Microarray-based, high-throughput gene expression profiling of microRNAs

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MicroRNAs (miRNAs) are small regulatory RNAs that serve fundamental biological roles across eukaryotic species. We describe a new method for high-throughput miRNA detection. The technique is termed the RNA-primed, array-based Klenow enzyme (RAKE) assay, because it involves on-slide application of the Klenow fragment of DNA polymerase I to extend unmodified miRNAs hybridized to immobilized DNA probes. We used RAKE to study human cell lines and brain tumors. We show that the RAKE assay is sensitive and specific for miRNAs and is ideally suited for rapid expression profiling of all known miRNAs. RAKE offers unique advantages for specificity over northern blots or other microarray-based expression profiling platforms. Furthermore, we demonstrate that miRNAs can be isolated and profiled from formalin-fixed paraffin-embedded tissue, which opens up new opportunities for analyses of small RNAs from archival human tissue. The RAKE assay is theoretically versatile and may be used for other applications, such as viral gene profiling.

miRNAs[1–6] have important roles in plant and animal development, apoptosis, fat metabolism, growth control and hematopoietic differentiation[6,4]. Dysregulation of miRNAs may contribute to human disease, including cancer[7–9]. Many individual miRNAs are conserved across widely diverse phyla, indicating their physiological importance. More than 200 miRNAs have been reported thus far from mammals and miRNAs are estimated to account for ~1.0% of expressed human genes[4]. Most animal miRNAs have the capacity to regulate multiple mRNA targets[4,10]. In mammalian cells, primary miRNA transcripts (pri-miRNAs) are cleaved sequentially in the cell nucleus and transported to the cytoplasm (as pre-miRNAs), where mature miRNAs are generated[11]. Mature miRNAs guide regulatory proteins to induce translational repression or degradation of specific target mRNAs[4,12].

Much is still unknown about miRNA biology. For example, the miRNA genes expressed in most tissues, species and cell lines are not known, and the physiological functions—and regulation—of almost all miRNAs remain to be determined. miRNAs may also have hitherto unexplored roles in human disease. These and other topics may be easier to address experimentally when miRNA gene expression studies become more feasible.

High-throughput miRNA gene expression analysis is a technical challenge. The short length and uniqueness of each miRNA render many conventional tools ineffective—very small RNAs are difficult to reliably amplify or label without introducing bias (see refs. 13,14). Earlier attempts at systematic gene expression analysis have involved dot blots[15] or northern blots (for example, see refs. 16,17). Additional assays for sensitive detection of miRNAs or their precursors have been developed, involving real-time quantitative PCR–based analysis of pre-miRNA expression[18] or a modification of the Invader assay for miRNA detection and quantification[19]. Northern blots are currently the gold standard of miRNA validation and quantification[20]. Higher-throughput techniques involving mature miRNAs may be helpful to further understanding of the role(s) of miRNAs in normal and disease tissues.

The focus of our study was a strategy for high-throughput miRNA gene expression analyses that we term the RNA-primed, array-based Klenow enzyme (RAKE) assay. We also demonstrated, for the first time, that a robust sampling of miRNAs can be made from formalin-fixed, paraffin-embedded (FFPE) pathological samples and subjected to expression analysis with the RAKE assay. The RAKE assay is a sensitive, specific technique for assessing DNA and RNA targets, offering unique advantages for specificity over northern blots or other microarray-based expression profiling platforms, and may have broader applications than for miRNA detection.

RESULTS

Assay development and validation

We developed a method to achieve high-throughput gene expression analyses of miRNAs. We sought to eliminate systematic bias associated with RNA ligation steps, amplification and cDNA intermediates, or separate fluorophore labeling. We used on-slide enzymatic reactions, which have been used previously for other purposes (see, for example, refs. 21,22). DNA oligonucleotide probes whose 3′ halves are complementary to specific miRNAs, and whose 5′ halves (spacer) are shared, are synthesized and covalently cross-linked at their 5′ termini onto glass microarray slides. Three thymidines separate the spacer from the remainder of the DNA probe, which is antisense to specific miRNAs. The RNA sample, containing miRNAs, is hybridized and treated with

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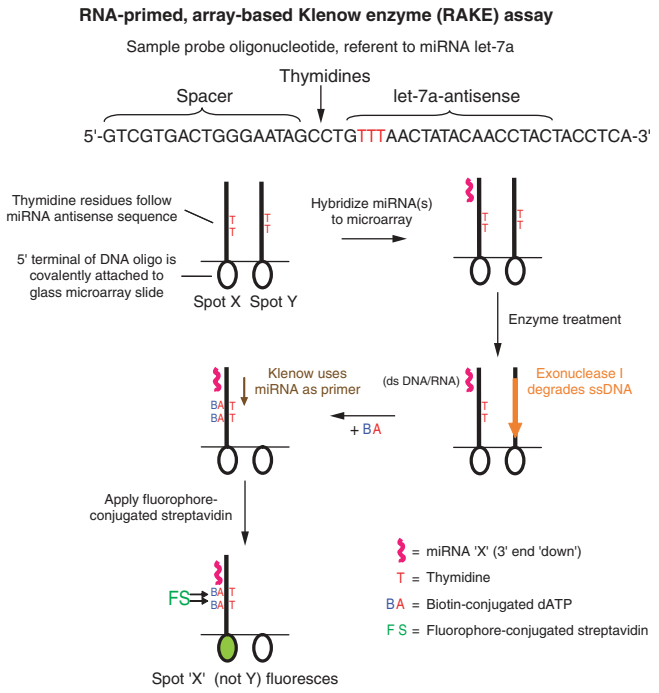

Figure 1 | Schematic diagram of RNA-primed, array-based Klenow enzyme (RAKE) assay. The sample probe at the top of the figure illustrates the generic structure of the DNA oligonucleotides used on the microarray. The nucleotides at the 5′ half comprise a spacer, which is constant for all the probes, followed by three thymidine nucleotides. The variable portion of each probe is at the 3′ end, which is the antisense sequence of various miRNAs. An RNA sample, containing miRNAs, is hybridized to a glass microarray on which all the DNA probes have been spotted. Next, the slide is washed and exonuclease I is applied to degrade unhybridized (single-stranded) DNA probes. The slide is washed again and the Klenow fragment of DNA polymerase I is applied to catalyze the addition of biotin-conjugated dATPs using the miRNA as a primer and the spotted probe as template. Finally, the biotins are labeled with streptavidin-conjugated fluorophore to specifically highlight spots (here, spot 'X') where miRNA hybridization occurred.

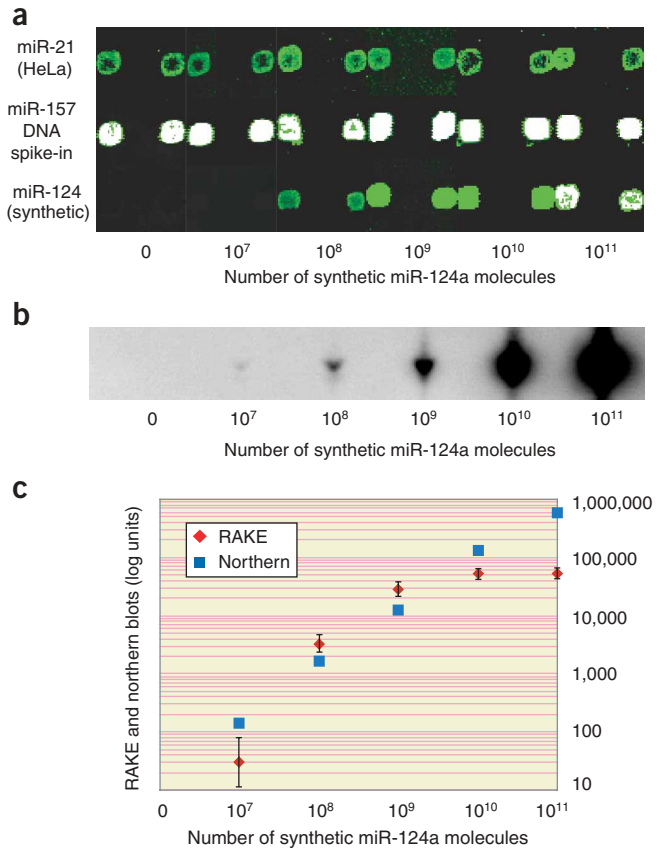

Figure 2 | Sensitivity and dynamic range of RAKE and comparison to northern blots. (**a**) Indicated molecules of a synthetic miRNA (miR-124a, not expressed in HeLa cells) were added to a 'complex RNA mixture' derived from low-molecular-weight HeLa RNAs (10^8 molecules, or 0.16 fmol). A DNA oligonucleotide corresponding to a plant miRNA (miR-157; not found in HeLa cells) was also added at a constant concentration, as a 'spike-in.' (**b**) The same number of miR-124a molecules were resolved on a 20% denaturing polyacrylamide gel and analyzed by northern blots. (**c**) Quantification and comparison of sensitivity between RAKE and northern blotting. For RAKE, signal was defined as the median of foreground spot fluorescence at 532-nm wavelength minus background (defined by surrounding pixel intensity); negative values were normalized to zero (these data are not otherwise normalized). Each concentration point (red diamonds) represents the mean, with standard deviation shown, from 12 spots (2 microarray slides with 6 spots per miRNA on each microarray). For northern blots, signal intensity was measured with a phosphorimager after overnight exposure; concentration spots (blue squares) represent the mean from two different experiments.

exonuclease I, which specifically degrades single-stranded, unhybridized probes. The Klenow fragment of DNA polymerase I is then applied along with biotinylated dATP (B-dATP). The hybridized miRNAs act as primers for the Klenow enzyme and the immobilized DNA probe as template, leading to incorporation of B-dATPs. The slide is washed and a streptavidin-conjugated fluorophore is applied to visualize and analyze the spots containing hybridized and Klenow-extended miRNAs (**Fig. 1**).

We developed a glass-slide microarray with probe spots corresponding to 239 miRNAs (sequences were obtained from the official microRNA registry[23]), including miRNAs from humans, mice, rats and *Arabidopsis thaliana*. These were spotted in three pairs throughout the slide for a total of 1,422 spots per microarray.

We included DNA probes complementary to plant miRNAs on the microarray both for negative controls and for future studies involving *A. thaliana*. We used three *A. thaliana* miRNA 'spike ins'. Synthetic DNA oligonucleotides corresponding to the three plant miRNAs (ath-miR-157, ath-miR-163, and ath-miR-169) were introduced in each hybridization step at concentrations of 10^{-9} M, 10^{-10} M and 10^{-11} M, respectively. These spike-in DNA oligonucleotides were used to assist normalization and to provide 'absolute' reference points for each study (see below and **Fig. 2**). Ath-miR-157, at 10^{-9} M, provided an internal control for the highest fluorescent signal level.

We investigated the sensitivity of RAKE using a synthetic RNA target oligonucleotide corresponding to the sequence of mature miR-124a (**Fig. 2a**). Each concentration represented duplicate arrays (12 data points each). RNA isolated from HeLa cells was included to compose a 'complex' RNA background. The abundance of miR-21 (a normal component of HeLa cells) and the miR-157 DNA 'spike-in' did not vary significantly at different concentrations of miR-124a (**Fig. 2a**). The dynamic signal of miR-124a spanned at least three orders of magnitude (**Fig. 2a,c**). These results are not normalized, and thus demonstrate the robust nature of the 'raw'

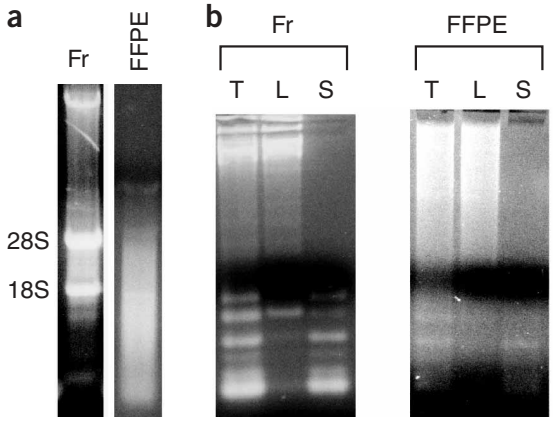

Figure 3 | Representative agarose gels to demonstrate RNA used in the RAKE assay. Fr, RNA from fresh brain tumor; FFPE, RNA from formalin-fixed, paraffin-embedded brain tumor. (**a**) Analysis of total RNA from fresh and FFPE anaplastic oligodendroglioma tissue on 1% agarose gels. Ribosomal RNAs (28S and 18S) are indicated. (**b**) Analysis of low-molecular-weight RNA from fresh and FFPE anaplastic oligodendroglioma tissue on 3% agarose gels. T, total RNA; L, larger RNA only; S, low-molecular-weight RNA, which is used for the RAKE assay. The lanes on the FFPE tumor represent one-fifth of the RNA isolated from a single 50-μm-thick section (~1 μg of RNA).

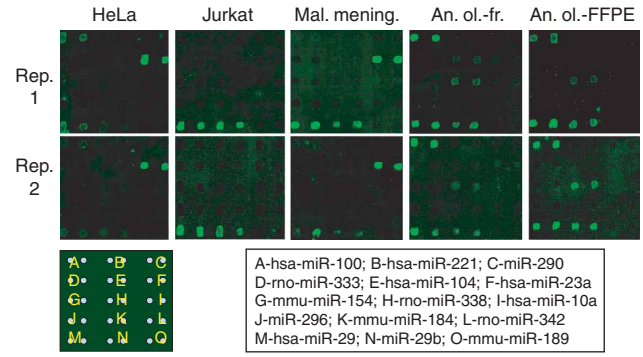

Figure 4 | Representative images from RAKE assays. Samples are indicated on top. 'Rep. 1' and 'Rep. 2' indicate replicates. Position and identity of spotted probes are shown on the bottom. Mal. mening., malignant meningioma; An. ol.-fr. and An. ol.-FFPE, fresh and FFPE-derived anaplastic oligodendroglioma, respectively.

data. The technique is comparable in sensitivity to northern blots (**Fig. 2b**,**c**), allowing detection of miRNA in the <10-pg range, which is consistent with earlier studies using northern blots on miRNAs[17]. As compared to northern blot data, the microarray data show slightly less sensitivity, some variability at very low concentrations, and slightly less linearity across the large scale of concentrations we evaluated (five orders of magnitude) (**Fig. 2c**). These differences are due to different saturation profiles inherent to microarray fluorescence in comparison to radiation detection.

RNA isolation and processing for RAKE

We evaluated RNA derived from human epithelial and hematopoietic cell lines and two human brain tumors (**Supplementary Table 1**). To minimize the likelihood of cross-hybridization, we used only low-molecular-weight RNA in our microarray hybridizations, isolated using a commercially available kit. We hypothesized that we could also isolate miRNAs from archival formalin-fixed, paraffin-embedded (FFPE) pathological material, as short segments of RNAs have been shown to be preserved in FFPE tissue[24] and siRNAs are relatively slow to degrade *in vivo*[25]. To directly compare the performance of RAKE with RNA isolated from fresh and FFPE material, we procured tissue from a surgically removed human brain tumor (anaplastic oligodendroglioma). We isolated RNA from half of the specimen and submitted the other half for routine FFPE processing. We subsequently isolated RNA from 50-μm-thick serial sections from the paraffin block. Consistent with earlier studies, RNA prepared from fresh tissue appeared less degraded than that of FFPE tissue (**Fig. 3**).

RAKE analysis on tissue-cultured, FFPE and fresh RNA

Specimens were analyzed using three replicates for all RNA samples. Most 'negatively' hybridizing spots showed less signal than background (**Fig. 4**). Signal was defined as the median of foreground spot fluorescence at 532 nm wavelength minus background (defined by surrounding pixel intensity); negative values were normalized to zero. Otherwise, no normalization was used, because there was only a single dye, the number of samples was only 239 and values were consistent across microarray slides (see below). A summary of the mean of the three replicates for all five samples (RNA from HeLa cells, Jurkat cells, malignant meningioma, and fresh and FFPE-derived anaplastic oligodendroglioma tissue) is presented in **Figure 5**.

The DNA spike-in oligonucleotides produced high signal, as expected. Some miRNAs showed high signal in multiple tissues (for example, miR-15, miR-16 and let-7f), whereas others were relatively restricted to certain tissue types (for example, miR-27b in HeLa cells, miR-148 in Jurkat cells, miR-199b in malignant meningioma and miR-9 in the fresh and FFPE anaplastic oligodendroglioma). All replicates were highly correlated (**Supplementary Fig. 1**).

Northern blot validation

Northern blots were used to evaluate selected data obtained by RAKE (**Fig. 6**). These experiments were carried out on total RNA. The results were generally the same for RAKE and northern blots. However, for hsa-miR-23b, which on northern blots showed robust signal for both HeLa and malignant meningioma RNA, RAKE showed no detectable amounts of hsa-miR-23b. We hypothesized that miR-23b expression was indeed low or absent in these tissues; however, in northern blots the miR-23b DNA oligonucleotide probe might cross-hybridize with the paralogous miRNA, miR-23a. By contrast, the RAKE assay discriminated between miR-23a and miR-23b because, like many paralogs, miR-23a and miR-23b differ at their 3′ end, and therefore could prime Klenow extension only when hybridized to the appropriate, specific probe.

Discrimination of paralogous miRNAs that differ at the 3′ end

To further test the ability of RAKE to discriminate between paralogous miRNAs, we carried out parallel microarray and northern blot experiments on three separate paralogous miRNA pairs (**Fig. 7a**) that, like most miRNA paralogs, differ primarily at their 3′ ends. Each paralogous miRNA was analyzed by RAKE and on northern blots (**Fig. 7b**,**c**). The mean signal intensity for each

Figure 5 | Profiling and relative abundance of different miRNAs determined using RAKE. H, HeLa; J, Jurkat; M, malignant meningioma; PO, FFPE tissue from anaplastic oligodendroglioma; FO, fresh tissue from anaplastic oligodendroglioma. Light green represents no signal, black is low signal, and red is highest signal.

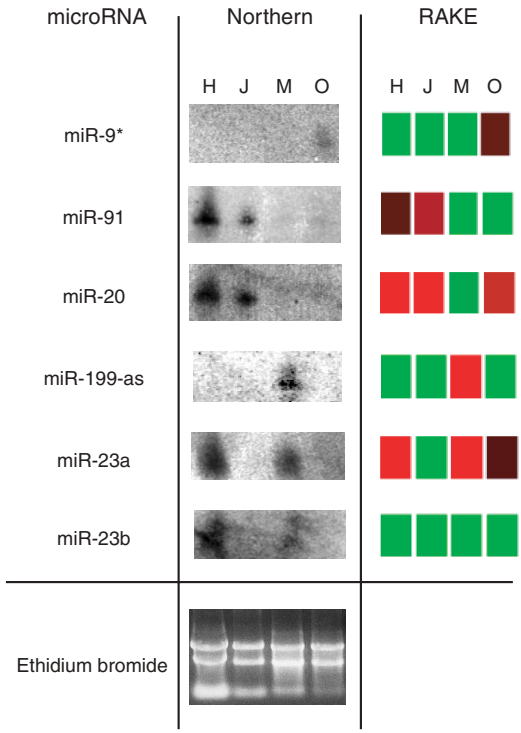

Figure 6 | Correlation between northern blots and RAKE assay for HeLa (H), Jurkat (J), malignant meningioma (M) and anaplastic oligodendroglioma (O). Five micrograms of total RNA is loaded per lane. Quality of total RNA used is assessed by electrophoresis on 1% agarose gel followed by ethidium bromide staining (a representative gel is shown). RAKE assay results corresponding to the northern blots are shown at right.

miRNA was determined in RAKE ($n = 12$) and in northern blots ($n = 2$) and a ratio between the paralogous miRNAs was calculated for each pair (**Fig. 7d**). The power of RAKE to discriminate between hsa-miR-23a/23b paralogs is 9.6 times (13.9/1.44) that of northern blotting in a sample containing hsa-miR-23a and 10.6 times higher in an hsa-miR-23b sample. The extent of the improvement in 3′ distinction for RAKE versus northern blotting varies between the miRNA paralogs. One possible explanation for this is that it relates to the identity and position of the nucleotide differences between the paralogous miRNAs, which in turn may influence their hybridization properties (see **Supplementary Results**). Nevertheless, it is clear that RAKE is superior to northern blotting in discriminating between paralogous miRNAs differing at the 3′ ends (**Fig. 7d**).

DISCUSSION

The purpose of this study was to develop a microarray platform that could enable high-throughput gene expression analyses of small RNAs. RAKE is a new tool with high sensitivity and specificity for miRNA profiling. A major advantage of RAKE is that there is no sample RNA manipulation. Certain possible biases that may be introduced during enzymatic labeling, or during cDNA generation or amplification of the sample RNA before hybridization to the glass microarray, are thus avoided. RAKE allows for rapid and simultaneous detection of all known miRNAs from the same sample. Another advantage of RAKE is the ability to completely automate all steps from sample hybridization to detection (as done in the present study). This is achieved by using existing technologies and equipment used for traditional mRNA microarrays, and allows for highly consistent performance.

On a technical level, our results are consistent with findings in earlier microarray experiments. For RNA targets analyzed using DNA oligonucleotide probe–based microarrays, probe-target cross-hybridization is seen only if the degree of 'theoretical' cross-hybridization is over 80% for 50–70-mer[26] probes, and

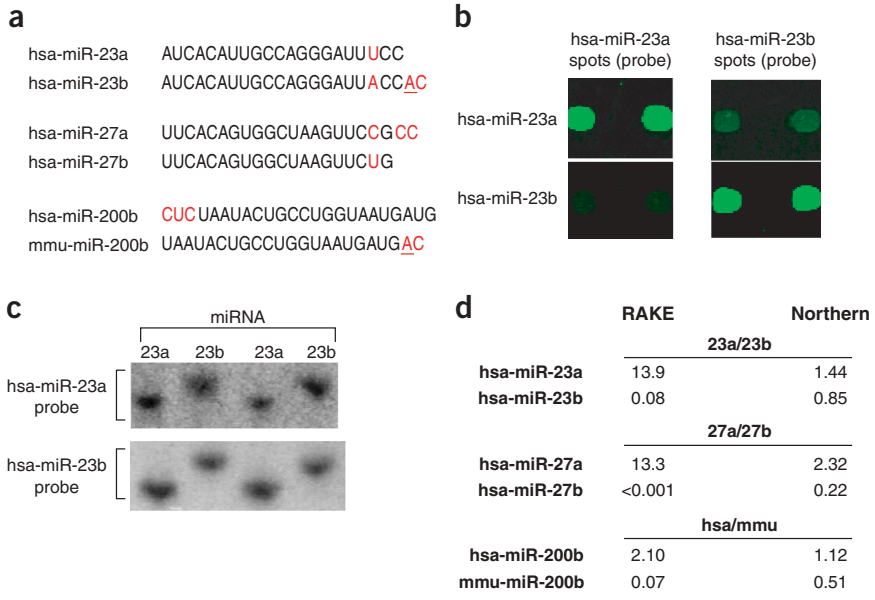

Figure 7 | RAKE is superior to northern blots in discriminating between miRNA paralogs. (**a**) Sequences of the three pairs of paralogous miRNAs tested. For each synthetic, paralogous miRNA, 0.1 pmol (6×10^{10} molecules) was analyzed by RAKE or northern Blots. (**b**,**c**) Representative experiments for the hsa-miR-23a–hsa-miR-23b pair for RAKE (**b**) and northern blots (**c**). (**d**) The mean signal intensity for each miRNA was determined in RAKE ($n = 12$) and northern blots ($n = 2$) and a ratio between the paralogous miRNAs was calculated for each pair and is indicated.

90% or more for a ~20-mer[27–31] probe, depending upon variables that include hybridization conditions, probe G-C composition, and the location of the 'mismatch' nucleotide(s).

Northern blotting is considered the standard method for miRNA validation and quantification[20]; it offers both 'quantitative' and 'qualitative' information and, unlike a microarray experiment, confirms the length of the hybridized transcripts. In contrast to RAKE, however, northern blotting is laborious and hence less well-suited for high-throughput expression profiling. The RAKE assay also gives unique qualitative data, because the 3′ end of the miRNA 'primer' should hybridize specifically to the oligonucleotide 'template'. For this reason, RAKE can discriminate the exact 3′ end of miRNAs. This advantage is significant because of the many mature miRNAs for which paralogs differ at the 3′ end. These miRNAs, derived from different genes, would be predicted to cross-react adversely in northern blots (and in standard microarray methods using labeled target pools) but not usually on RAKE assay. Our data from three pairs of paralogous miRNAs support the hypothesis that RAKE is superior to northern blotting in discriminating miRNA paralogs (**Figs. 6** and **7**).

The specificity of the northern blot technique has received scant critical review, considering the widespread use of this method. Short DNA-RNA hybrids show melting temperatures and binding dynamics that vary significantly with probe and target nucleotide composition, buffer contents, and the time and temperature of hybridization[26,30–33]. It is hence likely that signal intensity for northern blots varies from miRNA to miRNA (as it would on a microarray experiment) and from experiment to experiment. Also in common with microarrays, standard northern blotting does not provide an absolute quantification—each RNA queried must include a standard curve to be considered 'absolutely quantitative', and each standard curve must be run in parallel with each individual experiment[17]. Analogous RNA experiments on microarrays can be performed, but would be extremely labor intensive.

The RAKE assay was devised to exploit the known ability of the Klenow enzyme fragment to act as a DNA polymerase using an RNA primer on a DNA oligonucleotide template[34,35]. Earlier studies have demonstrated on-slide enzymatic reactions and primer extension (for example, see refs. 21,22). However, direct detection of RNA hybridization (using RNA-primed DNA polymerase) has not been reported on a microarray, nor has the special properties of the Klenow enzyme been used in microarray studies. It was also necessary to use exonuclease I, a 3′→5′, single-stranded DNA–specific exonuclease that is highly processive[36]. It is important to note that the activities of both Klenow enzyme and exonuclease I[36] are independent of the sequence of their substrates. Systematic bias is therefore not introduced. RNA ligases, in contrast, are prone to bias because the enzyme kinetics change with substrate sequence[13,14], producing an inaccurate representation of the miRNAs present in a target pool labeled by RNA ligase methods. Our results demonstrate sensitivity to the level of 10 pg of target miRNA, which is comparable to that of northern blots[17]. We may in the future obtain even greater sensitivity using 'sandwich'-type amplification or a more sensitive labeling technique (such as gold particles for resonance light scattering). Further work may also improve quantification of miRNA abundance and resolution of expression differences, perhaps through the incorporation of a standard reference that hybridizes to the spacer sequence and is detected with a second scanner channel.

Earlier studies have addressed the expression characteristics of miRNA genes using dot blots[15] and PCR[18]. Most pertinent is the work of Sempere *et al.* using northern blots[16]. Our results are broadly compatible with prior studies; in each tissue sampled, only a minority of miRNAs are expressed at detectable levels a given time in a given tissue. Some miRNAs seem to be widely expressed, including miR-98, let-7 paralogs, miR-16, miR-26a and miR-100. miR-124a and miR-9, which we find only in samples from anaplastic oligodendrogliomas, have been shown to be restricted to the central nervous system[16,17]. miR-92, which is present in cultured cells but not appreciably in tissues sampled[16], is also present in HeLa cells and Jurkat cells, but not in our primary tumor tissue. Finally, there is general agreement between the HeLa expression profile described in this study and the miRNAs evaluated previously in HeLa cells[3,37]. There are also aspects of our findings that are somewhat surprising. For example, miR-20 showed stronger expression than one might have expected from earlier studies. This expression was validated by northern blot (**Fig. 6**), and we emphasize that results of microarray studies must be validated by other means, such as northern blotting.

All of the tissues we evaluated derived from human tumors—two cancer cell lines and two brain tumors. It should be noted that the results from the brain tumors are not indicative of the biology of those tumors; more biological replicates are needed to make such a claim. Jurkat cells are derived from a T-cell lymphoma[38]. In earlier studies of chronic B-cell lymphomas, miR-15a and miR-16 were deleted or downregulated in more than two-thirds of cases[7], whereas miR-155 was highly expressed in Burkitt lymphoma[39]. By contrast, Jurkat cells show strong expression of both miR-15a and miR-16 (**Fig. 5**). Future studies may reveal whether the miRNA 'fingerprint' is unique or common for each type of tumor. It also remains to be seen whether these changes are of profound biological importance and whether they can be exploited for therapeutic purposes.

While this manuscript was under revision, two groups reported the use of microarrays with miRNAs. Croce and colleagues reported an oligonucleotide microarray for miRNA and pre-miRNA profiling using a biotinylated primer with a random octamer sequence at the 3′ end. This primer is used, along with reverse transcriptase, to generate a cDNA library from total RNA. The cDNA is isolated and applied to a microarray containing covalently linked DNA oligonucleotide probes corresponding to 245 human and mouse miRNAs[40]. Horvitz and colleagues prepared cDNAs from miRNAs using techniques previously used for the cloning of miRNAs, whereby adapters were ligated to miRNAs followed by RT-PCR using fluorescently labeled primers[41].

The RAKE assay, and these two recently reported microarray techniques[40,41], allow for sensitive, specific and high-throughput miRNA expression profiling. The sensitivity of RAKE is very similar to the sensitivity of the technique reported in ref. 41. However, in contrast to these two other microarray techniques, RAKE does not involve the generation of a cDNA library or amplification of the RNA sample and avoids sample RNA manipulation altogether. RAKE should be superior at discriminating paralogous miRNAs that differ at their 3′ ends, because these other techniques rely solely on hybridization to detect and discriminate between miRNA paralogs[40,41].

Because FFPE tissue can be assessed using the RAKE technique (or other miRNA tools), we should be able to analyze samples from the voluminous archive of human pathological specimens. The RAKE assay may also have applications besides miRNA gene expression profiling. The ability to apply the Klenow enzyme (with high sensitivity and specificity) as a RNA- or DNA-primed polymerase on a microarray slide may open the door to further interesting studies—including, for example, viral gene expression profiling.

METHODS

RNA isolation and northern blots Cell batches (from HeLa and Jurkat cells) and RNA were processed separately for biological replicates. RNA was purified initially using Trizol LS reagent (Invitrogen). Human tissue was procured in accordance with University of Pennsylvania Institutional Review Board policies. From archival FFPE tissue blocks, RNA was initially isolated using a previously described technique[42] (see also **Supplementary Methods**).

Microarray platform. Probe DNA oligonucleotides were synthesized at 600 pmol on 384-well plates (Qiagen), each containing a 5′-terminal C6-amino modified linker. Each probe had a sequence as shown in **Figure 1**, except for a control probe with the spacer only. Probes were suspended at 40 μM in 150 mM sodium phosphate buffer (pH 8.5; 200 U/ml print buffer) with 0.0005% Sarkosyl. A GeneMachines OmniGrid 100 robot printed probes onto CodeLink slides (Amersham) at 30–35% humidity at 24–27 °C. Each spot element measured ~120 μm in diameter and the center-to-center spacing was 400 μm. Each glass slide contained 6 spots (three spatially separated pairs) corresponding to each probe, for a total 1,422 spots including controls. Further chemical 'blocking' of the spotted glass slide was found to be unnecessary.

RAKE protocol. Small RNA hybridizations were found to optimally include >2 μg total mass per slide; less can be used, but the signal was weaker for less abundant miRNAs. Spotted microarray slides were processed using an automated hybridization apparatus (HS4800; Tecan), which greatly facilitated sample processing and allowed near-identical handling of all microarray slides throughout. The concentrated hybridization buffer was composed of 15% formamide and 15× SSC. DNA oligonucleotide spike-ins corresponded to plant (*A. thaliana*) miRNAs ath-miR-157, ath-miR-163 and ath-miR-169 prepared beforehand in a solution at 10^{-7} M, 10^{-8} M and 10^{-9} M, respectively, diluted in water. For all hybridization and enzymatic steps, RNasin (0.4 U/μl; Promega) was included. See **Supplementary Methods** for detailed protocol.

Validation steps. A concentration curve was generated using a synthetic target RNA oligonucleotide (miR-124a) in the background of a complex RNA mixture (low-molecular-weight RNA isolated from HeLa cells, a cell line that does not contain miR-124a).

Image analysis and data processing. Slides were scanned using a Genepix 4000B laser scanner (Axon) at a constant power level and sensitivity (550 PMT) using a single color channel (532-nm wavelength). Nonhybridizing and artifact-associated spots were eliminated by both visual- and software-guided flags. Image intensities were measured as a function of the median of foreground minus background. Negative values were normalized to zero, but other normalizations were not performed. Images were analyzed using the Genepix Pro5.0 software package (Axon). Excel and Genespring 6.2 were used for further data analysis.

Testing discrimination of miRNA paralogs. See **Supplementary Methods**

Database accession numbers. GEO accession numbers: RAKE microarray, GPL1464; microarray samples, GSM31764 through GSM31802; sample subsets, GSE1821.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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