In situ hybridization-based detection of microRNAs in human diseases

Abstract
MicroRNAs (miRNAs) are small non-coding RNAs that regulate various aspects of gene expression in physiology and development. Links between miRNAs and the initiation and progression of human diseases are becoming increasingly apparent. The development of methods to detect the subcellular and tissue localization of miRNAs is essential for understanding their biological role in homeostasis. In this review, we discuss how in situ hybridization can complement tissue-level miRNA expression profiling and its role as an investigational tool to better understand the etiology of human diseases. Furthermore, in situ hybridization of miRNAs represents a potent diagnostic assay that could be further refined and utilized for clinical applications.

Keywords
microRNA • in situ hybridization • human diseases • profiling

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Introduction

MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs (ncRNAs) that are highly conserved among species and are essential to many physiological and pathophysiological processes [1,2]. miRNAs are transcribed by RNA polymerase II or III, as longer primary transcripts that are further processed to form hairpin loop structures and undergo maturation through a series of catalytic steps. In the cytoplasm, mature miRNAs are integrated into the RNA-induced silencing complex (RISC) and are incorporated with messenger RNA (mRNA) [3,4]. Perfect or near-perfect complementarity between miRNA and its target mRNA has been found to induce mRNA degradation by RISC; partial complementarity has been found to repress mRNA translation by blocking ribosomal access to the mRNA [5]. Because of the “power” miRNAs have to control gene expression, they are involved in a wide range of developmental processes, cell-cycle regulation, and the control of cell fate [6]. Studies of miRNA expression have revealed tissue- or cell-specific expression patterns, and the deregulation of miRNA expression and activity has been correlated with the pathogenesis of a variety of human genetic diseases, e.g., cancer, cardiovascular disease, diabetes, and neurodegenerative disorders [6-11].

In situ hybridization (ISH) is a powerful tool that can provide invaluable insights into physiological and pathological processes by identifying the expression level and localization of specific miRNA within individual cells in tissue sections. In this review, we first provide an overview of miRNA ISH using locked nucleic acid (LNA)-modified probes and the recent progressions in this technology. Secondly, we briefly compare the different miRNA profiling methods associated with LNA-ISH. Finally, we discuss the recent use of ISH to research the pathology of various human diseases.

LNAs and miRNA ISH

Physicochemical properties of LNAs
LNAs comprise a class of bi-cyclic, high-affinity RNA analogues in which the furanose ring of LNA monomers is conformationally locked in an RNA-mimicking C3′-endo (N-type) conformation [12,13]. LNA is defined as an oligonucleotide containing at least one LNA monomer (i.e., one 2′-O,4′-C-methylene-β-d-ribofuranosyl nucleotide). LNA is a versatile tool possessing basic physicochemical properties that are essential for practical utilization, which include good aqueous solubility and a full compatibility with standard coupling chemistry and synthesis programs of commercial DNA synthesizers. Furthermore, LNA monomers are fully compatible with other monomers and can be readily combined with them (e.g., DNA, RNA, 2′-O-Me-RNA, and 2′-O-MOE-RNA) and/or with phosphorothioate or phosphodiester linkages [14,15].

Several studies have demonstrated that LNA-modified oligonucleotides exhibit unprecedented thermal stability when hybridized with their RNA target molecules [16,17]. An increase in melting temperature (Tm) of 2–10 °C per monomer against complementary RNA, compared with unmodified duplexes, has been reported. The observed relative increase in Tm per LNA monomer in LNA:DNA mixmers reaches a maximum for oligonucleotides containing less than 50% LNA monomers [18,19]. Importantly, LNA incorporation improves mismatch discrimination compared to unmodified reference oligonucleotides. LNA-modified oligonucleotides follow Watson-Crick base pairing rules and mediate high-affinity hybridization without compromising base pairing selectivity. In addition, LNA oligonucleotides display high stability and low toxicity in biological systems, efficient transfection into...
mammalian cells, good aqueous solubility, and potent antisense activity in vivo [20].

**miRNA IN SITU HYBRIDIZATION PROTOCOLS**

Detection of miRNA in tissue sections by ISH is required to understand the details of how individual miRNAs function in tissue homeostasis, development, and disease. ISH technology allows for the quantitative detection of expression at the cellular level. The generally fragile nature of RNA and the small size of miRNAs are two main parameters that make ISH a challenging technology. In contrast to ISH analysis of mRNA, which is most often performed using long antisense and sense probes obtained by in vitro transcription, in situ detection of miRNAs requires alternative methods for both detection and specificity analysis. The employed protocols generally include proteolytic digestion, post-fixation, acetylation, hybridization, or signal amplification and detection (Figure 1).

As was described above, the high affinity and excellent discrimination of 1-20 LNA oligonucleotides make them well suited for various miRNA detection and analysis techniques, including microarray analysis, qPCR, micromagnetic bead-based hybridization, and ISH. The use of LNA:DNA chimeric probes for ISH analysis has led to a series of detection strategies for chromogenic and fluorescence detection. Different types of tissues can be used, including paraffin sections [21-23], cryostat sections [24-27], whole-mount specimens [28,29], and cultured cells [26,30,31]. For successful detection of miRNAs, formalin-fixed paraffin-embedded (FFPE) sections 4-6 μm thick and frozen sections 8-12 μm thick are adequate. The efficiency of the system should be checked using a positive control probe and one or two negative control probes.

The two most critical steps in the miRNA ISH protocol are the target demasking step, often known as the “predigestion” or “proteinase-K” step, and the hybridization step. The proteolytic digestion step is based on the use of relatively unspecific cleavage of tissue components by proteases, such as proteinase K or pepsin. Proteolytic digestion of the tissue provides access to the miRNAs in the tissue matrix. The optimal level of proteinase K treatment depends on the extent of fixation. Specific probe hybridization requires a well-defined and stable hybridization buffer, a stable hybridization temperature, and an optimal probe concentration. The hybridization buffer must have an optimal level of salts, oligonucleotide stabilizers (i.e., inert RNA or DNA), viscosity-reducing agents (e.g., dextran and Denhardt’s solution), and denaturing agents (e.g., formamide). Successful miRNA ISH using LNA probes has been reported with concentrations of formamide ranging from 25% to 70% [23,30,32]. A formamide-free hybridization buffer has been developed which allows detection of miRNAs with LNA probes at a high signal-to-noise ratio [22].

**DEVELOPMENT OF miRNA IN SITU HYBRIDIZATION ASSAYS**

A few methods have been reported to be able to amplify the ISH signal when low copy numbers of miRNA need to be detected. The catalyzed reporter deposition (CARD) method is based on the deposition of biotinylated tyramine (BT) at the location of the probe label through the enzymatic action of horseradish peroxidase [33]. The BT precipitate can then be visualized with

**Figure 1. Principle of microRNA in situ hybridization**

**Fluorescence detection**

- T
- T
- T
- T
- DIG
- Anti-DIG
- Antibody incubation
- Hybridization and wash on slides
- LNA probe
- miRNA on tissue

**Chromogenic detection**

- AP, alkaline phosphatase; DIG, digoxigenin; HRP, horseradish peroxidase; T, tyramide.

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fluorochrome- or enzyme-labeled avidin. The tyramide can also be conjugated with highly sensitive Alexa Fluor dye to improve theISH signal.

Multiple color staining is very useful; combinations of ISH and immunohistochemistry (IHC) or sequential ISH protocols have been recently developed [23,34,35]. This technology makes it possible to assess the topographic relationship of the targets, that is, to determine whether targets are present in different cells, in the same cell, or even in the same cellular compartment. Information can also be obtained on possible cell-cell spatial contacts between different cell types. Furthermore, with an increasing demand for less invasive sampling techniques and smaller and fewer specimens available, multiple staining has an additional advantage of saving time and reagents. Chromogenic ISH is compatible with bright-field microscopy, and makes it easy to view signals simultaneously with tissue morphology. Double-color chromogenic ISH has been reported, but the differential detection of the probes in conjunction with counterstains can be problematic, so fluorescent ISH is ideal for detecting multiple probes. Application of tyramide signal amplification (TSA) allows for the identification of multiple targets by probes that are simultaneously hybridized. To prevent artifact staining, signal development using multicolored fluorescent tyramides must be carried out sequentially with a peroxidase inactivation step between each TSA reaction. One example of this is the work performed by Lorenzo Sempere’s group, who developed a very sensitive multicolor ISH/IHC assay to study a subset of cancer-associated miRNAs, including miRNAs frequently detected at low and high levels, in a panel of carcinomas of the breast, colon, lung, pancreas, and prostate [23].

ISH involves many steps and is quite time consuming. The development of automatic ISH/IHC equipment makes high-throughput analysis of gene expression, including miRNA expression, possible. The automatic system performs every step—from removing the paraffin to adding the primary and secondary antibodies, detecting the antigen, and adding the counterstain. Manually adding the probe and primary antibody is an option, which allows one to conveniently optimize the concentration of the primary antibody or probe. This optimization allows the processing of hundreds of samples within a few days.

Methods of miRNA expression profiling

miRNA expression profiling has been a key component of miRNA studies. It can be applied to disease prognosis, it may be used to define disease markers, and, in some cases, it can be used to predict cancer therapy outcome [36,37]. Numerous techniques are available for gaining information about miRNA signatures; the most frequently used methods are presented in Table 1. Northern blot analysis [38,39] was first used for gene expression detection and quantification of miRNA before the development of other techniques. The first high-throughput technology was miRNA microarray analysis, which was initially developed in Carlo Croce’s laboratory [40]. Other than microarray, frequently used strategies for miRNA profiling are quantitative real-time polymerase chain reaction (qRT-PCR) [41,42], bead-based hybridization [43], and deep sequencing [44,45] (Figure 2). However, miRNA expression is, in many cases, highly cell-type specific, and none of these methods identify which cells those miRNAs are expressed in. ISH technique is the only method that allows direct assessment of the distribution of particular miRNAs at the cellular and subcellular levels.

Deep sequencing

When applied to miRNA profiling, deep sequencing methods have high sensitivity and the potential to discover novel ncRNAs. Deep sequencing allows the simultaneous sequencing of several different RNA molecules, including small RNAs [46]. The existing deep sequencing platforms differ by the chemistry principles used, approach to amplification, efficiency, duration, expense, accuracy, error distribution, and tolerance of minimum sequence length (from 13 to 250 base pairs) [46,47]. Deep sequencing technologies are not subject to the problems of background noise and cross hybridization, compared with other high-throughput assays like microarray [47]. Information gained via deep sequencing reflects the actual miRNA profile, and de novo sequencing allows the discovery of miRNAs or ncRNAs not previously identified by cloning or sequencing [48]. Deep sequencing also allows for the detection of allele-specific expression of miRNA, and relative quantification of the guide vs. passenger strands of miRNAs. Key challenges of the deep sequencing method include the requirement for at least 10 ug of high-quality RNA, as the purification steps lead to a loss of material. Deep sequencing methods are also expensive for routine laboratory work. As they become more affordable, their use will become more widespread [44,48]. The “third generation” of sequencing, which will soon be available, is based on nanopore technology and will be able to read the diploid mammalian genome in approximately 24 hours at much lower costs than second-generation sequencing, which requires 4–5 days [49].

miRNA microarray analysis

Microarray analysis allows the simultaneous testing of multiple genes in numerous samples, provides a large amount of information, and saves time. It is now recognized that this method should be used only as a first step in miRNA expression screening and should be followed by other profiling techniques for validation purposes, such as qRT-PCR [40]. Many types of microarray are available that are based on distinct principles and technologies that vary in preparation steps, hybridization steps, and final application. Microarrays also differ by the surface used (glass or quartz); how probes are spotted (hand or machine), designed (a difficult task because of the small size of miRNAs), synthesized (possibly directly on the surface), and modified; and the amount of probe or sample that is needed. The aim of each experiment dictates the type of array used [40,50,51]. Finally, sample preparation and labeling vary, depending on the array used, on the need to recognize mature and precursor forms of miRNAs, and the fact that they may or may not require
## Table 1: Methods used for analyzing and profiling RNA expression.

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td><strong>Microarray</strong></td>
<td>• Sample preparation (RNA → cDNA) • cDNA labeling • Hybridization with solid-phase probes → signal • Washing • Detection of signal • Data analysis</td>
<td>• High throughput method (simultaneous testing of numerous samples) • More information gained in a single run • Simple method to standardize • Probes are spotted on solid surface by precise automated machine (thousands of probes on 1 cm²) • Genome-wide expression analysis</td>
<td>• Hard to differentiate expression of highly similar miRNA • Expression data presented in relative format • Background noise and cross-hybridization problems</td>
<td>[40]</td>
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<tr>
<td><strong>Bead-based array</strong></td>
<td>• Small RNA isolation • Adaptor ligation • Adaptor specific reverse transcription • PCR amplification • PCR product labeling • Signal detection with flow cytometry (hybridization of PCR amplicon with bead-fixed probes)</td>
<td>• High specificity (differentiation of closely related miRNA, sensitivity, and efficiency • High speed, low cost • Quantitative results • Classification of miRNA based on fluorescent dye mixtures • High-throughput analysis, but not the best one</td>
<td>Long procedure (adaptor ligation is transcription, target amplification) • Limited (but not low) number of miRNA can be analyzed in one assay • Competition of hybridization for target PCR amplicon between its own complementary strand and specific probe • Enrichment by fractionation is necessary</td>
<td>[43]</td>
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<td><strong>In situ hybridization (LNA probes)</strong></td>
<td>• Tissue or cell preparation • Digestion with protease (proteinase K) • Hybridization → washing • Conjugate incubation • Chromogenic or fluorescent detection • Reaction monitoring under microscope</td>
<td>• RNA preservation (no cell lysis is performed) • Actual localization of miRNA inside tissue or cell system • Multiplexing capability</td>
<td>Low-throughput method • Poor detection of miRNAs present in low copy numbers • Background signal problems in samples with a high number of miRNAs</td>
<td>[28,30,35]</td>
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<td><strong>qRT-PCR</strong></td>
<td>• RNA extraction from samples • RNA to cDNA reverse transcription (gene specific or total cDNA) • Target sequence amplification</td>
<td>• Low numbers of ncRNA detection • Qualitative and quantitative technique • Rapid, highly sensitive, specific, and affordable • Used as a confirmation method of microarray data • No postamplification detection is necessary</td>
<td>Low throughput method • Extraction of RNA and retro-transcription are steps where RNA can be degraded • Quality control checks are necessary for reliable quantitative results</td>
<td>[41,42]</td>
</tr>
<tr>
<td><strong>Northern blot</strong></td>
<td>• RNA extraction from sample • Size separation by electrophoresis • UV/heat fixation • Membrane hybridization by specific probe • X-ray RNA visualization</td>
<td>• Quantitative method • Separates RNA molecules by size and class • Quantity comparison among loaded samples • Simple, specific, cheap • Detection of amount, size, and quality of transcripts</td>
<td>Low throughput method • RNA degradation problem during electrophoresis • Unhealthy chemicals needed for performing assay (radioactive material, formaldehyde, DEPEC, ethidium bromide, UV light) • Difficult to perform using more than one probe • Low sensitivity</td>
<td>[38,39]</td>
</tr>
<tr>
<td><strong>Deep sequencing</strong></td>
<td>• Library generation • Genome/DNA of interest • Fragmentation • Adaptor ligation (DNAase, sonification) • PCR amplification (in situ polonies, emulsion PCR, bridge PCR) • Parallel sequencing</td>
<td>• Potential to discover novel ncRNA • High throughput method (simultaneous sequencing of many different RNA molecules) • Wide range of applications (polymorphism, mutation, and methylation detection, detection of structure variation, gene expression, and miRNA profiles) • Absolute quantification of miRNA profiles</td>
<td>Expensive (reagents, maintenance, bioinformatics software) • Loss of miRNA during necessary purification steps</td>
<td>[44,45]</td>
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need for large amounts of RNA. In fact, the PCR-based miRNA profiling method can detect miRNAs with very low copy numbers [41], which is important because the RNA from clinical samples may be very limited. Advantages of qRT-PCR-based techniques are its high levels of sensitivity, specificity, and affordability [53]. qRT-PCR also offers the possibility of early cancer diagnosis, detection of disease progression, cancer classification (when histopathological findings are indeterminate), screening for RNA genes responsible for diseases, and a new approach in monitoring treatment efficacy [53, 54]. miRNA expression analysis by qRT-PCR technique is also widely used to confirm the most significant results obtained from miRNA microarray genome-wide profiling [53]. However, qRT-PCR has some technical limitations. It requires the input of good-quality RNA, has a complicated reverse transcriptase step, and inter-batch variability.

miRNA ISH with LNA probes

miRNA ISH is the only method that renders the location of RNA visible on a histology section. Briefly, ISH detects miRNA by facilitating a reaction between the miRNA and the labeled miR-specific LNA probes. The greatest strengths of the technique are its ability to preserve RNA integrity (no cell lysis is performed) and identify the actual locations of miRNA inside tissues or cell compartments [35]. The efficiency of ISH depends on the miRNA copy number (the higher the better), probe length and concentration, and hybridization set-up conditions (i.e., stringency) [28, 35]. The several published protocols differ mainly in regard to the necessity of protease prehybridization, the molecule used for labeling the probes (fluorescein isothiocyanate or digoxigenin), probe concentration, hybridization temperature,

Bead-based hybridization

The advantages of the bead-based hybridization method for miRNA profiling are its high specificity, sensitivity, and efficiency [43]. The protocol starts by ligation of an adaptor molecule to a small RNA, which is followed by adaptor-specific reverse transcription (i.e., primers aligning to the adaptor) and then PCR amplification with primers antisense to the adaptor sequence is being subsequent (one of which is labelled with biotin). The biotinylated PCR product is then hybridized with miRNA-specific probes bound to fluorescent beads. Known miRNA-specific signals that are produced together with the beads and marked PCR products are measured and read by a flow cytometer. For each known miRNA, a complementary probe and mixture of fluorescent dyes have been made to distinguish and decipher the miRNA when its signal is detected [43, 52]. The bead-based hybridization technique is time-consuming and strenuous. As with miRNA microarrays, this method is limited in that only known miRNAs can be detected, but it has the advantage that all known miRNAs can be assayed in a single run [52], and the RNA input required can be as low as 100 ng. Although this technique is not as commonly used as other miRNA profiling techniques, the accuracy and reliability of the miRNA expression profiles generated by this technique are highly comparable to profiles gained with qRT-PCR or direct sequencing [43].

miRNA qRT-PCR

PCR-based profiling is the ideal solution for some of the problems encountered with microarray profiling, for instance the need for large amounts of RNA. In fact, the PCR-based miRNA profiling method can detect miRNAs with very low copy numbers [41], which is important because the RNA from clinical samples may be very limited. Advantages of qRT-PCR-based techniques are its high levels of sensitivity, specificity, and affordability [53]. qRT-PCR also offers the possibility of early cancer diagnosis, detection of disease progression, cancer classification (when histopathological findings are indeterminate), screening for RNA genes responsible for diseases, and a new approach in monitoring treatment efficacy [53, 54]. miRNA expression analysis by qRT-PCR technique is also widely used to confirm the most significant results obtained from miRNA microarray genome-wide profiling [53]. However, qRT-PCR has some technical limitations. It requires the input of good-quality RNA, has a complicated reverse transcriptase step, and inter-batch variability.

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and duration, and the requirement for signal amplification [35]. Common problems in LNA-based ISH are a strong background signal and/or a weak miRNA signal, which is especially prevalent in samples containing low miRNA copy numbers. These issues can be resolved by increasing the probe concentration or introducing an amplification step [35]. Although ISH is a low-throughput approach (one probe per miRNA), it is the only miRNA profiling technique that can evaluate miRNA expression in fixed tissues, cells, and circulating tumor cells and that can be used to screen for single miRNA molecules in human cancer tissue sections and animal embryos [28,30]. Furthermore, this method can be expanded to identify different ncRNAs.

**miRNA ISH application in human diseases**

**Cancer**

miRNA genes are most often located at fragile sites, which frequently undergo loss of heterozygosity (LOH), amplification, and breakages. This is one observation that supports the hypothesis that miRNAs may be dysregulated in human cancers [55]. Hundreds of expression profiling studies have also shown that tumors ubiquitously exhibit dysregulated miRNA expression patterns relative to corresponding normal tissue. Patterns of miRNA expression provide useful information for tumor classification and prognosis [56]. Nevertheless, as miRNA expression is, in many cases, highly cell-type specific, the results of such profiling studies must be interpreted cautiously because apparent dysregulation of miRNAs in cancer cells can be a manifestation of the distinct cell populations represented in tumors versus normal tissue. Measurement of miRNA by ISH allows for the visualization of miRNA expression within individual cells, thereby providing the means to characterize miRNA expression levels in cells of varying pathology. This method also allows for the large-scale specimen analysis on tissue microarrays (TMAs) with the previously described advantages related to standardization.

Some miRNA genes are overexpressed in cancers, indicating that they may have roles as oncogenes and accelerate the development of cancer. *mir-21* is one of the most well characterized miRNAs and is overexpressed in a number of solid tumors, including colon, breast, lung, pancreatic, prostate, bile duct, and stomach cancers [57,58]. Yamamichi et al. [59] found that *mir-21* was much more highly expressed in colorectal tumors than in normal colonic mucosa. They also observed, using ISH, that *mir-21* was expressed in cancer-associated stromal fibroblasts and that the frequency and extent of *mir-21* expression increased during the transition from precancerous colorectal adena to advanced carcinoma. Also using ISH, Dillhoff et al. [60] studied TMAs consisting of 80 resected pancreatic cancer specimens, 12 benign pancreas sections, and 45 samples with chronic pancreatitis. *Mir-21* was overexpressed significantly in 79% of pancreatic cancers and was seen preferentially in the tumor cells, and not in the surrounding stroma. Conversely, in the benign pancreas, *mir-21* was only weakly expressed in ductal epithelial cells. This strong expression of *mir-21* predicted limited survival in patients with node-negative disease and may prove be an important biologic marker for outcome. To better understand the role of *mir-21* in gliomas, Hermansen et al. [61] analyzed paraffin-embedded glioma tissue samples from 193 patients with grade I-IV tumors. ISH analysis showed that *mir-21* was expressed in tumor cells and tumor-associated blood vessels, but not in adjacent normal brain parenchyma. Furthermore, only *mir-21*-positive tumor specimens were associated with poor prognosis.

*mir-155* is another putative oncogenic miRNA that is overexpressed in several types of human solid tumors, including breast, colon, and lung cancer [57,58,62]. ISH analysis of solid tumors revealed that *mir-155* was predominantly confined to endothelial and immune cells, which suggests a differential effect of miRNAs on stromal, vascular, and immune responses [23]. Donnem et al. [63] did a large-scale study combining high-throughput TMA and ISH to evaluate the prognostic impact of *mir-155* expression in non-small cell lung cancer patients. They found that high *mir-155* expression was an independent negative prognostic factor in adenocarcinomas and an independent favorable prognosticator in squamous cell carcinomas patients with regional nodal metastasis. Ryu et al. [64] profiled three miRNAs (*mir-155*, *mir-21*, and *mir-221*) known to be overexpressed in invasive pancreatic cancer in a panel of pancreatic intraepithelial neoplasia (PanIN) lesions of varying histological grades. Using qRT-PCR combined with ISH, they identified *mir-155* deregulation as an early event in the multistep progression of pancreatic cancer, while *mir-21* and *mir-221* abnormalities occurred later, either at the stage of carcinoma-in-situ or in invasive adenocarcinomas.

Finally, *mir-10b* is a third putative miRNA oncogene. Using ISH, Preis et al. [34] identified that *mir-10b* was the most frequently and consistently overexpressed miRNA among characterized miRNAs in pancreatic ductal adenocarcinoma (PDAC) samples. *Mir-10b* expression was increased in cancer cells compared with CK19-positive epithelial cells in benign lesions. In patients with PDAC, lower levels of *mir-10b* were associated with improved response to multimodality neoadjuvant therapy, likelihood of surgical resection, delayed time to metastasis, and increased survival. These findings suggest that expression of *mir-10b* is predictive of response to neoadjuvant therapy and outcome in this disease.

Some miRNAs are downregulated in various human cancers, which suggests they function as tumor suppressors. As examples, *let-7b* and *mir-205* are frequently lost in a range of solid malignancies, including breast tumors. Quesen et al. [65] used semi-quantitative ISH to analyze a TMA of 2,919 FFPE archival breast tumors. The ISH results revealed that *let-7b* expression was associated with luminal tumors and had an independent significant positive prognostic value in this group, while *mir-205* expression was associated with tumors of ductal morphology and was of significant positive prognostic value within these tumors. This ISH approach provides a more direct and informative assessment of how altered miRNA expression...
contributes to carcinogenesis compared with miRNA expression profiling in gross tissue biopsies.

Studies using qRT-PCR and microarray analysis found that miR-205 is downregulated in primary and metastatic melanoma, compared to benign nevi. Hanna et al. [66] utilized quantitative ISH to evaluate the tumor suppressing properties of miR-205. They found that low levels of miR-205 expression in melanoma cells associated with a shorter survival outcome, suggesting a potential prognostic or predictive role for miR-205. A study by Hansen et al. [67] analyzed the possible predictive value of miRNA-126 in relation to first line XELOX therapy in patients with metastatic colorectal cancer, using a quantitative analysis of the miRNA-126 expression level based on ISH analyses of tumor sections from the primary tumor. A significant relationship between miRNA-126 expression levels and response to first line XELOX treatment was demonstrated, indicating that miRNA-126 may be an important predictive marker to chemotherapy applied in the clinical setting.

Taken together, these findings indicate that miRNAs have critical roles in the mechanism of human carcinogenesis and that aberrant expression of miRNAs may contribute to the development of human cancer. Further, these studies show that miRNA ISH allows for a far greater resolution of miRNA expression and is a necessary complementary technique to better understand the pathology of human cancer.

**Cardiovascular diseases**

miRNAs play an essential role in cardiovascular biology and are often dysregulated in several heart diseases, including myocardial hypertrophy, heart failure, and arrhythmia [68]. These deregulations can be caused by aberrant expression of genes from the miRNA biogenesis machinery. For instance, a very low level of Dicer expression was observed in patients with end-stage dilated cardiomyopathy and/or heart failure, and significantly elevated Dicer expression was found in recovering hearts after installation of a left ventricular assist device [69]. Dysregulation can also be caused by aberrant expression of miRNAs themselves (e.g., deregulation of miR-1 and miR-133 in cardiac hypertrophy and arrhythmia) [68].

Profiling studies have identified that some are miRNAs differentially regulated during cardiac development and disease, while their distinct cell-specific localization remains largely undetermined. In this regard, ISH analysis is the most powerful, as it can provide the specific localization of regulated miRNA and will also be prerequisite for subsequent unraveling of the importance and function of each miRNA in relation to cardiac development.

It is known that miR-1, miR-133a, miR-133b, miR-206, miR-208a, and miR-499 are muscle-specific miRNAs. Among them, miR-133b and miR-206 are only expressed in skeletal muscle and miR-208a is only expressed in cardiac muscle [70]. miRNA microarray analysis of the heart from a mouse model of myocardial infarction indicated that the levels of miR-1, miR-133a, and miR-208a were significantly reduced in the infarcted myocardium [71]. ISH of miR-133a also showed that miR-133a levels were very low in the infarcted and peri-infarcted myocardium [71]. Using ISH on carotid arteries *in vitro* and *in vivo*, Torella et al. [72] demonstrated that miR-133 is robustly expressed in vascular smooth muscle cells (VSMCs), whereas miR-1 is almost absent. Similarly, miR-33a is mainly expressed in VSMC cytoplasm, whereas miR-133b is not expressed at all in VSMCs. miR-133 expression is regulated by extracellular signal-regulated kinase 1/2 activation and is inversely correlated with VSMC growth. Wang et al. [73] also detected abundant expression of miR-195 in VSMCs by ISH and demonstrated its role in the cardiovascular system by inhibiting VSMC proliferation, migration, and proinflammation.

Gambardella et al. [74] detected overexpression of miR-206 in the skeletal muscle from myotonic dystrophy type 1 (DM1) patients. ISH localized miR-206 to the nucleus in both normal and DM1 tissues. However, in DM1 muscles, a strong signal was detected also in correspondence to centralized nuclei and nuclear clumps, which are pathological hallmarks of dystrophic muscles. Larsson et al. [75] also identified the expression of miR-145 in pericytes using ISH and found that miR-145 targets the hematopoietic transcription factor *Fli1* and blocks migration in response to growth factor gradients.

ISH has not been routinely performed in studies on cardiac miRNA function, likely due to the challenging and tedious nature of the protocols. Schneider et al. [76] reported the development of a markedly improved approach combining fluorescence-based miRNA-ISH with IHC. They applied this protocol to differentiating embryoid bodies (EBs), as well as to embryonic and adult mouse hearts, to detect miRNAs that were upregulated during EB cardiomyogenesis, as determined by array-based miRNA expression profiling [70]. The authors found specific cell-localization of miR-1 in myosin-positive cells (cardiomyocytes) of EBs of developing and mature hearts. In contrast, miR-125b and miR-199a did not localize to cardiomyocytes, as previously suggested for miR-199a, but were rather expressed in connective tissue cells of the heart. More specifically, by co-staining with α-smooth muscle actin (α-SMA) and collagen-I, they found that miR-125b and miR-199a localize to perivascular α-SMA(+) stromal cells [76]. Their findings highlight the importance of determining exact cell-specific localization of miRNAs by sequential miRNA-ISH and IHC for all studies aiming at understanding the role of miRNAs and their targets.

**Neurodegenerative diseases**

Recent miRNA expression profiling studies have revealed that some miRNAs are aberrantly expressed in the brains of patients with neurodegenerative disorders, such as Huntington’s disease (HD), Parkinson’s disease (PD), and Alzheimer’s disease (AD) [77]. Findings from those studies suggest that miRNAs play critical roles in neurodegeneration, as well as cardiovascular disease and cancer. Specific miRNAs may regulate the expression of their target proteins that are involved in the development of neurodegenerative diseases. However, tissue-level miRNA profiling has pitfalls because these profiling methods do not describe which cells those miRNAs are expressed in. The
central nervous system contains 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. ISH, an important tool in the context of studying neurodegenerative diseases [78, 79], is a complementary experimental technique for assessing directly the cellular and subcellular distribution of particular miRNAs and to understand the specific roles of those miRNAs in the human brain.

Kapsimali et al. [80] analyzed the neuroanatomical expression profiles of 38 miRNA in developing and adult zebrafish brain using ISH analysis. They found that miRNAs have a wide variety of different expression profiles in neural cells. For example, miR-92b is expressed in neuronal precursors and stem cells; miR-124’s expression is associated with transition from proliferation to differentiation; miR-9 is expressed in both proliferative cells and their differentiated progeny; and miR-218a is only expressed in motor neurons. Other cell-specific studies of miRNA for human brain are still very limited.

AD is the most common dementia among aged people. A number of specific miRNAs were found to be dysregulated in AD, and also were implicated in the regulation of key genes involved in AD, including APP and BACE1. miRNA expression patterns have been found to be altered not only in the AD brain but also in blood and cerebral spinal fluid of persons with AD [81-83]. Researchers investigated miRNA expression in post-mortem brain tissues, mouse models, cells, human blood, and cerebral spinal fluid of AD patients. The most recent profiling data showed that a subset of miRNAs seemed to be specifically altered in the AD brain. These “AD-specific” miRNAs include miR-29, miR-15, miR-16, miR-107, miR-9, miR-181, miR-101, miR-146, and miR-106 [78, 84, 85]. Hebert et al. [86] reported that miR-29a and miR-29b-1 can regulate BACE1 expression in vitro. ISH analysis demonstrated these miRNAs are coexpressed with BACE1 in all regions of adult brain. Liu et al. [87] found that miR-16 was one of the post-transcriptional regulators of APP in the age-associated AD mouse model, senescence-accelerated mouse prone 8 (SAMP8). ISH analysis further revealed that miR-16 expression was mainly concentrated in the hippocampus and cortex, and that miR-16 and APP displayed complementary expression patterns in SAMP8 mice and BALB/c mice embryos, suggesting that an abnormally low expression of miR-16 could potentially lead to APP protein accumulation in AD mice. 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 [79]. 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 enriched in neurons. Perhaps most importantly, unlike other miRNAs, the expression of miR-107 decreased disproportionately in particular laminae of neurons that were most affected by an AD pathology. Nelson et al. [88] performed ISH with special focus on the human entorhinal cortex (EC) and transentorhinal cortex (TEC), as the TEC is the area of the cerebral cortex that first develops neurofibrillary tangles in AD. The ISH staining showed that miR-107, miR124, miR-125b, and miR-320 have characteristic expression patterns in the TEC and EC.

PD is the second most common neurodegenerative disorder and is characterized by the presence of protein inclusions, called Lewy bodies, and a progressive loss of dopaminergic neurons in the midbrain. Minones-Moyano et al. [89] have evaluated miRNA expression deregulation in PD brain samples and found decreased expression of miR-34b and miR-34c in brain areas with variable neuropathological affects at different clinical stages of PD. The downregulation of miR-34b/c was detected in pre-motor stages of PD, but the cell-specific expression of these microRNAs in human brain remains uncharacterized.

HD is an autosomal dominant neurodegenerative disease caused by CAG trinucleotide repeat expansion in huntingtin, which encodes Huntingtin (Htt). A putative mechanism that results in these transcriptional changes is the aberrant cellular distribution of the transcriptional repressor RE1-silencing transcription factor (REST). Packer et al. [90] have reported that levels of several miRNAs with upstream RE1 sites are decreased in HD patient cortices relative to healthy controls. One of these, miR-9 and miR-9*, which are decreased early in HD, are processed from the same primary transcript from three genomic loci (miR-9-1, miR-9-2, and miR-9-3), miR-9-1 and miR-9-3 both have upstream RE1 sequences that can be occupied by REST. Interestingly, miR-9/miR-9* targets two components of the REST complex: miR-9 targets REST and miR-9* targets CoREST. These data provide evidence for a double negative feedback loop between the REST silencing complex and miR-9/miR-9* [90]. ISH analysis also found that miR-9 is highly expressed in the ventricular zone of the developing brain, which contains neural stem cells, but it is not expressed in the roof plate or the floor plate [91]. Over-expression of miR-9 negatively regulates the Hes1 protein expression by interacting with the 3’-UTR of Hes1 mRNA, thereby inducing cell cycle exit and neuronal differentiation.

It should be noted that the field of miRNAs in neurodegeneration is still in its earliest stages [78, 92] and ISH studies of the miRNAs at play in human brain physiology are still infantile. There are some limitations to the previous ISH studies that have been performed, as ISH is a relatively low-throughput technique and only a handful of miRNA probes can be evaluated at a given time. Also, post-mortem degradation of miRNAs in the human brain can be rapid and can affect different miRNAs at different rates. Finally, ISH is not a rigorously quantitative technique for evaluating gene expression because there are many variables that are difficult to control for. Recently, the development of high-throughput ISH and improved quantitation methods make it possible to analyze a larger number of human samples within several days. The wide application of this technique will provide more information to understand the specific roles of miRNAs in the human brain.
Conclusions and perspectives

Although the involvement of miRNA in human diseases was discovered only recently, these small ncRNAs have emerged as extremely important regulators of gene expression most diseases studied to date. The regulatory networks orchestrated by miRNA are complex, because they can act as either positive or negative modulators and bind to hundreds of different targets. In an added layer of complexity, each target message may be regulated by several miRNAs. Computational target site predictions in vertebrates indicate that miRNAs may be responsible for regulating up to 30% of human protein-coding genes [33]. The chemical and biological features of miRNA make them very suitable as diagnostic biomarkers or therapeutic targets in human diseases, and many studies have been published on this matter in recent years. Microarray analysis and other miRNA expression profiling methods have revealed the distinct connection between aberrant expression of miRNAs and human diseases, and many studies have been published in this manner. Microarray analysis and other miRNA expression profiling methods have revealed the distinct connection between aberrant expression of miRNAs and human diseases.

In conclusion, both theory and practical experience identify ISH as an important validation tool for interpreting the biological basis and clinical relevance of altered expression of disease-associated miRNAs and other non-coding RNAs. ISH should be routinely applied to increase the quality of miRNA and other ncRNA studies. The development of robust protocols and automated staining platforms will speed the transition of this technique from the bench to the clinic.

References


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In situ hybridization-based detection of microRNAs in human diseases


