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Analysis of microRNA expression by *in situ* hybridization with RNA oligonucleotide probes

Robert C. Thompson [a,b], Monika Deo [a], David L. Turner [a,c,*]

[a] *Molecular and Behavioral Neuroscience Institute, University of Michigan, 5062 BSRB, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200, USA*
[b] *Department of Psychiatry, University of Michigan, Ann Arbor, MI 48109-2200, USA*
[c] *Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109-2200, USA*

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Abstract

In situ hybridization is an important tool for analyzing gene expression and developing hypotheses about gene functions. The discovery of hundreds of microRNA (miRNA) genes in animals has provided new challenges for analyzing gene expression and functions. The small size of the mature miRNAs (~20–24 nucleotides in length) presents difficulties for conventional in situ hybridization methods. However, we have described a modified in situ hybridization method for detection of mammalian miRNAs in tissue sections, based upon the use of RNA oligonucleotide probes in combination with highly specific wash conditions. Here, we present detailed procedures for detection of miRNAs in tissue sections or cultured cells. The methods described can utilize either nonradioactive hapten-conjugated probes that are detected by enzyme-coupled antibodies, or radioactively labeled probes that are detected by autoradiography. The ability to visualize miRNA expression patterns in tissue sections provides an additional tool for the analyses of miRNA expression and function. In addition, the use of radioactively labeled probes should facilitate quantitative analyses of changes in miRNA gene expression.

Keywords: MicroRNAs; Gene expression; Nervous system; Brain; Development; Embryos; Fluorescein; Mouse; Rat; Human

1. Introduction

MicroRNAs (miRNAs) are short endogenous RNAs, ~20–24 nucleotide (nt) long, that were first identified in the nematode *Caenorhabditis elegans*, and subsequently found in most animals and plants [for review see 1–3]. miRNAs have been shown to function as post-transcriptional negative regulators of gene expression in a variety of systems [reviewed in 4,5]. There are about 500 known miRNAs in humans, although prediction algorithms suggest that the total number of miRNAs could be substantially larger [6], and experimental approaches suggest that new miRNAs still remain to be identified [7,8]. Thus, miRNAs appear likely to be a major component of gene regulation. miRNA synthesis is a multiple step process (reviewed in [1,2,9]). miRNAs are initially synthesized as longer primary RNA transcripts, and some miRNAs are clustered in genomes, where they can be transcribed as polycistronic primary transcripts. The nuclear microprocessor complex, which includes the endonuclease Drosha, recognizes secondary structures in primary transcripts and cleaves the primary transcripts to release short stem-loop miRNA precursors (~65 nt long), which are then exported to the cytoplasm. In the cytoplasm, the Dicer endonuclease cleaves a stem-loop precursor to release both the mature miRNA and its complementary strand. In most cases, one strand (arm) of the stem-loop precursor accumulates as a stable miRNA after Dicer processing, while the complementary strand (referred to as the "*" strand) is degraded. Mature miRNAs are incorporated into ribonucleoprotein (RNP) complexes that contain a member of

* Corresponding author. Address: Molecular and Behavioral Neuroscience Institute, University of Michigan, 5062 BSRB, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200, USA. Fax: +1 734 936 2690.
E-mail address: dlturner@umich.edu (D.L. Turner).

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the Argonaute protein family as well as other proteins, and these RNP complexes function as negative regulators of mRNA translation and/or mRNA level (reviewed in [10]).

For several years after the discovery that large numbers of miRNAs were present in animals, a major limitation for understanding miRNA function was the difficulty in determining detailed spatial expression patterns for specific miRNAs. However, two approaches for detection of miRNAs by in situ hybridization (ISH) have been developed recently. Plasterk and colleagues have used an ISH method based on Locked Nucleic Acid (LNA) oligonucleotide probes to detect mature miRNAs in whole mounts of zebrafish and mouse embryos [11,12]. The LNA-based ISH methodology has been applied to whole-mount embryos from a variety of vertebrate species, including chicken and medaka [13,14], and for the analysis of adult human brain sections [15] as well as mouse tissue sections [16,17]. Independently, we have developed a miRNA ISH method based upon RNA oligonucleotide probes and have used this method for the detection of mature miRNAs in tissue sections from embryonic and adult mice [18], as well as from rat and human brains (this article) and zebrafish (our unpublished observations). We have also detected miRNAs by ISH in cultured cells [18]. The LNA-based miRNA ISH method derives a high degree of sequence specificity from the base-pairing properties of LNA probes. In contrast, we have used high-stringency wash conditions based on tetramethylammonium chloride (TMAC) in combination with RNase A treatment to remove unhybridized probe to generate highly sequence specific conditions for miRNA ISH with RNA probes. Both methods appear to generate similar results based on the comparison of published expression patterns. One potential advantage of RNA probe based ISH is that the use of TMAC washes allows probes of different sequence compositions (with potentially different melting temperatures) to be processed under identical conditions [19,20].

Although miRNAs are generated by processing from longer precursor and primary transcripts that contain the same miRNA sequence, miRNA ISH using either RNA or LNA probes appears to primarily detect mature miRNA [12,18]. Most likely, the discrimination between mature miRNA and precursor or primary transcript RNAs reflects the accumulation of mature miRNAs to high levels in cells, relative to mRNAs [21], and the location of the target miRNA sequences within a stem-loop secondary structure in miRNA precursors and primary transcripts, which seems likely to reduce access of probes for hybridization during ISH. It also seems possible that miRNAs base-paired with a target mRNA in RNP complexes are less accessible to hybridization during ISH, in which case miRNA ISH may preferentially detect mature miRNAs that are not bound to a target mRNA, but this possibility has not been tested.

To date, most published studies on miRNA ISH have used probes labeled with nonradioactive haptens (digoxigenin or fluorescein). Bound hapten-labeled probes are detected by histochemical enzymatic reactions after application of alkaline phosphatase (AP)-conjugated anti-hapten antibodies. For RNA oligonucleotide probes, we have used fluorescein as a hapten, since it is convenient to commercially synthesize RNA oligonucleotides with a 5′ end fluorescein. Nonradioactive ISH methods are rapid and permit the precise localization of mRNA or miRNA expression to specific cells or even cellular compartments, as well as facilitate whole-mount analyses, but these methods are less amenable to quantitative analysis of miRNA levels. However, ISH with radioactively labeled probes allows accurate semi-quantitative or quantitative comparisons of mRNA expression levels [22,23] using autoradiographic methods (e.g. X-ray film, photographic emulsions, phosphorimagers). As described below, we have modified our miRNA ISH method to allow the use of ^{33}P 5′ end-labeled RNA probes for the detection of miRNAs in tissue sections. This approach should allow comparisons of miRNA levels among different cells or structures (e.g. brain nuclei). In the following sections, we provide detailed methods for detection of miRNAs by ISH using RNA oligonucleotide probes labeled with either a fluorescein hapten or ^{33}P.

2. Experimental design considerations

2.1. Probe design and controls

In most cases, we have used synthetic RNA oligonucleotide probes with 20 nt of complementarity to a specific target miRNA, and the conditions presented were optimized for this probe size. However, probes with 21 or 22 nt complementary to a target miRNA appear to function similarly [18]. Most of our fluorescein-labeled RNA probes also include two additional nucleotides at the 5′ end that do not match the target miRNA. These bases are not required for probe function, but they were included to potentially improve access of the anti-fluorescein antibody to the 5′ end fluorescein. Since the TMAC-based wash conditions are not sensitive to sequence composition, the choice of which 20 nt sequence within a miRNA longer than 20 nt should be used for probe generation appears to be arbitrary. Since many miRNAs are part of gene families that are composed of miRNAs that differ at only one or a few positions, all probe designs should be compared against miRBase (http://microrna.sanger.ac.uk/) [24] to identify potential unanticipated miRNA matches. We have observed that our conditions can prevent significant hybridization of an RNA oligonucleotide probe with two mismatches to an abundant miRNA [18], and in some cases it may be possible to distinguish miRNAs that differ by a single nucleotide near the center of the miRNA. In general, if it is desirable to distinguish between closely related miRNAs, probes should be positioned on the target miRNAs so as to maximize the number of mismatches with non-target miRNA(s), and so that potential duplexes between probe and non-target miRNA(s) will be as short