miChip: an array-based method for microRNA expression profiling using locked nucleic acid capture probes

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MicroRNAs (miRNAs) represent a class of short (22 nt) noncoding RNAs that control gene expression post-transcriptionally. Microarray technology is frequently applied to monitor miRNA expression levels but is challenged by (i) the short length of miRNAs that offers little sequence for appending detection molecules; (ii) low copy number of some miRNA; and (iii) a wide range of predicted melting temperatures (Tm) versus their DNA complementary sequences. We recently developed a microarray platform for genome-wide profiling of miRNAs (miChip) by applying locked nucleic acid (LNA)-modified capture probes. Here, we provide detailed protocols for the generation of the miChip microarray platform, the preparation and fluorescent labeling of small RNA containing total RNA, its hybridization to the immobilized LNA-modified capture probes and the post-hybridization handling of the microarray. Starting from the intact tissue sample, the entire protocol takes ~3 d to yield highly accurate and sensitive data about miRNA expression levels.

INTRODUCTION

MicroRNAs (miRNAs) are a family of short regulatory RNAs that negatively control gene expression at the post-transcriptional level[1,2]. Base pairing between the miRNA and the 3′-UTR of target mRNAs mediates specific translation inhibition and/or degradation of mRNA targets[3,4]. miRNAs regulate numerous cellular processes as diverse as differentiation[5,6], proliferation[7] and apoptosis[8]. Moreover, cellular miRNAs are also important for the replication of pathogenic viruses (e.g., miR-122a facilitates replication of human hepatitis C)[9], and small RNAs are also encoded by the genomes of several viruses to facilitate viral replication by suppressing cellular genes[10,11].

Detection of differential expression of miRNAs in many cases has established the basis for miRNA functional analysis and the characterization of the important roles played by miRNAs. In addition, specific miRNA expression patterns can provide valuable diagnostic and prognostic indications in the context of human malignancies such as solid tumors[12–15] and leukemias[16,17]. Thus, accurate profiling of miRNA expression represents an important approach to investigate and compare both physiological and pathophysiological states.

Different methodologies have been applied to profile miRNA expression. These include miRNA cloning approaches[18], northern blotting with radiolabeled probes[19], quantitative PCR-based (QPCR) amplification of precursor or mature miRNAs[20] as well as 'bead'-based profiling technologies (e.g., Luminex xMAP[21]). However, if a large number of miRNAs are screened for in a parallel fashion, microarray-based miRNA profiling assays often constitute the most effective methodology. Although microarray-based miRNA expression profiling may be less sensitive in detecting low abundant miRNAs (e.g., compared to QPCR), its application is often less reagent and/or time consuming (e.g., compared to northern blotting or cloning approaches) or less expensive (e.g., compared to quantitative reverse transcriptase PCR).

Specificity and accuracy of standard mRNA expression profiling techniques applied to miRNA expression profiling protocols are challenged by the fact that mature miRNAs (i) are short, (ii) display a wide range of predicted melting temperature (Tm) versus their (DNA) capture probes (CPs), (iii) only represent a small fraction (~0.01%) of total cellular RNA and (iv) often occur in families that in some cases differ by as little as a single nucleotide[19]. These features complicate the design of suitable capture probes across the complete 'miRnome' and the optimization of hybridization conditions that are unbiased regarding an accurate detection of all miRNAs. To meet these requirements, we have developed a microarray platform (miChip[22,23]) that accurately and sensitively monitors expression of miRNAs without previous need for RNA fractionation or miRNA amplification and that can discriminate between closely related miRNA family members[22]. miChip technology is based on the use of modified nucleotides termed LNA that are incorporated within the oligonucleotide capture probes immobilized on the array surface. LNA is a synthetic RNA analog characterized by increased thermostability of nucleic acid duplexes when LNA monomers are introduced into oligonucleotides[24]. Each incorporated LNA monomer increases the Tm of an LNA/RNA hybrid by 2–10 °C (ref. 25). As a consequence of this property, LNA-modified capture probes can be designed such that, despite the short length of the miRNA target, a uniform Tm can be applied to a genome-wide set of probes, allowing the establishment of normalized hybridization conditions.

Here, we provide detailed step-by-step protocols for (i) the generation and maintenance of the miChip microarray platform, (ii) isolation of small RNA-containing total RNA, (iii) miRNA labeling and (iv) hybridization and post-hybridization handling of the microarray.

Experimental design

All experimental steps included in this protocol are carefully optimized. It is therefore advisable to exactly follow the outlined protocols. Exchange of the spotting robot, spotting technology or

array surface coating may affect the data obtained from an expression profiling experiment. Likewise, alterations in the protocol for total RNA preparation (e.g., the use of column-purified RNA), miRNA labeling (e.g., the use of an alternative T4 RNA ligase or a modified labeling buffer), hybridization buffer composition, the hybridization procedure (e.g., the use of an automatic slide processor) or the stringency of the post-hybridization washes may affect miRNA expression profiles.

Choice of Tm normalized probe sets

In initial experiments, we spotted a test set of capture probes on *N*-hydroxysuccinimide (NHS)-coated (CodeLink, GE Healthcare) slides, which consisted of either unmodified DNA oligonucleotides or LNA-modified and unmodified nucleotides. The hybridization conditions were separately optimized for the two capture probe test sets. Overall, the miRNA profile obtained from DNA-based capture probes was similar to the one generated by LNA-modified capture probes. However, the hybridization signal obtained from LNA-modified capture probes yielded a several-fold-increased sensitivity that was most obvious when lower amounts (2.5–5 mg) of total input RNA were used[22]. To further exploit the biophysical properties offered by the LNA modification, we then designed capture probes with a uniformly normalized Tm of 72 °C. These Tm-normalized LNA-modified capture probes were able to distinguish between closely related sequences under optimal hybridization conditions. miRNAs that yield high signal intensities after hybridization to LNA-modified capture probes with 100% complementarity in most cases showed a drastically reduced signal when hybridized to a capture probe with a single mismatch close to the central position. These and related data[22] demonstrated that Tm-normalized LNA-modified capture probes hybridize their miRNA targets in a highly specific manner and that a single nucleotide mismatch suffices for destabilization of the heteroduplex. Therefore, LNA-modified capture probes enable efficient discrimination between miRNA family members, at least when they differ in nucleotides close to the central position. It is for these properties that we opted for LNA-modified capture probes for use in microarray-based miRNA detection.

Choice of slide surface

In preliminary experiments, we compared slide surfaces with different properties (e.g., CodeLink, GE Healthcare; GAPS, Corning) and concluded that NHS-coated (CodeLink, GE Healthcare, cat. no. 30001100) slides possess superior properties regarding the binding of short (20 mer) oligodeoxynucleotides. CodeLink slides are designed to covalently bind to amino-modified oligonucleotides. The NHS ester generates a covalent bond with the C6-amino group of the modified oligonucleotides simply reacting to a humidified environment. Therefore, it is important to store CodeLink slides desiccated.

The experimental protocols described here were not tested on commercially available miRCURY LNA-arrays (Exiqon). In case miRCURY LNA-arrays are used, we suggest to follow the protocols recommended by the manufacturer.

Choice of total RNA isolation and purification protocols

miRNA expression profiling data are strongly affected by the quality of the total RNA used as input material. Thus, a reliable and robust method for RNA isolation is essential. In our standard protocol, we prepare total RNA using organic solvent (such as Trizol, Invitrogen), which efficiently recovers all RNA species, including miRNAs. We follow the manufacturer's protocol with one exception: to assure efficient precipitation of miRNAs, we have substituted the isopropanol RNA precipitation step by the addition of three volumes of 100% ethanol. During our establishment phase, we have tested several RNA extraction methods that selectively enrich for small RNAs (e.g., combination of Qiagen's RNeasy and Minielute, MirVana (Ambion), differential precipitation using Polyethylene glycol (PEG) and small RNA extraction after separation on PAGE-Urea gels). However, in our hands, miChip analysis of small RNA-containing total RNA yields comparable signal intensities to small RNA-enriched total RNA, suggesting that the additional small RNA enrichment step is not required (data not shown).

Choice of RNA-labeling protocols

miRNAs do not share common features such as Poly A tail and/or Cap structures to append an universal anchor for labeling or amplification. In addition, miRNAs are too short (~22 nt in average) to allow for cDNA synthesis and efficient incorporation of fluorescently labeled nucleotides. Here, we further optimized a previously published method: ligation of a short ribonucleotide conjugated Cy-dye to the miRNA 3′-end using T4 RNA ligase[26].

Controls

To monitor the binding efficiency of capture probes in different print runs, a defined concentration of Cy3-labeled capture probe is printed on each array.

MATERIALS

REAGENTS

- miRCURY LNA probe set (Exiqon, http://www.exiqon.com) ▲ CRITICAL miRCURY capture probes are synthesized with a C6-amino linker attached to their 5′-end. The C6-amino linker anchors the capture probes to the active amino groups present on NHS glass surfaces as commercially available (CodeLink, GE Life Science). The miRCURY probe set is commercially available, desiccated in a 384-well plate format from Exiqon.
- Small RNA-containing total RNA extracted from cell lines or tissues
- Trizol reagent (Invitrogen, cat. no. 15596-018) ! CAUTION Trizol should be used with appropriate safety measures such as protective gloves, glasses and clothing and appropriate ventilation.
- 20× salt sodium citrate (SSC; Fluka, cat. no. 85635)
- 10% (vol/vol) dodecylsulfate sodium salt (SDS; Serva, cat. no. 20765)
- 100% ethanol (Merck, cat. no. 1.00983.2500)
- 75% ethanol (dilute from 100%; Merck, cat. no. 1.00983.2500)
- Chloroform (Merck, cat. no. 1.02445.1000)
- T4 RNA ligase (Ambion, cat. no. 2141)
- RNase inhibitor (Ambion, cat. no. 2682)
- ATP disodium salt (Grade I, Sigma, cat. no. A2383-1G)
- PEG 50% aqueous solution (PEG 6000, Fluka, cat. no. 81304)
- RNA linker (5′-)p-rUrUrUrU-Cy3(-3′) (Biospring, http://www.biospring.de)
- 3 M sodium acetate, pH 5.5 (Ambion, cat. no. 9740)
- Herring sperm DNA (Promega, cat. no. D181B)
- Ethanolamine (Sigma, cat. no. E9508)
- Trizma base (Sigma, cat. no. T6791)
- Sodium phosphate monobasic anhydrous (Sigma, cat. no. S2554)
- Sodium phosphate dibasic heptahydrate (Sigma, cat. no. S9390)
- Linear acrylamide (Ambion, cat. no. 9520)

TABLE 1 | Composition of the miChip master labeling mix for miRNA labeling.

Component	Volume (μl)
T4 RNA ligase buffer (10×)	3.0
RNase-free water	1.3
PEG (50%)	10.0
ATP (200 mM)	0.2
RNA linker (100 μM)	2.0
RNase inhibitor (Ambion, 40 U μl^{-1})	0.5
T4 RNA ligase (Ambion, 5 U μl^{-1})	3.0

TABLE 2 | Composition of the miChip precipitation master mix for post-labeling sample cleanup.

Component	Volume (μl)
RNase-free water	59.5
Sodium acetate (3 M, pH 5.5)	10.0
Linear acrylamide (5 μg μl^{-1})	0.5

- RNase-free water (Accugene, cat. no. 51200)
- 100% formamide, deionized (Ambion, cat. no. AM9342) ! CAUTION Formamide is harmful if inhaled, made in contact with skin or if swallowed; it also causes burns. Formamide should be used with proper safety measures such as protective gloves, glasses and clothing and appropriate ventilation.

EQUIPMENT

- Lucidea array spotter (GE Life Science)
- Water bath (50 °C)
- Heat block (95 °C)
- Microscope glass slide staining trays
- Tabletop centrifuge (Eppendorf, model 5810R) fitted with rotor adaptors for microplate carriers (cat. no. A4-62) or rotor adaptors for 50-ml tubes
- Tabletop centrifuge (Eppendorf, model 5417) located at 4 °C (cold room)
- Tabletop centrifuge (Eppendorf, model 5415D)
- Tissue lyzer (Qiagen)
- Humidity chamber (container filled with a 1-cm-deep slurry of saturated solution of sodium chloride)
- Tungsten carbide beads, 3 mm (Qiagen, cat. no. 69997)
- NanoDrop ND-100 spectrophotometer (http://www.nanodrop.com/)
- Agilent 2100 bioanalyzer (Agilent Technologies)
- Microarray hybridization oven (Agilent Technologies, cat. no. G2545A) fitted with rotor racks
- Microarray chambers (Agilent Technologies, cat. no. G2534A)
- Microarray gasket slides (Agilent Technologies, cat. no. G2534-60005)
- GenePix 4200AL microarray scanner (Molecular Devices, http://www.moleculardevices.com/pages/instruments/gn_genepix4200al.html)
- 1.5- and 2-ml safe-lock Eppendorf tubes (Eppendorf, cat. no. 0030120.086 and 0030120.094)
- NHS glass surface (CodeLink, GE Life Science, cat. no. 30001100)
- Genepix Pro 6.0 software (Axon Instruments)
- Microsoft Excel (or similar)
- TIGR MultiExperiment Viewer (TMeV, http://www.tigr.org/software/)
- GeneSpring 7.3 (Agilent Technologies, optional)

REAGENT SETUP

3× printing buffer 150 mM sodium phosphate (pH 8.5). Mix 0.5 M of sodium phosphate monobasic to 0.5 M of sodium phosphate dibasic solutions until the desired pH is reached. Adjust concentration to 150 mM by addition of deionized water. Filter-sterile and store at room temperature (25 °C) for up to 6 months.

Post-coupling blocking solution 50 mM ethanolamine, 0.1 M Trizma (pH 9) and 0.1% SDS. Prepare fresh before use.

Post-coupling washing solution 4× SSC, 0.1% SDS. Filter-sterile and store at room temperature (25 °C) for up to 6 months.

Labeling master mix For each sample to be analyzed, prepare 20 μl of labeling master mix (see **Table 1**) by mixing T4 RNA ligase buffer (Ambion, 1×, final dilution), ATP (1.3 mM, final concentration), RNase inhibitors (20 U, final amount), RNA ligase (10 U, final amount), Cy3-conjugated RNA linker (6.67 μM, final concentration) and PEG (15.5%, final concentration). Prepare fresh before use. ▲ CRITICAL The T4 RNA ligase used within this protocol is not thermostable and should not be kept at 37 °C for an extended period.

Precipitation master mix For each sample to be analyzed, prepare 70 μl of precipitation master mix (see **Table 2**) containing sodium acetate (300 mM, final concentration) and linear acrylamide (2.5 μg, final amount). Prepare fresh before use. ▲ CRITICAL To minimize variability between experiments, the preparation of labeling and precipitation master mixes are recommended (See **Tables 1** and **2**).

1.5× hybridization buffer 7.5× SSC, 0.15% SDS, 37.5% formamide and 150 μg ml^{-1} of herring sperm DNA in RNase-free water. Store aliquots at 4 °C. Filter-sterile and store in a cold room (4 °C) for up to 6 months; warm up to 54 °C before use.

Washing buffer 1 (W1) 2× SSC, 0.1% SDS, filter and store at room temperature, 25 °C for up to 6 months.

Washing buffer 2 (W2) 0.2× SSC, filter and store at room temperature, 25 °C for up to 6 months.

Washing buffer 3 (W3) 0.1× SSC, filter and store at room temperature, 25 °C for up to 6 months.

Saturated solution of sodium chloride Add as much solid NaCl to deionized water as needed to form 1-cm-deep slurry on the bottom of a container. Prepare fresh before use. ▲ CRITICAL All solutions and buffers are prepared using RNase-free water.

PROCEDURE

Spotting of miRCURY capture probes on CodeLink slides ● TIMING 3.5 h working time

1| Dilute miRCURY array probes (Exiqon) to a final concentration of 20 μM in 3× printing buffer at least 12 h before spotting. ▲ CRITICAL STEP The 12 h incubation assures that the capture probes are completely and uniformly dissolved into the 3× printing buffer. Following the printing, plates are sealed and stored at −20 °C.
▲ CRITICAL STEP For long-term storage (more than a month), it is advisable to store the plates dried.

2| Load NHS-coated slides (CodeLink, GE Life Science) into the Lucidea array spotter (GE Life Science).

3| Carry out printing in a controlled environment (65% of humidity; 21 °C). The Lucidea printing head is configured such that each spot measures 120 μm in diameter with a center-to-center spacing between spots of 300 μm. To increase the statistical value of a single microarray experiment, each capture probe is printed in quadruplicate using a redip every five slides.
▲ CRITICAL STEP Quadruplicate spots were routinely used for the miRCURY probe set up to version 8.0. Starting with miRCURY probe set version 8.1, spot duplication is used to accommodate the increasing number of capture probes that are now available on the slide.

4| After spotting is finished, remove the printed slides from the spotter, place into microscope slide racks and process immediately (see below).

Coupling of C6-modified oligos to NHS-coated slides ● TIMING 30 min working time + overnight incubation

5| The coupling of the C6-modified oligos to the NHS-coated slides takes place during overnight incubation (25 °C). Spotted glass slides are subjected to post-processing as recommended by the manufacturer. In brief, following the printing process, remove slides from the spotter, return to the original Codelink boxes and place in humidity chambers. Seal the chamber with saran wrap or airtight lids and incubate overnight (25 °C).

Post-coupling treatment of printed arrays ● TIMING 2 h

6| Following overnight coupling of C6-modified oligos to NHS-coated slides, transfer the printed arrays into microscope glass slide staining trays and inactivate residual reactive groups on the CodeLink surface by incubation with post-coupling blocking solution; submerge arrays into prewarmed (50 °C) post-coupling blocking solution and incubate for 15 min in a water bath (50 °C). Rinse arrays twice with deionized water (25 °C).

7| Incubate slides in post-coupling washing solution for 15 min to 1 h at 25 °C. Rinse arrays twice with deionized water (25 °C).

8| Place racks into microplate carriers and centrifuge at 450*g* for 2 min at room temperature.

▲ **CRITICAL STEP** Alternatively, slides can by individually dried using nitrogen gas or by centrifugation of the slides at 450*g* for 2 min at room temperature in 50-ml tubes.

■ **PAUSE POINT** Ready to hybridize slides can be stored for up to 6 months in desiccators (25 °C).

Isolation of total RNA from cells and tissues ● TIMING Day 1, 1 h

9| The extraction of total RNA can be achieved from cultured cells using option A and from mouse tissues using option B (**Fig. 1**).

(A) Extraction of total RNA from cultured cells

(i) Maintain cells under appropriate culture conditions.

(ii) Rinse tissue culture cells twice with ice-cold PBS and directly add Trizol (the required amount of Trizol reagent varies according to the surface size of the culture dish; use 1 ml of reagent to isolate total RNA from 10^7 cells) to the cells according to manufacturer's instructions.

(iii) Collect the cell lysate into a 2-ml safe-lock Eppendorf tube and homogenize the samples by passing the cell lysate several times through a pipette or a 22-gauge needle.

(B) Extraction of total RNA from mouse tissues using tissue lyzer

(i) Submerge fresh or frozen tissue in a 2-ml safe-lock Eppendorf tube in Trizol, as suggested by the manufacturer (use 1 ml of reagent to isolate total RNA from 100 mg of tissue).

(ii) Add to the tube two carbide beads and secure the tubes in the tissue lyzer tubes holder.

(iii) Use the tissue lyzer to disrupt the samples by homogenizing for 2 min with an impulse frequency set to 30.

(iv) Visually inspect the sample for complete disruption of the tissue.

10| Add 200 µl of chloroform per ml of Trizol used to the homogenate.

11| Shake tubes vigorously for 15 s. Incubate at 25 °C for 5 min.

12| Separate the organic from the aqueous phase by centrifugation at 20,000*g* for 15 min at 4 °C.

13| Transfer the aqueous phase (upper phase) to 2-ml safe-lock Eppendorf tubes.

14| Precipitate total RNA by adding one-tenth volume of 3 M sodium acetate and three volumes of 100% ethanol. Incubate for at least 60 min at −80 °C.

▲ **CRITICAL STEP** If more than 750 µl of Trizol are used during homogenization, then the final volume of Step 4 may exceed 2 ml.

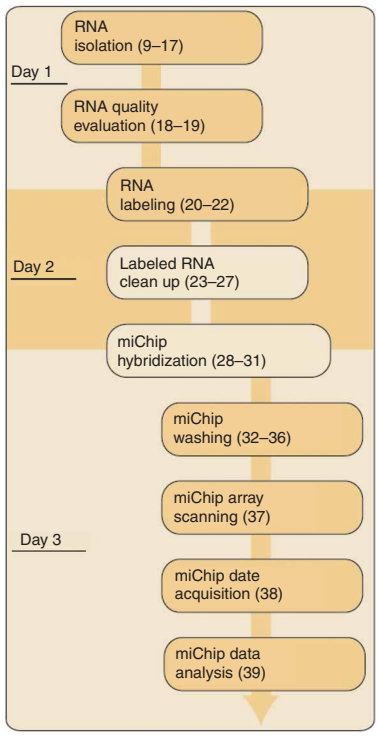

Figure 1 | Schematic representation of the miChip protocol. Within the flowchart, individual steps are shown within boxes. Steps that are typically performed on the same day are grouped by day.

15| Pellet RNA by centrifugation at 20,000*g* for 30 min at 4 °C.

16| Rinse pellets with 1 ml of 75% ethanol and centrifuge at 16,000*g* for 5 min at 25 °C.

17| Air-dry RNA pellets and dissolve in RNase-free water.
■ **PAUSE POINT** Total RNA can be stored at −80 °C for several months.

RNA quality control ● TIMING Day 1, 45 min
18| In our experimental setup, RNA concentration is measured by using a Nanodrop spectrophotometer (ND-1000, NanoDrop Technologies). For the measurement of nucleic acid concentration, Nanodrop uses a small volume (1 µl of solution) and it reliably measures concentrations in the order of 50–100 ng.
▲ **CRITICAL STEP** Alternatively, RNA quantity can be determined using conventional UV spectrophotometer.

19| RNA integrity is determined using an Agilent 2100 Bioanalyzer (Agilent Technologies). The profile obtained gives an estimate of RNA integrity, which is indicated by the RNA integrity number (RIN). RIN numbers cover a range from 1 to 10, with 10 indicating the best quality. Our test experiments show that the quality of expression profiles diminishes if the RIN number is below 7 (data not shown).
▲ **CRITICAL STEP** If a Bioanalyzer is not available, RNA integrity can be determined by gel electrophoresis. Advantages of Nanodrop and Bioanalyzer compared to conventional methods include the requirement of lower amounts of total RNA for analysis.

miRNA labeling protocol for miChip hybridization ● TIMING Day 1, 45 min working time + overnight incubation
20| Dissolve 2 µg of total RNA in 10 µl of RNase-free water.

21| To each sample, add 20 µl of labeling master mix (for details see REAGENT SETUP). Vortex tubes briefly and spin for 1 min at no more than 80*g* (or 800 r.p.m. in an Eppendorf table top centrifuge).
▲ **CRITICAL STEP** Avoid centrifugation of tubes faster than 80*g*, as this may cause salt precipitation. If it happens, dissolve pellets by brief incubation at 37 °C and vortex.

22| Place samples into a dark box and incubate overnight (12–16 h) at 4 °C.

Sample precipitation ● TIMING Day 2, 1 h and 45 min working time
23| Inactivate the enzymes (T4 RNA ligase and RNase inhibitor) by heating samples at 95 °C for 5 min. Spin briefly to collect the condensate at the bottom of the tube and store samples on ice until further use.

24| Add 70 µl of precipitation master mix containing sodium acetate (300 mM, final concentration) and linear acrylamide (2.5 µg, final amount per reaction) (see REAGENT SETUP) to each sample.

25| Precipitate the RNA by adding 300 µl of 100% ethanol to each sample followed by 1 h incubation at −80 °C (or overnight at −20 °C).

26| Spin samples at 20,000*g* for 30 min at 4 °C. Discard supernatant and rinse pellets with 1 ml of 75% ethanol (avoid vortex). Centrifuge samples at 16,000*g* for 5 min at 25 °C.

27| Air-dry pellets for 5–10 min at 25 °C.
■ **PAUSE POINT** Labeled RNA can be stored at −80 °C for several weeks.

Hybridization of labeled miRNAs to miChip ● TIMING Day 2, 30 min + overnight incubation
28| Dissolve labeled RNA pellets in 150 µl of RNase-free water and add 300 µl of 1.5× hybridization buffer (see REAGENT SETUP).

29| Denature samples for 5 min at 95 °C (do not chill the samples).

30| Centrifuge samples at 16,000*g* for 5 min at 25 °C and store tubes in a dark box until further use (25 °C).
▲ **CRITICAL STEP** Do not refrigerate the denatured samples, as this may cause SDS precipitation.

31| For each sample to be analyzed, prepare a microarray chamber with a microarray gasket slide. Place the microarray gasket slide into the chamber, pipette the samples on top of the gasket slide and sandwich it between the gasket slide and the array. Place the secured microarray chambers into the Agilent oven and hybridize for 16 h at 54 °C with a rotation speed of 4 (equivalent to 15–20 r.p.m.).

Post-hybridization washing of miChip ● TIMING **Day 3, 1 h**

32| Following hybridization, individually remove microarray chambers from the hybridization oven. Release the chamber security, submerge the gasket slide-array sandwich in W1 (25 °C) and separate the array from the gasket using forceps.

33| Store hybridized array in W1 (25 °C) until all arrays are removed from the oven and separated from the microarray gasket slides. Successively, transfer arrays into fresh W1 (25 °C) and rinse them on a rocking shaker for 10 min.

34| Next, wash arrays in W2 and W3, each time for 10 min, and once in W3 for 5 min (all washes are performed at 25 °C on a rocking shaker).

35| Finally, rinse arrays for 30 s in deionized water (25 °C, on the shaker).

36| Centrifuge the rinsed arrays at 450*g* for 2 min at 25 °C in 50-ml Falcon tubes.

■ **PAUSE POINT** Processed arrays can be stored in a dark box at 25 °C for several days without any appreciable decrease in signal intensities.

Scanning of miChip arrays ● TIMING **Day 3, 10 min per array**

37| In our experimental setup, array images are generated using the Genepix 4200AL laser scanner (Auto Loader, Axon Instruments). For this purpose, miChip arrays are scanned in batches using the Genepix auto PMT (Photo Multiplayer) algorithm, with pixel saturation tolerance set to 0.2% following the supplier's protocol.

▲ **CRITICAL STEP** Although miChip was originally developed for competitive 2-dye hybridization assays[22], problems associated with Cy5-dye stability in the presence of high levels of ozone has induced us to switch to Cy3-based single-color arrays[27]. Thus, hybridization images are acquired using the A532 channel.

miChip image analysis ● TIMING **Day 3, 10 min per array**

38| Load the tiff images generated by the Genepix 4200AL scanner into Genepix 6 microarray analysis software. Remove artifact-associated spots by both software- and visual-guided flags. Genepix measures signal intensities according to the local background subtraction method as a function of the median of foreground pixels minus median of background pixels. Note that Genepix software saves signal intensities into a TAB-delimited file using the '.GPR' file extension.

▲ **CRITICAL STEP** Alternatively, tiff images can be generated individually from manual scans of single arrays by using Genepix 4200 (or a similar laser scanner).

Further data analysis

39| Perform the analysis of miChip data by one of the following options: (A) using Microsoft Excel for data normalization in combination with the TMeV tool for data clustering or (B) using Genespring software for both data normalization and clustering.

▲ **CRITICAL STEP** Genespring and Excel are proprietary software and valid licenses need to be acquired.

(A) Data analysis using Excel and TMeV

(i) Import GPR files obtained from the Genepix6 microarray analysis software into Excel.

(ii) Equalize quality-flagged values (indicated by Genepix 6 as a negative flag) and values with signal intensities below 50 up to '0'.

(iii) Then calculate the median intensity of the four replicated spots for each capture probe (note that the correct signal is contained in the column 'F532 Median—B532'; from miChip v8.1 onward, only two replicate spots are present).

(iv) Import median values of the background-corrected replicas for each capture probe into the TMeV microarray analysis software for hierarchical clustering and/or advanced statistical analyses.

(B) Data analysis using Genespring

(i) Import the signal intensities contained in the GPR files directly into Genespring.

(ii) Normalize the data (note that the correct signal is contained in the column 'F532 Median - B532') to the 50th percentile.

(iii) Use Genespring build-in tools to visualize box plots, scatter plots and carry on hierarchical clustering.

? TROUBLESHOOTING

● TIMING

Steps 1–8 (capture probe spotting, coupling and post-coupling treatment) are performed in a batch process (e.g., once every 6 months): the time required for these steps is not included in the timeline. Printing, coupling and post-processing of 150 Codelink slides requires 3 working days.

Steps 9–22, day 1: ~3 h work time and overnight incubation

Steps 23–31, day 2: ~3.5 h work time and overnight incubation

Steps 32–39, day 3: ~7 h work time

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Problem	Possible reason(s)	Solution
Detection of a white pellet following centrifugation of the labeling master mix	If the labeling master mix is centrifuged higher than 80*g*, the PEG contained within the mix may precipitate	To dissolve the precipitate, incubate the tube briefly at 37 °C and vortex until pellet dissolves
A white, instead of a pink, pellet is detected following precipitation of the labeling reaction. This suggests a failure of the RNA labeling step	The total RNA used as starting material does not contain small RNA	Recheck the total RNA preparation protocol
	The total RNA used as starting material is contaminated by phenol that may inhibit T4 RNA ligase activity	A low 230/260 ratio of the total RNA as measured by the nanodrop may be indicative of phenol contamination. In this case, extract the total RNA using vol/vol of chloroform followed by an ethanol precipitation
	The T4 RNA ligase used for labeling is inactive	Note that Ambion T4 RNA ligase is not thermostable and it should not be kept at 25/37 °C for an extended period of time
	The RNA linker used in the labeling reaction does not contain a phosphate group at its 5′-end or displays any other problems related to design or preparation	Ask the company providing your linker for quality information
Pink pellet color is lost during the 75% ethanol wash following precipitation of the labeling reaction	The presence of a white pellet suggests that RNA labeling has failed (see also above)	The RNA linker used in this manuscript cannot be precipitated if it is not attached to high molecular weight, ethanol-insoluble molecules (Biospring technical communication). Therefore, low molecular weight, free RNA linker only was initially co-precipitated with unlabeled RNA and it has now dissolved during the 75% ethanol wash
Weak or no signals are detectable on the hybridized array, suggesting that hybridization has failed	The absence of small RNAs (including miRNAs) within the total RNA input material	Recheck the total RNA preparation protocol
	Labeling has failed	See above for more information
	Inappropriate hybridization buffer composition or hybridization temperature	Check hybridization buffer composition and the hybridization oven settings
	Inefficient capture probe binding to the array surface	These can be monitored by analyzing the signal intensities of the spotting controls
High nonspecific background signal is detected on the hybridized array, suggesting problems with the hybridization conditions	Washing conditions are not sufficiently stringent	Check the washing buffer composition
	Dirt or dust may have deposited on the surface of the cover slip or the microarray	

ANTICIPATED RESULTS

MiChip analysis will provide a list of miRNAs that are detectably expressed in a given tissue or cell type. In a successful experiment, miRNAs already known to be expressed within the tissue analyzed (positive controls) will be contained within this

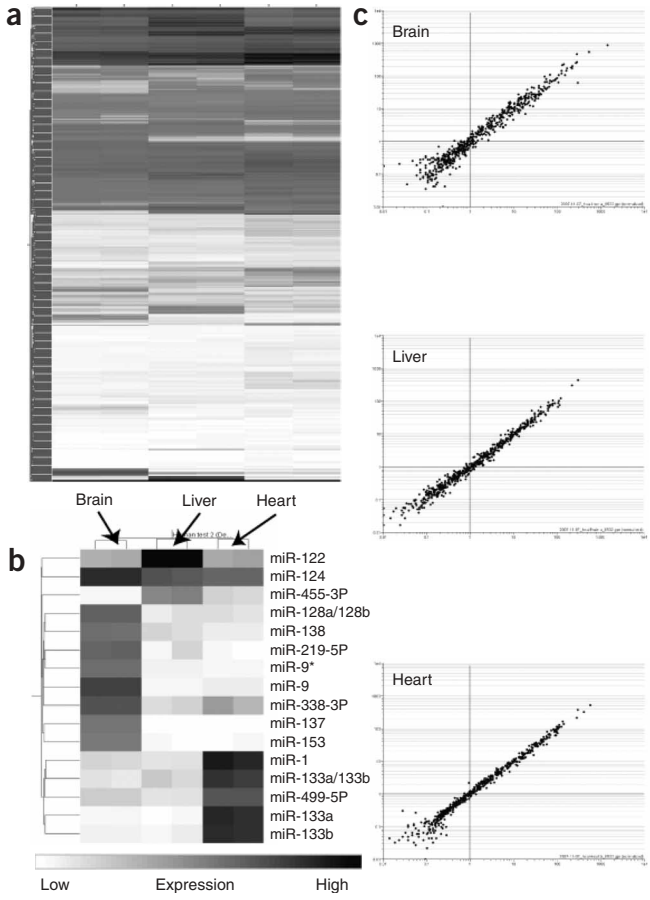

Figure 2 | An example of a classical miChip experiment that compares miRNA expression between human heart (Ambion FirstChoice total RNA, AM7966), brain (Ambion FirstChoice total RNA, AM7962) and liver (Ambion FirstChoice total RNA, AM7960). For each tissue, 2 μg of small RNA-containing total RNA were labeled (see Steps 20–27) and hybridized to miChip (based on miRbase v 9.2; containing 558 human specific miRNAs; Steps 28–31). Signal intensities were captured (Steps 32–38) and the GPR files generated by the Genepix 6.0 software were introduced into Genespring (v 7,3, Agilent Technologies; Step 39B). Then data were normalized to the 50th percentile. (**a**) Heat map generated from miChip hybridization data (capture probes correspond to miRbase release v 9.2) reflecting miRNA expression in human total RNA from liver, heart and brain (Ambion). (**b**) Heat map generated from a selection of miRNAs differentially expressed in human liver, heart and brain. The clustering of miRNAs expressed across the different tissues (using Gene Tree, which applies distance as similarity measure to cluster miRNAs within each samples, and Condition Tree, which uses distance as similarity measure to cluster samples to each other) reveals miRNAs that are expressed in a tissue-specific manner. For example, miR-122 expression is only detected in the liver[28], whereas miR-124, a nervous system-specific miRNA[1], is mainly found in the brain. By contrast, several muscle-specific miRNAs (miR-1, miR-133a and miR-133b) are detectable in the heart. (**c**) Scatter blot analysis representing technical replicas generated for each tissue total RNA indicates high reproducibility of miChip experiments.

list, whereas miRNAs known to be expressed in different tissues (negative controls) will not be contained within this list. Such information may be used to define criteria to analyze the remaining list of miRNAs, e.g., to estimate a cutoff value for experimental noise.

Differential miRNA expression between two or more physiological or pathophysiological states or at different time points after the start of a specific treatment compared to an untreated condition may be of interest. To define those miRNAs that are differentially expressed in a statistically significant manner, at least three biological repeats should be analyzed. By contrast, technical replicas are not necessarily required because of the high reproducibility of miCHip experiments (**Fig. 2**). After data normalization, the cutoff for significant differential miRNA expression is often defined arbitrarily. We routinely trust differential expression as low as 1.7-fold. Once stringent filtering has narrowed down the list of miRNAs that are interesting for further experimentation and/or clinical diagnosis/prognosis predictions, the results need to be validated by an alternative technical approach (e.g., qPCR) to further assess the enrichment/depletion of miRNAs. Bioinformatic searches of relevant databases (e.g., miRbase, PicTar, Argonaute, TarBase or miRNAMAP) may then help to identify putative miRNA target genes. It is important to note that miRNA/mRNA interaction sites are often specified by imperfect base pairing that may make them difficult to predict. Experimental validation of predicted target mRNAs therefore is essential.

Figure 2 shows a classical miChip experiment that compares miRNA expression between human heart (Ambion FirstChoice total RNA, AM7966), brain (Ambion FirstChoice total RNA, AM7962) and liver (Ambion FirstChoice total RNA, AM7960).

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