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Multiplexed Detection Methods for Profiling MicroRNA Expression in Biological Samples

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The recent discovery of short, non-protein coding RNA molecules, such as microRNA molecules (miRNAs), that can control gene expression has unveiled a whole new layer of complexity in the regulation of cell function. Since 2001, there has been a surge of interest in understanding the regulatory role of the hundreds to thousands of miRNAs expressed in both plants and animals. Significant progress in this area requires the development of quantitative bioanalytical methods for the rapid, multiplexed detection of all miRNAs that are present in a particular cell or tissue sample. In this Minireview, we discuss some of the latest methods for high-throughput miRNA profiling and the unique technological challenges that must be surmounted in this endeavor.

1. Introduction

MicroRNA molecules (miRNAs) are a class of small (approximately 19–23 nucleotides), non-protein coding RNA molecules found in a broad range of plants, viruses, and mammals. miRNAs are initially transcribed in the cell nucleus as long precursors (pri-miRNA) that are enzymatically processed into approximately 70-nucleotide-long stem-loop structures (pre-miRNA), which are then exported into the cell cytoplasm. A second enzymatic processing step creates approximately 22-nucleotide-long mature miRNAs. These mature miRNAs can regulate gene expression following incorporation into an active RNA-induced silencing complex (RISC) where they interact with complementary sites on messenger RNA molecules and result in translational repression and sometimes degradation of the target messenger RNA. Although identification of the characteristic stem-loop structure of pre-miRNA is important for miRNA classification,[1] it is the expression level of the mature form that is of most interest to researchers investigating the biological function of miRNAs. There are a number of good reviews available on the mechanism of gene regulation and silencing by miRNAs.[2–6]

Although the first published description of a miRNA appeared more than a decade ago,[7] the importance of miRNA in gene regulation and cell function is just beginning to be understood. Studies in model organisms have shown that miRNAs are involved in the regulation of many critical biological processes such as development, differentiation, metabolism, and immunological response.[8–10] Recently, several research groups have established links between alterations in the expression levels of miRNAs and the initiation and development of human cancers.[11,12] Comparative analyses between various malignant and normal tissue samples revealed characteristic patterns, in which some miRNAs are overexpressed and others strongly repressed, that depended on the cancer type, disease stage, and response to treatment.[12] Also, miRNA expression levels have been shown to play a controlling role in tumor growth rates, suggesting possible new strategies for therapeutic treatment.[13] A key approach emerging in the investigation of the cellular roles of miRNAs is to profile the mature miRNA expression levels in specific tissue types at various developmental or disease stages.

For this reason, considerable effort has been devoted to the development of new methods for high-throughput analysis of multiplexed miRNA gene expression. Currently, over 4500 miRNA sequences are listed in the miRNA registry,[14,15] including 475 human miRNAs. The total number of miRNAs in the human genome is not yet known, but estimates based on computational analyses range up to 1000.[2,16] Recently, Tuschl and co-workers published one of the most comprehensive studies of mammalian-miRNA expression patterns to date in which they used a variety of experimental and computational methods to analyze miRNA expression pro-

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files from 26 different organ systems and cell types of humans and rodents.[17] A timeline for miRNA discovery and detection is shown in Figure 1. The earliest attempts at systematically profiling miRNA expression were performed by using a large number of single-miRNA-detection experiments such as northern blotting.[18] Although laborious and sample-intensive, multiplexed northern blotting continues to be widely used as the standard method against which data from newer, more-sensitive detection techniques are validated. Other multiplexed single-miRNA approaches such as a modified invader assay[19] and quantitative RT-PCR (reverse transcriptase/polymerase chain reaction) of precursor miRNAs[20] or mature miRNAs[21,22] are very sensitive and require low amounts of starting material. The successful application of RT-PCR is the result of innovative approaches towards the design of RT primers with high specificity towards individual mature miRNA species;[22] potentially, multiple primers could be combined in a single pool that would enable much-higher-throughput profiling than is currently possible with individual sample analyses.

In recent years, these multiplexed single-miRNA methods have been supplanted by the use of oligonucleotide microarray-based detection platforms as the most-efficient approach to miRNA high-throughput profiling.[23–32] cDNA microarrays for the multiplexed detection of messenger RNA molecules have proven to be extremely valuable for the study of gene expression in biological samples;[33] in a similar fashion, the population of known miRNA molecules should in principle be detected and characterized by creating DNA microarrays with hundreds to thousands of complementary probe sequences immobilized on a single chip surface. However, the short sequence length of miRNAs (19–23mers) means that the methods used to detect messenger RNA with oligonucleotide microarrays cannot be used. In addition, direct PCR amplification of mature miRNAs cannot be used, making the ultrasensitive detection of miRNA at femtomolar concentrations particularly difficult. Herein, we focus on the latest developments that overcome the various difficulties in the application of microarray-based detection platforms for the profiling of miRNA expression in biological samples.

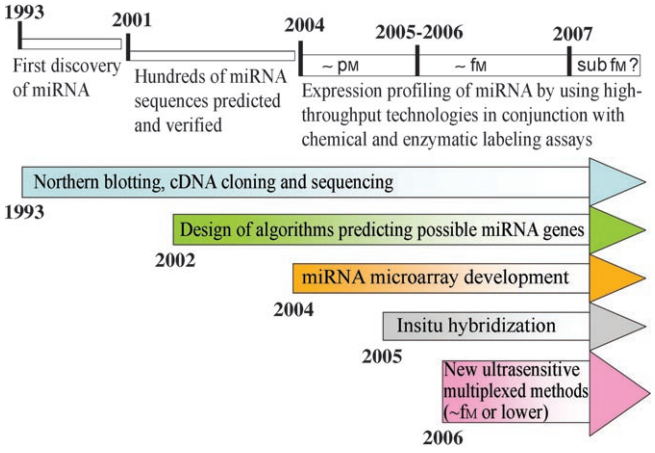

Figure 1. Timeline for microRNA discovery and detection. Initially, expression analysis was performed by using northern blotting. Cloning and sequencing methods were later used to discover hundreds more miRNAs. The improved understanding of miRNA properties has enabled the development of computer algorithms that search for possible miRNA gene locations and targets. Experimental methods have progressed through several generations of microarray-based strategies with improved sensitivity and accuracy. cDNA = complementary DNA.

Alastair W. Wark was born in Scotland in 1974 and obtained a PhD in Chemistry from the University of Strathclyde in 2000 with Dr. Len Berlouis and Dr. Frank Cruikshank. After carrying out postdoctoral research at the Ecole Polytechnique Fédérale de Lausanne, Switzerland, he was awarded a Lindemann Trust Fellowship to investigate new surface-sensitive spectroscopic techniques at the University of Wisconsin-Madison. In 2004, he moved with Prof. Corn's group to his current position as a project scientist at the University of California, Irvine.

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2. Challenges in miRNA Profiling

Microarray-based techniques are particularly attractive for miRNA profiling as they are able to screen large numbers of miRNAs simultaneously. However, miRNAs present several unique challenges that make them more difficult to analyze than conventional oligonucleotide microarray targets such as genomic DNA and messenger RNA. The short length of mature miRNAs provides very little sequence length to work with for the design of complementary microarray probes. The duplex melting temperature (T_m) of each known miRNA and its complementary DNA strand varies widely,

and normalization of the hybridization efficiency for each microarray element cannot be achieved by simply reducing the length of some of the probe sequences. The short lengths of miRNAs also make it difficult to reliably amplify or label each miRNA without introducing signal bias. Furthermore, miRNAs represent only a small fraction (ca. 0.01 %) of the mass of a total RNA sample, with the relative expression levels between different miRNAs varying by as much as four orders of magnitude from a few copies to over 50000 copies per cell.[2] It is also important to ensure that the non-active pri-miRNA and pre-miRNA precursor species do not contribute to the array detection signal.

Efforts to improve the accuracy of miRNA profiling measurements have primarily focused on developing new strategies for 1) complementary probe design and 2) miRNA labeling. Most miRNA studies reported to date have employed DNA capture probes, resulting in conditions that are more reliable for comparing expression levels of the same miRNAs in different samples than actually for comparing the relative levels of different miRNAs in the same sample. To equalize the hybridization conditions without compromising specificity, several researchers have proposed incorporating modified nucleotides into the probe sequences[31,32] or extending the length of the probe-sequence–miRNA-duplex interaction with additional base pairs that are introduced upon miRNA labeling.[34] Recently, Castoldi et al.[31] demonstrated the application of locked nucleic acids (LNAs) in place of conventional DNA probes. LNAs are commercially available nucleic acid analogues that contain one or more modified nucleotide monomers in which the ribose moiety is modified with an extra bridge that connects the 2′-O and 4′-C atoms. By controlling the number and location of the modified nucleotides, the thermostability of each miRNA–LNA duplex can be significantly increased as well as making it possible to design a complete set of T_m-normalized probes, which results in more-accurate profiling. The increased binding affinity of LNA probes not only yields a tenfold increase in sensitivity compared with unmodified DNA probes, but also improves hybridization discrimination among closely related miRNAs.[31,35]

Another challenge in miRNA profiling is that conventional methods that are used to amplify or label longer RNA and DNA targets prior to detecting a microarray hybridization signal cannot be directly applied. To produce an accurate miRNA profile, it is extremely important that the labeling method quantitatively reflects the abundance of different miRNAs present in the sample while maintaining the biological integrity and hybridization performance of the target miRNA. The specificity of the labeling assay towards mature miRNAs can also be improved through the use of commercially available miRNA enrichment kits (e.g. Ambion, Qiagen), which remove the precursor miRNAs and other larger RNA species. In the following sections, we discuss a variety of chemical and enzymatic methods (a summary of which is shown in Table 1) designed to directly attach a label or tag, allowing sensitive detection. For comparison with labeling methods, examples of detection methods that do not involve miRNA modification prior to hybridization are also described.

3. Direct Detection of miRNA

Despite the challenges described in the previous section, there are a handful of reports demonstrating direct multiplexed detection of miRNA with sensitivities comparable with or greater than northern blotting. By direct, we mean here the detection of miRNA without the need for chemical or enzymatic modification of the target molecules prior to analysis. Another very active area of nonmultiplexed direct detection, which we will not discuss herein, is the area of in situ hybridization in which fluorescently labeled complementary LNA probes are used to directly visualize the specific location and evolution of a mature miRNA target within different organisms and tissue sections.[36–38]

A number of direct detection methods have been developed for miRNA profiling applications and are listed in Table 1. In this section, we describe two examples of direct multiplexed miRNA detection: 1) a solution-based sandwich assay format in which the target miRNA simultaneously hybridizes to a pair of spectrally distinguishable fluorescent oligonucleotide probes, which are each complementary to one half of the target miRNA, and 2) a surface-based assay that directly measures sequence-specific adsorption (termed "hybridization adsorption") onto complementary probe array elements created on the surface of a supported gold thin film.

3.1. Solution-Based Direct Multiplexed Detection of miRNA

A dual-probe hybridization scheme that is capable of directly detecting single miRNA molecules in solution was recently demonstrated by Neely et al.[39] Each probe is labeled with a different fluorescence tag and specifically binds to one half of the target miRNA (see Figure 2). This measurement was only made possible through the use of LNA probes, whose higher binding affinity to miRNA compared with conventional DNA enabled the use of short 10-mer probe lengths. Following hybridization, the sample solution is flowed through a microfluidic capillary in which multiple lasers at different wavelengths excite the fluorescent probes that pass through a very small focused volume. Individual miRNA molecules are easily identified by a coincident burst of photons at different color wavelengths from both hybridized fluorophore probes. The number of miRNAs counted over a given time period was then used to determine the target concentration. As a demonstration, the expression levels of 45 human miRNAs within 16 different tissues were quantified with a detection limit of 500 fM. High specificity was also achieved with the LNA probes, enabling miRNAs that differ by only a single nucleotide to be clearly distinguished. Only one miRNA is targeted in each sample volume, however, a single assay can be performed with as little as 50–100 ng of total RNA. This is an order of magnitude lower than standard microarray methods, which usually involve additional miRNA-labeling/amplification preparative steps.

Table 1: Summary of methods for miRNA-expression profiling.

	Method	Notes	Ref.
Direct detection	Northern blotting	Hybridization of complementary ^{32}P- or digoxigenin-labeled oligo probes after gel separation.	[18, 35, 52]
	Single-molecule fluorescence	Dual hybridization of two fluorescent-labeled LNA probes in a sandwich assay format.	[39]
	Signal-amplifying ribozymes	Hybridization of miRNA to a ribozyme or molecular beacon induces separation of the fluorophore and quencher, thus increasing signal.	[53]
	In situ hybridization	In situ tissue and cell analysis: hybridization of fluorescent-labeled complementary probes containing chemically modified nucleotides (e.g. LNAs).	[36–38]
Solution-based chemical modification	Fluorescence imaging	Coordination of the Pt–fluorophore complex to G base residues (Ulysis Alexa Fluor).	[23]
		Alkylation of N heteroatoms on any nucleotide base (Label IT reagent).	[43]
		3′ End labeling: 1) $NaIO_4$ and 2) formation of an amine derivative, which is reacted with NHS–Cy5.	[32]
		3′ End labeling: 1) $NaIO_4$, 2) biotin hydrazide, and 3) binding of streptavidin–quantum-dot conjugates to hybridized miRNA.	[45]
	Electrochemistry	3′ End labeling: 1) $NaIO_4$ and 2) isoniazid-conjugated OsO_2 nanoparticles.	[46]
Solution-based enzymatic modification	Cloning	Large-scale cDNA cloning and sequencing.	[54]
	Fluorescence imaging	T4 RNA ligase reaction that covalently attaches labeled nucleotides directly to the 3′ end of miRNA.	[24, 31, 34, 55]
		1) Poly(A) polymerase reaction to create a 3′ tail containing amine-modified nucleotides that 2) react with a dye of an NHS ester (*mir*Vana labeling kit).	[47]
		1) Poly(A) polymerase tailing, 2) T4 DNA ligation of tag DNA, and 3) hybridization of a labeled DNA dendrimer (Ncode labeling kit).	[29, 48]
		1) T4 RNA ligation of 3′ and 5′ adaptor oligonucleotides and 2) RT-PCR incorporating labels.	[27, 28, 30]
	Invader assay	Binding of two overlapping hairpin probes to miRNA forming a site recognized for cleavage and subsequent fluorimetric analysis.	[19]
	Quantitative RT-PCR	Various strategies for creating primers that target individual precursor or mature miRNA species.	[20–22]
	Rolling-circle amplification	miRNA detection protocol based on padlock probes and enzymatic amplification.	[56]
	Fluorescent-bead cytometry	1) Ligation of 3′ and 5′ adaptor oligos to miRNA, 2) RT-PCR with biotin-labeled primer, and 3) bead assay.	[27, 57]
Surface-based enzymatic modification	SPR imaging	1) Poly(A) polymerase 3′ extension of hybridized/adsorbed miRNA and 2) complementary hybridization of T_{30}-coated gold nanoparticles.	[49]
	RAKE assay (fluorescence imaging)	1) Klenow enzyme extension of hybridized/adsorbed miRNA with biotin-conjugated nucleotides and 2) detection with streptavidin–dye conjugate binding.	[50, 51]

3.2. Surface-Based Direct Multiplexed Detection of miRNA

The detection and identification of short, unlabeled RNA and DNA oligonucleotides can also be achieved in a microarray format by using surface plasmon resonance imaging (SPRI). This is a surface-sensitive optical technique that has been widely applied to the real-time monitoring of various bioaffinity interactions (e.g. DNA–DNA, RNA–DNA, and peptide–protein) at biopolymer layers formed on a thin gold film.[40] Although the SPRI detection limit for DNA and RNA analysis lies in the low nanomolar range,[41,42] which is not as sensitive as fluorescence microarray measurements, SPRI analysis does provide valuable insights into the kinetics and thermodynamics limitations of the miRNA surface hybridization adsorption reaction.

An example of the detection of RNA by hybridization adsorption onto DNA microarrays with SPRI is shown in Figure 3. A two-component single-stranded DNA (ssDNA) microarray is exposed to target RNA, with only the perfectly matched array elements forming duplexes through hybridization adsorption (see inset of Figure 3b). A positive increase in percent reflectivity ($\Delta\%R$) owing to selective hybridization adsorption is observed. If the increase in the SPRI signal upon hybridization adsorption remains below 10%, then it is directly proportional to the relative surface coverage (θ) of complementary RNA. The binding affinity between RNA and the surface-immobilized DNA probe can be quantitatively evaluated by creating a Langmuir isotherm plot such as that shown in Figure 3b. The symbol θ is related to the bulk RNA concentration (C_{RNA}) by the Langmuir adsorption isotherm, as shown in Equation (1) where K_{ads} is the Langmuir adsorption coefficient.

$$\theta = K_{ads} C_{RNA}/(1 + K_{ads} C_{RNA}) \quad (1)$$

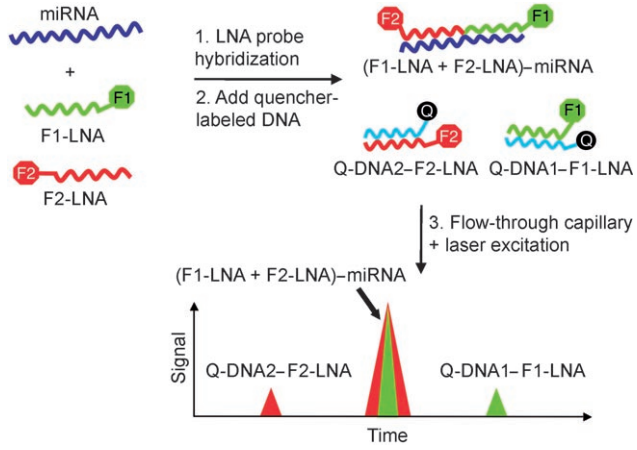

Figure 2. Direct single-molecule detection of miRNA. Step 1: Two spectrally distinct fluorescent LNA probes (F1-LNA and F2-LNA) are hybridized in solution to each target miRNA in a sandwich-assay format to form the duplex labeled (F1-LNA + F2-LNA)–miRNA. Step 2: Complementary DNA probes modified with a quencher molecule (Q-DNA1 and Q-DNA2) are then added, which hybridize to the remaining "free" LNA probes and form two different duplexes (Q-DNA2–F2-LNA and Q-DNA1–F1-LNA). Step 3: The sample solution is subsequently flown through a microfluidic capillary within which light from lasers operating at the F1 and F2 excitation wavelengths is focused closely together. A signal corresponding to the target miRNA is observed as two coincident spikes when both probes F1 and F2 are excited simultaneously. Adapted from reference [39].

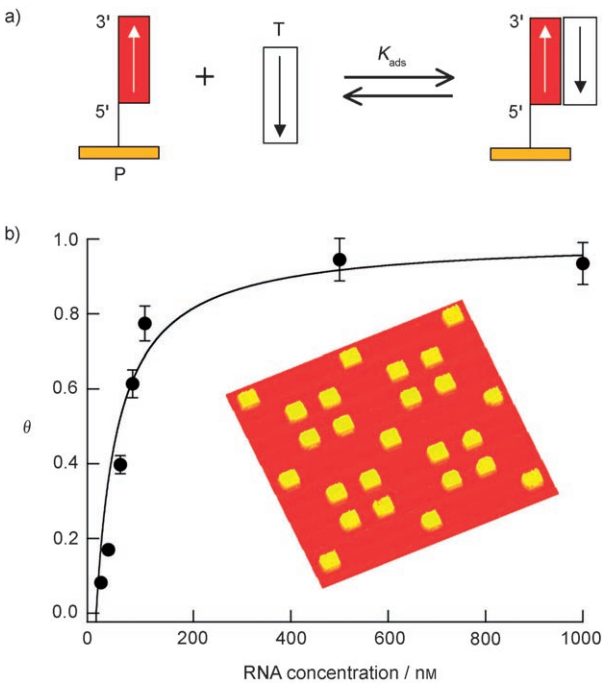

Figure 3. a) Target (T) single-stranded RNA hybridization adsorption onto a probe (P) ssDNA microarray element. b) A representative plot of relative surface coverage as a function of target-RNA concentration. The solid line is a Langmuir isotherm fit to the data from which a value of $K_{ads} = 2 \times 10^7\ M^{-1}$ was determined. The inset is a SPRI difference image obtained by subtracting images acquired before and after sequence-specific RNA hybridization adsorption on a two-component DNA microarray.

For RNA hybridization adsorption onto a DNA microarray, K_{ads} is around $10^7\ M^{-1}$. This value can be increased approximately by a factor of ten by replacing the ssDNA probes with LNAs that bind more strongly to the RNA target. This improves both the sensitivity and specificity of the surface hybridization measurement.[31]

The SPRI data in Figure 3 show that at biologically relevant concentrations (pM and lower), the equilibrium fractional surface coverage of miRNA on each microarray element will be extremely small. For example, given a K_{ads} of approximately $10^8\ M^{-1}$ and a bulk miRNA concentration of 10 fM, the equilibrium fractional surface coverage of miRNA is only 10^{-6}. A good approximation of the surface density of LNA probes on a microarray element is 1×10^{12} molecules cm^{-2}, so that the surface density of LNA–miRNA duplexes is 10^6 molecules cm^{-2} at 10 fM. For a 500-μm array element used in SPRI, this corresponds to 2500 miRNA molecules; for a 50-μm array element size, which is typically used in fluorescence imaging measurements, this surface density corresponds to only 25 miRNA molecules! This demonstrates the difficulty in detecting very low concentrations and the importance of designing probe sequences that have a high binding affinity towards the target miRNA.

A second reason that it is difficult to detect low miRNA concentrations with microarrays is the kinetics of hybridization adsorption. The rate constant k_a for hybridization adsorption of miRNA is approximately $10^4\ M^{-1}s^{-1}$. As the velocity of the adsorption reaction can be described by $k_a C_{RNA}$, the time required to reach an equilibrium surface coverage increases significantly as the concentration is lowered. In addition, there is also a diffusional contribution that becomes significant at lower concentrations owing to the time required for the miRNA molecules to reach the surface array elements. For detection at 10 fM, a minimum reaction time of approximately 4 h is necessary to reach a steady-state coverage provided a circulating flow cell is used to minimize diffusion effects. At even lower concentrations and if a circulating flow cell is not used, then longer reaction times will be required.

Both the Langmuir adsorption equilibrium arguments and adsorption kinetics clearly demonstrate that the application of microarray-based techniques to perform miRNA profiling requires the use of an amplification technology. A variety of chemical and enzymatic assays designed to enhance the sensitivity of both fluorescence and SPRI-based microarray measurements are discussed in the following sections.

4. Chemical Modification Strategies for Multiplexed miRNA Detection

To achieve higher sensitivity in a miRNA assay, it is necessary to chemically or enzymatically modify the target miRNA species prior to detection. A list of the chemical modification strategies that have been used to date is given in Table 1. The simplest method of chemical modification is to attach a fluorescent label directly onto the miRNA molecules. Figure 4 outlines three exemplary strategies that have re-

cently been adapted for fluorescence-based miRNA detection with chemical labeling.

The use of a platinum dye complex that forms a stable adduct at the N^7 position of any guanine base in the miRNA sequence (see Figure 4a) was first demonstrated by Babak et al.[23] The cisplatin derivative (Ulysis Alexa Fluor, Molecular Probes) is covalently bound to a fluorophore that has

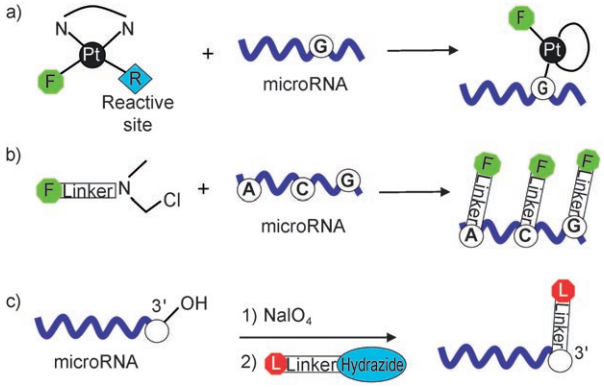

Figure 4. Chemical strategies for miRNA labeling. a) Reaction of a platinum fluorophore complex (Ulysis Alexa Fluor reagent) to form a coordinative bond at the N^7 position of any guanine (G) base. b) An alkylation reaction (Label IT reagent) that targets any reactive N heteroatom on the miRNA with some preference for the guanine (G), cytosine (C), and adenine (A) bases. c) A two-step end-labeling reaction in which the 2′,3′-diol on the ribose ring at the 3′ terminus of the miRNA is first oxidized to a dialdehyde by sodium periodate followed by a condensation reaction with a hydrazide derivative that is linked to a labeling moiety.

only one reactive coordination site available. Upon incubation in a solution with miRNAs at an elevated temperature, a ligand-exchange reaction takes place, with a labile nitrate at the reactive site being replaced by a coordinative bond, firmly coupling the fluorophore to the miRNA target. This approach assumes that all miRNAs contain at least one G residue.

A second approach, which also targets nucleotide bases in the miRNA sequence, is shown in Figure 4b. In this case, an aromatic nitrogen mustard reactive group linked to a fluorophore (Label IT, Mirus Bio) directly alkylates any reactive N heteroatom in the miRNA with possible preference for N^7 of guanine, N^3 of adenine, and N^3 of cytosine.[43] Because this labeling reaction is based on the modification of nucleotide bases, care must be taken to remove excess reagent to prevent false labeling of oligonucleotide probes on the microarray surface. The major drawback of both nucleotide modification strategies is that they lack specificity for miRNA as compared with other RNA or DNA species in the sample. Further specificity towards small RNA species can be obtained by size fractionating to create a sample enriched in mature miRNA prior to labeling. In addition, there is a possibility that the binding affinity between modified miRNA and oligonucleotide probes may be partially impaired.

A third approach that has better control over the total number of labels attached to each miRNA, and which was developed originally for labeling mRNA by Weiler and coworkers,[32,44] is shown in Figure 4c. Here, the 2′,3′-diol on the ribose ring at the 3′ terminus of the miRNA is first oxidized to a dialdehyde by sodium periodate. A condensation reaction with a hydrazide derivative can then be performed to covalently couple a single label onto the 3′ end of each miRNA. As this approach requires an intact 2′,3′-diol, possible background-signal contributions from contaminant RNA bearing 3′-phosphate groups or DNA can be minimized. In addition to dye labeling,[32] this reaction has also been used to biotinylate miRNA samples prior to microarray analysis.[45] The hybridized miRNA was then detected by using fluorescence measurements of streptavidin–quantum-dot conjugates, which bind to the miRNA through the streptavidin–biotin interaction. The reported detection limit of approximately 40 pM (0.4 fmol) was similar to a comparable approach, by the same authors, that was based on colorimetric measurements by using streptavidin-modified gold nanoparticles.[45] A further example of this chemical labeling method is the direct attachment of OsO_2 nanoparticles to miRNAs hybridized onto a modified electrode surface.[46] Although limited to a single miRNA per electrode, amperometric measurements involving the OsO_2 electrocatalyzed oxidation of hydrazine could be applied to detect 80 fM concentrations.

5. Solution Enzymatic Modification Strategies

The most successful miRNA modification strategies to date for multiplexed miRNA detection employ enzymatic modification reactions, either in solution or directly on the microarray surface. Nucleic acid enzymes are often used in biotechnological applications as highly efficient tools for the amplification and site-selective manipulation of genomic DNA and RNA. One of the most widely used essential enzymatic reactions for miRNA modification is the lengthening of the target miRNA by catalyzing the addition of nucleotides to either the 3′ or 5′ ends; these extension reactions enable conventional techniques such as cloning and PCR amplification to be employed for miRNA discovery and detection.

Several solution-based enzymatic assays have been proposed for high-throughput miRNA profiling by using microarrays. A common approach used by several researchers is to ligate adapter oligonucleotide sequences to both the 3′ and 5′ ends of miRNA followed by the use of reverse transcriptase (RT) to create a cDNA library. Further PCR amplification incorporating fluorescent probes is then performed prior to microarray analysis.[27,28,30] Although highly sensitive, this indirect approach to miRNA profiling requires extensive sample manipulation. We describe herein three more-convenient enzymatic assays that are designed to directly attach one or more labels to each target miRNA in solution.

One of the most powerful enzymatic modification reactions applied to miRNA analysis is the use of T4 RNA ligase to covalently couple the 5′ end of a polynucleotide sequence of any desired length to the end with the 3′-OH group of miRNA. This reaction was first applied by Thomson et al.[24] to directly attach a fluorophore-conjugated dinucleotide to the miRNA pool prior to microarray hybridization. Figure 5a

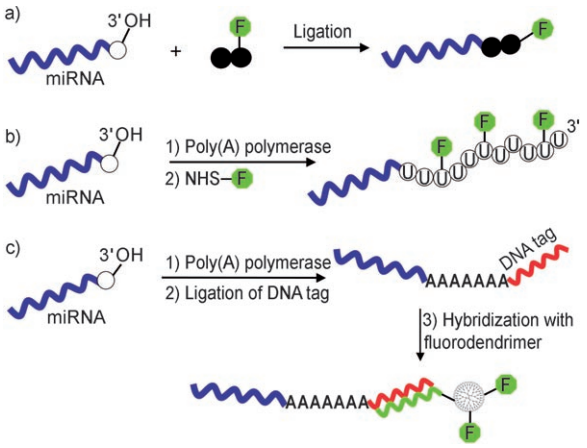

Figure 5. Enzymatic strategies for miRNA labeling. a) Ligation of a fluorophore-conjugated dinucleotide to the 3′-OH group end of miRNA by using T4 RNA ligase. b) *E. Coli* poly(A) polymerase is used to catalyze the multiple addition of nucleotides to create a poly(U) tail at the 3′ end of each miRNA. A fraction of the added nucleotides are amine-modified and are then covalently coupled to a fluorophore containing an *N*-hydroxysuccinimide (NHS) ester reactive group. c) The enzyme-catalyzed formation of a 3′ poly(A) tail is followed by a second enzymatic reaction in which a DNA tag sequence is ligated to the 3′ end of the tail. The tagged miRNA is then hybridized to a microarray and subsequently detected through a DNA-modified dendrimer that is complementary to the tag sequence and which also incorporates up to several hundred fluorophores for amplified detection.

shows a schematic diagram of the reaction indicating the position of the fluorophore, which blocks the 3′ end and ensures that only one label is attached to each miRNA. Recently, the enzyme reaction conditions were further optimized by Wang et al.[34] to minimize (but not completely remove) the effect of variations in miRNA sequences and the secondary structure on the ligation efficiency. Wang et al. also claimed a detection limit of approximately 5 fM (0.2 amol in 45 μL) by also using an improved probe-design strategy.

Further enhancements in sensitivity have been investigated by using combined tailing/labeling procedures instead, such as that shown in Figure 5b and c, which allow the attachment of multiple signaling species. The method using the *mir*Vana miRNA labeling kit (Ambion), shown in Figure 5b, appends a 20–50 polynucleotide to each miRNA by using the enzyme poly(A) polymerase to create a 3′ tail that is a proprietary mixture of standard and amine-modified bases.[47] The amine-labeled miRNAs are then covalently coupled to an amine-reactive dye to produce a multiply labeled miRNA sample that is subsequently introduced to the microarray and detected by fluorescence imaging. The NCode (Invitrogen) enzymatic method described in Figure 5c also entails the creation of a 3′ poly(A) tail. A second enzymatic reaction with T4 DNA ligase is then used to covalently couple a unique oligonucleotide tag sequence to the 3′ end of the poly(A) tail. Following cleanup of the RNA sample solution and hybridization to the microarray, each tagged miRNA is detected by hybridizing to a single sequence-specific dendrimer that contains as many as 900 fluorophores, thus greatly amplifying the detection signal. Recently, this method was further extended to achieve greater sensitivity by using a combination of reverse transcriptase with a poly(dT) primer and T7 polymerase reactions to comparably amplify all miRNA species prior to dendrimer-labeling detection.[48]

6. Surface-Based Enzymatic Modification Strategies

There are several significant advantages if enzymatic modification strategies used to specifically label miRNA can be implemented directly on a microarray surface instead of in solution. One major advantage of a surface-based approach is that there is no sample manipulation before miRNA hybridization to the microarray. This avoids possible biases that could be introduced owing to differential labeling between various miRNAs or sample loss during the labeling reaction and cleanup. Furthermore, multistep reactions are easier to perform sequentially on a surface than in solution, and only miRNAs that are hybridized to the surface are modified, whereas in solution all RNA species are potentially labeled. Note that surface labeling will not work for the reactions shown in Figures 4a and 4b (see Section 4), as they are not specific to RNA and will also label the oligonucleotide probes attached to the microarray surface. In principle, the sodium periodate chemical reaction described in Figure 4c should be amenable to the specific labeling of surface-attached miRNAs but has not yet been demonstrated in a microarray format. In this section, we describe two recent examples of miRNA detection based on surface enzyme reactions.

Recently, we developed a novel approach that combines both a surface poly(A) polymerase reaction and DNA-modified nanoparticle enhancement for the ultrasensitive microarray detection of miRNAs by using SPR imaging.[49] As outlined in Figure 6, target miRNA is first hybridized from solution onto a single-stranded LNA microarray (step 1). The surface-bound miRNA is then polyadenylated with poly(A) polymerase (step 2). The SPRI response is then further

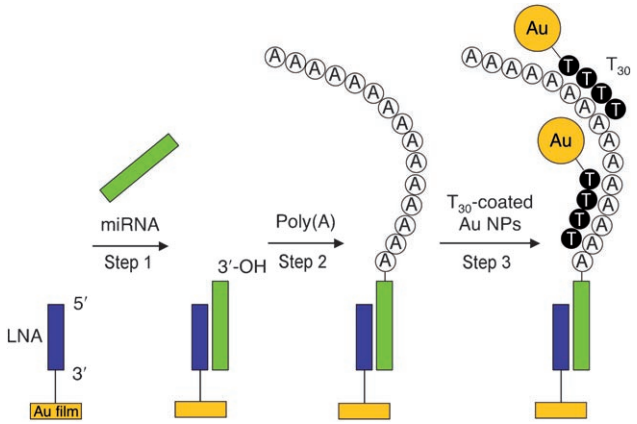

Figure 6. Detection of microRNAs by using a combination of surface polyadenylation and nanoparticle-amplified SPRI. Step 1: hybridization adsorption of miRNA onto a complementary LNA array element. Step 2: poly(A) tail addition at the 3′ end of surface-bound miRNAs by using poly(A) polymerase. Step 3: hybridization adsorption of T_{30}-coated Au nanoparticles (NPs) to poly(A) tails. Reprinted with permission from reference [49].

amplified by the hybridization adsorption of poly(T)-coated nanoparticles onto the poly(A) tails (step 3).

The surface polyadenylation–nanoparticle amplification methodology was demonstrated with the multiplexed detection of three different miRNAs present in a total RNA sample that was extracted from mouse-liver tissue. A four-component microarray was constructed containing three LNA probes designed to bind to the known miRNA sequences, miR-16, miR-122b, and miR-23b, with a DNA probe that was used as a negative control.[49] A total RNA sample (250 ng) in 0.3 M NaCl/10 mM phosphate buffer (500 μL) was circulated over the microarray surface repeatedly for 4 h followed by surface amplification. Analysis of the resulting SPRI difference image (Figure 7a) and corresponding line profile (Figure 7c) clearly

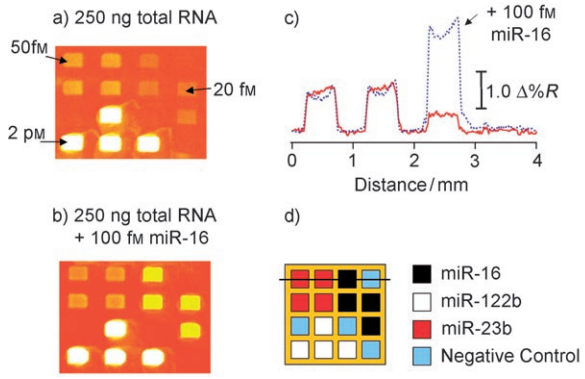

Figure 7. Quantitative analysis of miRNAs of mouse-liver total RNA (250 ng) by using polyadenylation-nanoparticle amplified SPRI measurements. a) SPRI difference image obtained by subtracting images acquired before and after the nanoparticle amplification step. b) An SPRI difference image obtained from a separate chip by using the same total RNA concentration as the top image plus the addition of 100 fM synthetic miR-16. c) Comparison of line profiles taken from both SPRI difference images with the solid and dashed lines corresponding to top and bottom images, respectively. d) Representation of the four-component LNA probe microarray and the location of the line profile (indicated with a black line). Reprinted with permission from reference [49].

shows the miR-122b sequence as the most abundant. Having already calibrated the SPRI response signal by using synthetic analogues of the target sequences, it was possible to estimate miRNA concentrations of 20 fM, 50 fM, and 2 pM for miR-16, miR-23b, and miR-122b, respectively. Further verification of the concentration of the least-abundant miRNA (miR-16) was obtained by repeating the measurement with the addition of 100 fM synthetic miR-16. As shown in Figure 7b and c, a fivefold increase in the SPRI signal was observed only at the miR-16 probe array elements. The detection limit of this amplified SPRI methodology was found to be 5 attomoles (10 fM in 500 μL), which is at least 50 times more sensitive than the solution-based enzymatic tailing/labeling fluorescent measurements mentioned in the previous section.

Another example of a surface-based enzymatic amplification reaction for miRNA detection is the RNA-primed array-based Klenow enzyme assay (RAKE) approach.[25,50,51] This assay is based on the ability of a miRNA molecule to act as a primer for a Klenow polymerase extension reaction when the miRNA is fully base paired with a ssDNA probe. Providing there is no mismatch, the 3′ end of the miRNA is extended with biotin-conjugated nucleotides, the number of which depends on the DNA probe template. Streptavidin-conjugated fluorophores are then used to visualize and quantify the microarray elements containing hybridized and Klenow-extended miRNAs. The sensitivity of the RAKE assay was found to be comparable with other fluorescent-based microarray assays involving the direct attachment of a single label.[24] However, RAKE avoids sample-manipulation procedures associated with labeling or amplification prior to hybridization and is also highly specific with superior discrimination between miRNAs that differ at their 3′ ends.

7. Summary and Outlook

There is no doubt that the demand for rapid, high-quality miRNA profiling will continue to increase exponentially as the complex roles of miRNAs in regulatory networks and human diseases become more understood. The ideal miRNA profiling technique will be easy to multiplex, have negligible sequence bias towards particular miRNAs, involve simple experimental protocols with minimum sample manipulation prior to hybridization, and have a large measurement dynamic range from subfemtomolar to nanomolar concentrations. Although this ultimate technique does not yet exist, recent developments utilizing microarray-based detection platforms in combination with various chemical and enzymatic miRNA-labeling strategies are very close to achieving this idealized goal. To date, most of the miRNA-labeling reactions are performed in solution and require one or more reaction steps; more-recent methods employ surface enzymatic reactions that can be easily cleaned up as the selective labeling of miRNAs occurs after hybridization adsorption onto the probe microarray surface. In addition, the new surface-based labeling strategies will most likely employ either multiple fluorophores or multiple nanoparticles with a single miRNA; however, some care will have to be taken as the signal levels of these detection methods are less controlled and quantifiable. In general, we expect that in the not-too-distant future a variety of new, quantitative, ultrasensitive approaches for miRNA-expression profiling by using microarrays will amply fill the needs of researchers interested in the study of miRNA in biological systems.

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