linearized such that there is no longer any quenching of the fluorochrome. In this way, the beacon-bound target is detectable by live-cell fluorescence microscopy. The beacon must be able to hybridize to its target and be stable within the cell in order to be effective. An improvement on this method involves the use of molecular beacons directed against adjacent sequences in the target mRNA and containing fluorophores at the free ends that, when hybridized to the target, will lead to fluorescent resonance energy transfer (FRET). This enhanced technique has been used to follow the trafficking of viral RNAs in cells (Santangelo et al., 2006). One limitation of the molecular beacon approach is that the signal is not very bright, since usually only one fluorochrome is present.

Microinjecting *in vitro*-transcribed, fluorescently labeled transcripts

Another method for visualizing transport of exogenously introduced mRNAs is by microinjection of *in vitro*-transcribed, fluorescently labeled transcripts in cells. Labeled transcripts can be generated by including nucleotides coupled to fluorophores (e.g., with cyanine dyes or Alexa flours) in the *in vitro* transcription reaction. The fluorescently labeled mRNAs can then be microinjected into neurons, preferably into the nucleus where they can potentially bind appropriate RNA-binding proteins necessary for their cytoplasmic localization, and be tracked by live-cell microscopy. This approach has been used to track RNAs moving in granules along microtubules (Tübing et al., 2010), and, in
MS2 and lambda N systems

Whereas molecular beacons and in vitro–transcribed RNAs must be introduced into cells by microinjection or by cell-penetrating peptides, the MS2 (Bertrand et al., 1998) and lambda N (Daigle and Ellenberg, 2007) systems provide a means of genetically encoding fluorescent markers of specific mRNAs in cells and animals. Both approaches contain two components, which we will describe for lambda N. The first component encodes the RNA of interest, into which a series of 15-hairpin-loop RNA elements, called box B elements, are engineered into the 3’ untranslated region (UTR). It is useful to have this construct encode a fluorescent protein in order to visualize the efficient expression in transduced, transfected, or microinjected cells. The second component encodes the 22-amino-acid-long bacteriophage protein lambda N, fused to a nuclear localization signal (NLS) and to three copies of enhanced green fluorescent protein (eGFP, or any other fluorescent protein). When the second protein is expressed in a cell, the NLS causes it to accumulate within the nucleus. When, however, it binds to the target RNA containing the box B binding sites, the high binding affinity overrides the nuclear localization, and one can follow the labeled mRNA as it is transported into the cytoplasm. The lambda N and MS2 approaches have gained widespread use for tracking RNA movement in a number of cell types, including neurons.

Several variables must be optimized for the system to work in a particular experimental preparation. We have found, for example, that a single NLS is not very efficient for nuclear import in primary neurons because it leads to background signal in the cytoplasm even in the absence of box B–containing RNA. However, three copies of the NLS decrease cytoplasmic background. In addition, one can test the efficiency of distinct NLSs, for example, the M9 NLS or the SV40 large T-antigen NLS. Another variable to optimize is the number of fluorescent proteins fused to the lambda N or MS2 protein, with the aim of achieving a signal that is bright enough to detect and follow over time but that does not add excessive molecular weight to the localized transcript. It may also be necessary to change the location of the box B sites in the 3’UTR. Finally, one can experiment with increasing the number of box B sites in the target RNA in order to increase the signal, again, without imposing an excessively large molecular weight on the transported RNA.

Methods for Visualizing Local Translation in Living Cells

Novel methods have been developed to determine whether a specific mRNA is locally translated in neurons. Here we will focus on translational reporters encoding photoconvertible fluorescent proteins, which we have used to visualize translation at Aplysia sensorimotor synapses during long-term neuronal plasticity (Wang et al., 2009). Our aim here is to discuss some of the critical variables that apply when performing and interpreting this type of experiment.

Whether or not to include the entire RNA transcript

When designing translational reporters for local translation, one must first determine which regions of the RNA are required for mRNA localization and regulated translation. One possibility is to include the entire transcript, fusing a photoconvertible fluorescent protein (the reporter) to the coding region. Another possibility is to fuse the 5’ and 3’UTRs of the localized mRNA to the reporter. The rationale for the latter is that many studies have indicated that localization elements are often (but not always) contained within the 3’UTR, whereas translational regulation is often encoded, at least in part, by the 5’UTR. To confirm that a reporter accurately reflects the localization of the endogenous RNA, one can perform double-label FISH for the endogenous and reporter RNAs. It is worth noting that this approach can be used in conjunction with mutational analyses in order to determine the specific RNA elements involved in RNA localization.

Detecting new translation using photoconvertible fluorescent proteins

Having a translational reporter that encodes a photoconvertible fluorescent protein provides a means of monitoring new, local translation because one can photoconvert all preexisting protein from green to red and then monitor new translation as the appearance of new green signal. A critical concern in these experiments is to verify that the newly synthesized (green) protein is synthesized locally, as opposed to being made in the soma, and then being transported to a distal locale. This can be challenging because the protein takes some time to fold and mature before it actually emits fluorescence, and during this time it can diffuse or be transported within the cell. Further, since there is always more translation in the soma than in neuronal processes,
the signal from somatically synthesized protein can saturate local translation.

The time it takes for a fluorescent protein to fold and fluoresce within a cell is not easy to measure. In our hands, in Aplysia neurons at room temperature, we can detect the appearance of newly synthesized (green) reporter within approximately 10 min. One way to limit the diffusion of protein from its site of synthesis during this time is to attach a signal for membrane anchoring, e.g., for myristoylation or palmitoylation (Aakalu et al., 2001). This approach has the advantage of concentrating the signal and making it more easily detectable. Of note, however, we have found that, unlike soluble proteins, myristoylated reporter proteins in Aplysia neurons do not colocalize with the reporter RNA and in fact are often separated by tens of microns; this characteristic suggests that the protein may be transported following synthesis to distinct membrane domains.

In Aplysia, the problems with somatically synthesized proteins diffusing can be directly addressed by simply severing and removing the cell body, photoconverting the remaining neurites, and visualizing new (green) protein synthesis, which can have occurred only locally. This is a major advantage of this system over vertebrate neurons, where neuronal processes deprived of their cell bodies do not survive in culture.

Controls in these types of experiments include inhibition of protein synthesis with translational inhibitors such as anisomycin, cycloheximide, or emetine to ensure that any newly detected green protein results from local translation. It is also important to include volume-filling controls to ensure that any increases in signal do not result simply from changes in the local volume of a structure; we have found that the photoconverted, soluble red dendra2 protein serves this purpose well.

An increasing number of photoconvertible fluorescent proteins can be used for these experiments. Each has slightly distinct characteristics (Huang et al., 2009; McKinney et al., 2009). Some, such as Kaede, are tetrameric; others, such as dendra2 and mEos, are monomeric. Dendra2 is advantageous for certain experiments because a high-affinity anti-dendra2 antibody is available; mEos2 is advantageous because it is more photostable than dendra2, and because, unlike its precursor mEos, it photoconverts efficiently at 37°C. As new photoconvertible fluorescent proteins are being generated, it is worth investigating these before generating constructs for imaging, taking into consideration several features: whether they are monomeric or multimeric, how bright and photostable they are, what wavelengths they emit, how much light is required to photoconvert them, how stable the photoconverted product is, and whether or not there are any antibodies available to detect the expressed fusion protein.

**Conclusion**

mRNA localization and regulated translation have been found to play critical roles in a wide range of physiological processes in the brain. The spatial restriction of gene expression that mRNA localization affords allows individual synapses and processes to autonomously regulate their protein composition in response to distinct stimuli, greatly expanding the computational capacity of neural circuits. However, this spatial restriction of gene expression also poses technical challenges to the study of mRNA localization and local translation because one cannot simply analyze mRNAs and proteins in whole-cell or tissue homogenates. Rather, genomic-scale approaches to identifying the population of localized transcripts will require new techniques for purifying subcellular neuronal compartments. Similarly, understanding how mRNAs localize and how their translation is regulated within specific compartments will require the development of single-cell-level imaging techniques for visualizing each process within neuronal subcellular compartments (Weil et al., 2010).

**References**


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