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In situ hybridization is a necessary experimental complement to microRNA (miRNA) expression profiling in the human brain

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Abstract

MicroRNAs (miRNAs) play fundamental roles in human brain neurochemistry. However, much remains to be learned in this fast-paced field. To understand how miRNAs contribute to normal biologic functions and disease states, it is critical to understand the miRNAs that are expressed in particular cell types under a range of conditions. Many tools have been developed to help describe the repertoire of miRNAs present at the tissue level in a given sample. However, tissue level miRNA profiling is inadequate to pinpoint the cellular and sub-cellular distribution of individual miRNAs. Such knowledge is especially important in the nervous system with its many cell types, microscopic heterogeneity with regard to functionally distinct cell groups, and extreme geometrical complexity in cellular shapes. We have found that in situ hybridization shows important cerebral cortical lamina-specific patterns of miRNA expression that would be lost on most tissue level expression studies, and these lamina-specific patterns can be directly relevant to human brain disease. Thus, in situ hybridization is an important experimental complement to tissue-level miRNA expression profiling. Technical and theoretical aspects of this important technique are described, especially those pertinent to studying the human brain.

Introduction

MicroRNAs (miRNAs) are non-coding RNAs, ~22 nucleotides in length. In the relatively brief period since their discovery, research has demonstrated that miRNAs play important roles in plant and animal biology [18, 20]. Nonetheless, we are still very much in the “Pre-Copernican” era of non-coding RNA research, and an open-minded perspective is appropriate as we make our initial steps in this fascinating field, and also as we establish the ground-rules for future studies.

miRNA expression profiling has been a key component of miRNA studies -- we cannot know the roles played by particular miRNAs without knowing the tissues in which they are expressed. Through miRNA profiling techniques, we know that the expression of brain miRNAs changes across different neuroanatomical areas and also during brain development. However, tissue-level miRNA profiling has pitfalls because these methods do not describe

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which cells those miRNAs are expressed in. The central nervous system (CNS) has many different cell types and tissue level miRNA profiling is “blind” to how cell types are distributed in the tissue sample from which the RNA is isolated. This shortcoming can seriously hamper the ability of researchers to draw accurate conclusions from the results of tissue-level miRNA profiling experiments.

Thus, to understand the specific roles for miRNAs in the human brain, there must be a complementary experimental technique. The method to assess directly the distribution of particular miRNAs at the cellular and sub-cellular levels is in situ hybridization (ISH). Compared to other RNA expression profiling techniques, ISH is more labor-intensive and “low-throughput”, but allows far greater resolution of a given RNA’s expression.

Like many other aspects of miRNA research, miRNA ISH was published first in plants [2]. A key breakthrough in miRNA ISH was the introduction of locked nucleic acid (LNA) probes [9, 35], which enabled extreme apparent specificity despite the small RNA probe and target sizes. Since then, a number of excellent methods papers have been published about miRNA ISH [10, 12, 25, 26, 30, 34, 38]. Here we describe a method for performing miRNA ISH in the human brain, which is similar to our prior published protocols [21, 36].

Materials and methods

Brain tissue was obtained from University of Kentucky ADC Brain bank using appropriate IRB protocols. Post mortem human brain sample was obtained via autopsy within 3 hrs of death. Brain tissue was fixed in 4% paraformaldehyde (overnight at 4C) and then immersed in 20% sucrose overnight (4C). Tissue was cut to 25 microns on a freezing microtome and mounted onto pre marked Superfrost® Plus slides. The cut tissue sections were allowed to air-dry for 30 minutes. The slides were then transferred to a –80C freezer, until subsequent processing as described below.

The slides were thawed and incubated in 40µg/ml proteinase K in PBS (phosphate buffered saline) for 20 minutes. The slides were then quickly washed twice with Glycine (0.2% w/v) in PBS, each washes of 1 minute duration, followed by two additional PBS washes (1min). The tissues then were fixed in 10% formaldehyde for 10 minutes. This step was followed by two washes of PBS (1 min each). The sections were next acetylated in a mixture of acetic anhydride and triethanolamine (48 ml of water, 250 µl HCl, 300 µl Acetic anhydride, 670 µl Triethanolamine) for 10 minutes. This step was followed by a series of washes in PBS, each one of 2 minutes duration. Next, two additional washes of 5 minute duration were performed in 5XSSC (Sodium Chloride Sodium Citrate buffer). The slides were then kept immersed in pre warmed (37C) pre-hybridization buffer (650 ml ultrapure formamide), 250 ml 20XSSC, 1 ml Tween-20 (Fisher scientific)) supplied with 100µg/ml yeast RNA. The slides were subjected to pre-hybridization at 37C for 3 hrs, in a humid hybridization chamber. For hybridization, the same pre-hybridization buffer was used, with LNA probes (Exiqon Inc) labeled with Digoxigenin at their 5’ ends. The tissues were subjected to hybridization overnight at 37C, in a humid chamber.

On the second day, the sections were washed twice with pre-warmed 5XSSC. This step was followed by stringency washes at 37C in pre-warmed 50% formamide and 2XSSC. Sections were subjected to three such high-stringency washes, each of 20 minute duration. These washes were followed by five PBST (PBS/0.1% Tween@20) washes of 3 minute duration at room temperature. This step was followed by 2 hrs of blocking in 2% sheep serum (Jackson labs) and 2mg/ml BSA (bovine serum albumin) in PBST. Following blocking and pre-adsorption of anti Digoxigenin antibody, the tissue was immersed in pre-adsorbed (1:1500)

anti-Digoxigenin Antibody, conjugated with Alkaline peroxide (Boehringer/Roche) for overnight binding at 4 C.

On the third day, tissue sections were washed four times with PBST, 10 minutes each, followed by two 10 minute washes in staining solution (10mM Tris-HCl pH-9.0, 50mM MgCl₂, 100mM NaCl, 0.1% Tween-20). This step was followed by incubation of the tissues in staining solution containing alkaline phosphatase substrate, NBT (Nitro blue tetrazolium Boehringer/Roche; 50mg/ml) and BCIP (5-Bromo-4-chloro-3-indolyl phosphate EMD, Omnipur®; 50mg/ml).

The staining was continued until good visibility obtained under a light microscope. Staining was stopped by immersing the tissue in PBS for 5 minutes twice. Then the tissues were washed in distilled water for 5 minutes, followed by dehydration in alcohol (5 min steps starting in 50% ethanol). This step was followed by two 5 minute washes in xylenes and then the tissues were mounted in a xylene based medium. The sequences of LNA® probes used in this study:

hsa-mir-320 - AAAAGCUGGGUUGAGAGGGCGA

hsa-mir-107- AGCAGCAUUGUACAGGGCUAUA

hsa-mir-124a - UAAGGCACGCGGUGAAUGCC

hsa-mir-125b – UCCCUGAGACCCUAAUUGUGA

hsa-mir-9 – UCUUUGGUUAUCUAGCUGUAUGA

hsa-let7b – UGAGGUAGUAGGUUGUGUGGUU

Results and Discussion

miRNAs play key roles in neurodevelopment, synapse function, and many other neurobiological contexts [13, 31, 39]. The diverse and powerful roles for CNS miRNAs led us to hypothesize that miRNAs and other noncoding RNAs may provide “complexity multipliers” enabling the relatively small genetic repertoire of humans (<10⁶ genes) to produce the staggeringly complex human brain (>10¹⁴ synapses) [22]. Decreasing cellular miRNAs impairs cellular chromatin remodeling and DNA methylation, although the exact mechanisms for this are still unknown [5, 16, 20]. miRNA pathways are now also known to play roles in many human diseases including in neurodegenerative diseases. MiRNAs may contribute directly to Alzheimer’s disease, Fragile X mental retardation syndrome, Parkinson’s disease, and other brain diseases [3, 6, 8, 22, 23]. A recurring paradigm is that miRNAs provide vital neuroprotective functions, because neurodegeneration ensues after neuronal miRNA levels are decreased [1, 3, 11, 28]. Exact details of these neuroprotective functions are still mostly uncharacterized.

Although there are many tools to analyze miRNA neurochemistry, ISH provides critical additional information about the cellular and sub-cellular distribution of miRNAs. A number of prior human studies have indicated the importance of ISH for miRNA research (for example, see [4, 27, 29]). MiRNAs and their processing proteins are also evidently processed to different sub-cellular compartments in neurons, which may be an extra incentive to perform ISH [15, 31, 32]. There are additional compelling theoretical reasons why ISH is of special importance in the human CNS [21, 23, 36], particularly when comparing diseased and normal tissue.

In evaluating tissues from disease brains, it is often incorrect to view abnormal tissue to merely reflect changes in the existing cells. Rather, “diseased” cells may be replaced by

other cells of minor direct relevance to the disease process. For example, in Alzheimer's disease, the normal constituents of the brain (neurons, resting astrocytes) may be completely replaced by activated glial cells and inflammatory cells (Fig 1A and B). Tissue level profiling in this context can be misleading if coupled with inappropriate assumptions.

There are formidable potential obstacles to interpreting RNA expression studies of "normal" brains as well, aside from the intrinsic difficulties in confidently separating "normal" from "abnormal" clinical cases. The human CNS comprises many distinct cell types in close proximity to each other, some of which can be difficult to dissect from each other in the best of circumstances. Neurons, astrocytes, oligodendrocytes, microglia, leukocytes, vascular cells, and subtypes thereof have distinct functions and need to be evaluated separately. Cell groups that are quite near to each other can have large differences in cell geometry, lineage, and neurotransmitter repertoire with important differences in RNA distribution (see below).

The contradistinction between cerebral cortical gray matter and white matter is very important—the former comprises many neurons and astrocytes, with a lush vascularity, whereas the latter contains few neurons and many oligodendrocytes. This difference is critically relevant because samples that are denoted as "cerebral cortex" can have distinct miRNA repertoires merely because there was a different proportion of gray versus white matter in the samples [24, 37]. We and others have shown that neurons and astrocytes express different miRNAs, as one might expect [14, 33, 37]. Some of these results, comparing primary rat brain culture miRNAs, is shown in Table 1 (these data should be interpreted with the caveat that cells in tissues may express quite differently than cultured primary cells). Cell type-specific miRNA expression is certain to introduce a key bias in many tissue-level expression profiling experiments that do not start with an attentive study design including careful dissection of brain tissue.

Even within the gray matter, where most of the CNS neurons reside, there can be important differences in miRNA expression, even between closely-related cells such as neuronal subtypes. Figure 2 shows the ISH staining patterns of five different miRNAs on an individual case. Note that the human cerebral cortex is characterized by multiple layers of neurons (classically described as six discrete layers, but the layering characteristics change across anatomical areas of cerebral cortex). Different miRNAs are expressed in neurons of different cerebral cortical laminae. A similar finding was recently published by Mellios et al [17].

In addition to the theoretical caveats about tissue-level miRNA profiling, specific examples have been found in which miRNA ISH was a critical complement to tissue-level miRNA expression profiling in studying diseases of human brain. For example tissue level miRNA profiling reveals that both miR-124 and miR-9 are changed in gliomas [19, 21]. We extended the findings from the microarray to ISH and found that the changes seen in miR-124 at the tissue level are probably an artifact of the decreased numbers of neurons in glial tumor specimens[21]. By contrast, ISH revealed that miR-9 is itself expressed robustly in tumor cells, so this miRNA is a better candidate for further studies as a potential cancer-related gene. Since both of these miRNAs are expressed at high levels, ISH was performed on formalin-fixed and paraffin-embedded tissue for this study.

We also found ISH to be important in the context of studying neurodegenerative diseases [23, 36]. At the tissue level, miR-107 is decreased in the pathogenesis of AD, starting even before clinical disease is apparent, in parallel with the pathological hallmark lesions of amyloid plaques and neurofibrillary tangles [36]. When ISH was performed for miR-107 in human brains, it was found that miR-107 is expressed in both neurons and astrocytes but is neuronally-enriched. Perhaps most importantly, unlike other miRNAs, the expression of

miR-107 is decreased disproportionately in particular laminae of neurons that are most affected by pathology in AD [36]. It should be noted that the field of miRNAs in neurodegeneration is still very much in its earliest stages [6–8, 23].

In summary, both theoretical and practical experience indicates that miRNA ISH is important complement to tissue-level miRNA expression profiling in the human brain. This practice should be routinely applied to increase the quality of brain miRNA studies. ISH studies may be most important in the context of experimental approaches that involve comparing normal versus disease brain tissue.

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Figure 1.

In the human brain, cellular heterogeneity introduces important potential sources of bias for tissue-level gene expression profiling. In the course of brain disease, tissues can be depopulated for a given cell population and repopulated with another, in a manner that is not necessarily instructive about the disease process. For example, the normal cellular constituents of cerebral cortex, that includes neurons (green arrow, 1A), can be lost in AD (1B). AD brain can contain reactive astrocytes (blue arrow, 1B). Scale bar = 50 microns in A and B. In addition to heterogeneity linked to disease, the normal brain components should be taken into consideration. Specifically, the gray matter can have a distinct miRNA repertoire from white matter as a reflection of the different cell populations that inhabit those areas (1C).

Figure 2.

Near serial sections from the same portion of temporal isocortex of a human brain (AD patient). Note the individual cortical cell layers show the different staining patterns using different in situ hybridization (ISH) miRNA probes. These results indicate that nearby human cortical neurons have distinct miRNA expression repertoires.

Table 1

MiRNAs enriched in primary neuronal and astrocyte cultures (Source: Ref [37])

Neuron-enriched	Ratio neurons/astrocytes
miR-124a	208.9
miR-129*	14.2
miR-128	12.5
let-7gL	5.0
miR-181c	4.3
miR-219	4.2
Astrocyte-enriched	Ratio astrocytes/neurons
miR-193	39.7
miR-21	17.0
miR-23a	9.6
miR-143	9.4
miR-221	8.9
miR-23b	8.5
miR-27	8.4
miR-24	7.9
miR-29	7.2
miR-22	6.3
miR-143a	5.8
miR-193	5.0