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Short Communication

Development of a low-cost detection method for miRNA microarray

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MicroRNA (miRNA) microarray is a powerful tool to explore the expression profiling of miRNA. The current detection method used in miRNA microarray is mainly fluorescence based, which usually requires costly detection system such as laser confocal scanner of tens of thousands of dollars. Recently, we developed a low-cost yet sensitive detection method for miRNA microarray based on enzyme-linked assay. In this approach, the biotinylated miRNAs were captured by the corresponding oligonucleotide probes immobilized on microarray slide; and then the biotinylated miRNAs would capture streptavidin-conjugated alkaline phosphatase. A purple-black precipitation on each biotinylated miRNA spot was produced by the enzyme catalytic reaction. It could be easily detected by a charge-coupled device digital camera mounted on a microscope, which lowers the detection cost more than 100 fold compared with that of fluorescence method. Our data showed that signal intensity of the spot correlates well with the biotinylated miRNA concentration and the detection limit for miRNAs is at least 0.4 fmol and the detection dynamic range spans about 2.5 orders of magnitude, which is comparable to that of fluorescence method.

Keywords microRNA; miRNA microarray; enzyme-linked detection method of miRNA

Introduction

MicroRNAs (miRNAs) are single-strand non-coding RNAs of about 22 nucleotides in length. They are generated from hairpin structure precursors (pre-miRNA) through the cleavage of Dicer enzyme [1,2]. miRNAs exist widely in animals and plants and play important regulatory roles through targeting mRNAs for cleavage or translational repression [1,3]. They are known to participate in many biological processes, including development, differentiation, apoptosis, etc. [4–7]. Many miRNAs have been

shown to be associated with human diseases, such as breast cancer [8], cardiac disease [9], leukemia [10], etc. All these studies indicated that miRNA expression levels are closely associated with developmental stages and physiological states as well as disease processes and thus miRNA expression profiling plays an important role in miRNA studies. And among all the miRNA expression profiling approaches, the microarray method has been widely used because of its high throughput, small sample volume requirement, etc. [11–19]. In the current field of miRNA microarray, fluorescence detection method has been mainly used, in which a costly detection instrument such as laser confocal microarray scanner is required [11,18,19]. The high cost will limit the wide applications of miRNAs microarray, especially in clinical diagnosis. Recently, we established a low-cost detection method for miRNA microarray using an enzyme-linked assay that is similar to Joos' ELISA detection method used in protein microarrays [20]. In our method, alkaline phosphatase was introduced through its streptavidin conjugate binding to biotinylated miRNA, which was specifically captured by its corresponding oligonucleotide probes immobilized in a spot on microarray slides. The purple-black precipitation of the enzyme catalytic product on the spot could be easily detected by a charge-coupled device (CCD) digital camera mounted on a microscope and the detection limit for miRNAs was at least 0.4 fmol and the detection dynamic range was from 70 to 35 nM spanned about 2–3 orders of magnitude. Using this method, we analyzed the expression of eight miRNAs in epididymis of 9-week-old rat and found that this method had good reproducibility and the detection results were consistent with that of the fluorescence detection method.

Materials and Methods

Materials
Standard microscope glass slides (25 mm × 75 mm) and cover glass were purchased from Sigma-Aldrich (St. Louis, USA). BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/

nitro blue tetrazolium) pre-mixed solution was purchased from EMD Chemicals, Inc. (Darmstadt, Germany). Streptavidin-coupled alkaline phosphatase was purchased from R&D Systems (Minneapolis, USA). The amine-modified oligonucleotide probes antisense to miRNAs were designed as described previously [14] and synthesized by Invitrogen (Carlsbad, USA). Total RNA was first extracted from epididymis of 9-week-old Sprague–Dawley (SD) rat with Trizol reagent (Invitrogen) and then was fractionated on the 15% denaturing polyacrylamide gel. The miRNA fraction was recovered, precipitated, and stored at $-80°C$ for further labeling, hybridization, etc.

Principle of the enzyme-linked detection for miRNA microarray

Figure 1 shows the schematic diagram of enzyme-linked detection method of miRNA microarray. The isolated miRNAs from biological samples were first biotinylated at the 3′ terminal and then were captured, respectively, by the corresponding miRNA probes in hybridization process. The miRNA probes were pre-spotted on the slides to form a miRNA microarray. After the removal of the unbound miRNAs, streptavidin-conjugated alkaline phosphatase was added to react with the biotinylated miRNAs captured at each spot on the microarray slides. Finally, the microarray is incubated with BCIP/NBT, in which BCIP can be hydrolyzed by the catalysis of alkaline phosphatase to produce an intermediate that is then oxidized by NBT to form insoluble dark-purple materials precipitated on the spots of the slides. In this process, the enzyme as an amplifier converts the trace miRNA into a large number of precipitations. Alkaline phosphatase is a highly active enzyme, and even at very low quantity it can still induce enough precipitation signals to be detected by a commercial digital CCD camera mounted on a microscope (**Fig. 2**). Because the precipitate formation is stoichiometrical to the amount of alkaline
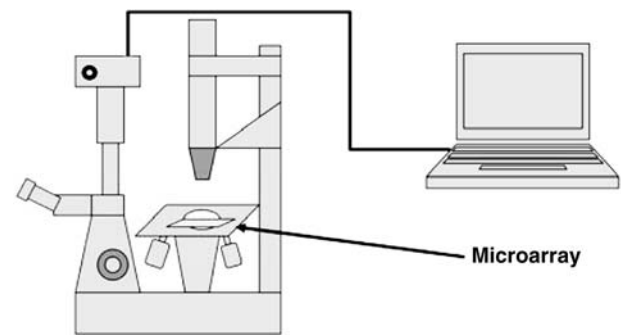

Figure 2 Schematic detection device used in the enzyme-linked detection method of miRNA microarray.

phosphatase, which again strictly depends on that of miRNA captured by the probe, the precipitate amount detected by CCD camera can be used to analyze the miRNA level on each spot in microarray.

Microarray fabrication

Eight specific miRNAs from rat were chosen for a model miRNA microarray system. The sequences of chosen miRNA were obtained in Sanger database [2,21]. The eight target miRNA sequences, the oligonucleotide probes, and the negative control probe in the model microarray were listed in **Table 1**. The sequences of the eight oligonucleotide probes are complementary to the corresponding target miRNAs. All probes contain an amino modified 10-deoxyadenosines linker in the 5′ terminal for immobilization on the glass. The fabrication of the model miRNA microarray was the same as described in our previous paper [14]. In brief, the probes were dissolved in $3\times$ SSC (standard saline citrate) solution at $24 \mu M$, and then printed on a home-made activated slides under 60% humidity with PixSys 5500 spotting robot (Cartesian Technology, Irvine, USA) according to a designed pattern
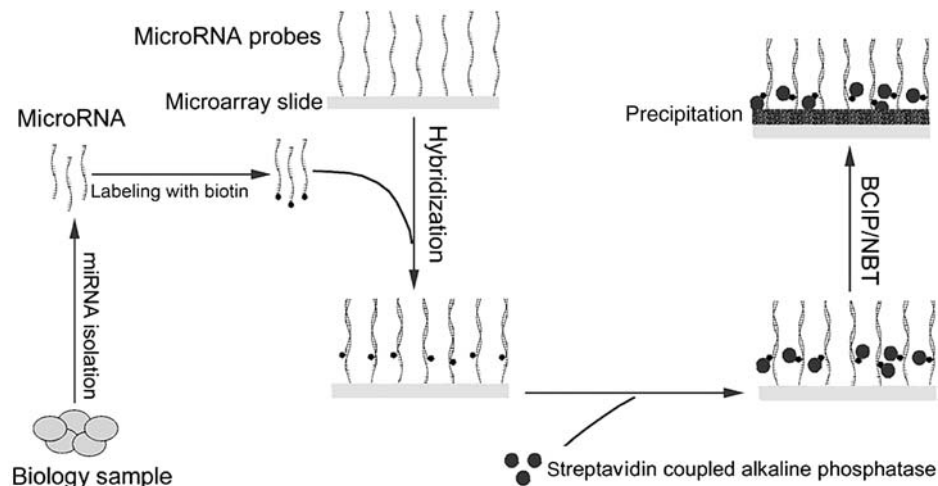
Figure 1 Schematic principle of the enzyme-linked detection method of miRNA microarray.

Table 1 Oligonucleotide probes used in the model microRNA microarray

ID	MicroRNA sequence	Probe
Rno-miR-136	ACUCCAUUUGUUUUGAUGAUGGA	NH_2-5′-(A)$_{10}$-TCCATCATCAAAACAAATGGAGT-3′
Rno-miR-141	UAACACUGUCUGGUAAAGAUGG	NH_2-5′-(A)$_{10}$-CCATCTTTACCAGACAGTGTTA-3′
Rno-miR-188	CAUCCCUUGCAUGGUGGAGGG	NH_2-5′-(A)$_{10}$-CCCTCCACCATGCAAGGGATG-3′
Rno-miR-190	UGAUAUGUUUGAUAUAUUAGGU	NH_2-5′-(A)$_{10}$-ACCTAATATATCAAACATATCA-3′
Rno-miR-21	UAGCUUAUCAGACUGAUGUUGA	NH_2-5′-(A)$_{10}$-TCAACATCAGTCTGATAAGCTA-3′
Rno-miR-326	CCUCUGGGCCCUUCCUCCAGU	NH_2-5′-(A)$_{10}$-ACTGGAGGAAGGGCCCAGAGG-3′
Rno-miR-33	GUGCAUUGUAGUUGCAUUGCA	NH_2-5′-(A)$_{10}$-TGCAATGCAACTACAATGCAC-3′
Rno-miR-378	ACUGGACUUGGAGUCAGAAGG	NH_2-5′-(A)$_{10}$-CCTTCTGACTCCAAGTCCAGT-3′
Negative Ctrl.		NH_2-5′-(A)$_{10}$-GTGTGTGTGTGTGTGTGTGTGT-3′

(shown in later text). The activated glass slides were covered with a self-assembling monolayer of epoxy, which were prepared as described in our previous paper [22]. The diameter of the printed spots on the slides was about 140 μm and the distance between spots was 250 μm. The pattern of model microarray was shown in later text. The model microarray contained four similar sub-arrays, and each sub-array consisted of nine probes with quadruplicate. After printing, the probes were allowed to react with the slide surface overnight at 20°C and humidity of 60%. To examine the miRNA detection limitation and dynamic range for the current method, a 21-nt single-strand biotinylated oligonucleotide (5′-GATAATGGACCCCAATCAAA-C-3′) and its corresponding probe (NH_2-5′-(A)$_{10}$-GTTTG-ATTGGGGTCCATTATC-3′) were also synthesized (Invitrogen).

MiRNA labeling

In the current method, the miRNAs to be detected require labeling with biotin. The labeling was carried out as previously described [14]. Briefly, the enriched miRNAs of 90 μg from epididymis of 9-week-old SD rat were dissolved in 30 μl diethylpyrocarbonate-treated water and diluted with 10 μl of 0.25 M sodium acetate (pH 5.6). Then 1 μl of 5 mM sodium peroxide was added and the mixture was incubated in the dark at 37°C for 1.5 h. To remove the excessive sodium peroxide, 1 μl of 10 mM sodium sulphite was added followed by incubation at room temperature for 30 min. Finally, 20 nmol biotin-X-hydrazide was added and the mixture was incubated at 37°C for 3 h. The biotinylated miRNAs were precipitated with ethanol and stored in −80°C for further experiments.

Microarray hybridization and detection

The biotinylated miRNAs were dissolved in 4× SSC, 0.1% SDS, and hybridized with miRNA microarray as previously described [14]. The hybridization reaction was performed at 45°C overnight. And then the hybridized microarray was washed with 1× SSC/0.5% SDS at 37°C for 10 min. After this, the miRNA level on the microarray was detected by either fluorescence or enzyme-linked method. In the fluorescence method, the microarray was incubated with 10 μl of 2 nM streptavidin-conjugated Qdot 655 (Invitrogen) at room temperature for 1 h. After washing, the microarray was scanned on a ScanArray 5000 Scanner (PerkinElmer, Waltham, USA), with 100% power, 80% photomultiplier and scan resolution of 5 μm. In the enzyme-linked method, the microarray was incubated with 10 μl of 1 μg/ml streptavidin-conjugated alkaline phosphatase at room temperature for 1 h. After washing, the slides were incubated with BCIP/NBT at 37°C in dark for 1 h. Finally, the microarrays were washed and then detected with commercial C-4000Z digital CCD camera (Olympus, Tokyo, Japan) mounted on an Olympus microscope.

Data analysis of microarray

The signal intensity of miRNA microarrays detected with the current method was expressed as the gray level of each spot in the images obtained with CCD camera. In the data processing, the original image from CCD camera was first transformed into gray-scale image, which was further processed with the Photoshop 9.0 (Adobe System, San Jose, USA) to map the lightest and darkest pixels into black and white. The mean value of the gray levels of all the pixels inside a spot was defined as the signal intensity of the spot. The gray level of background image was subtracted from the signal intensity. In the fluorescence method, the obtained images from laser confocal scanner were processed with QuantArray software (PerkinElmer).
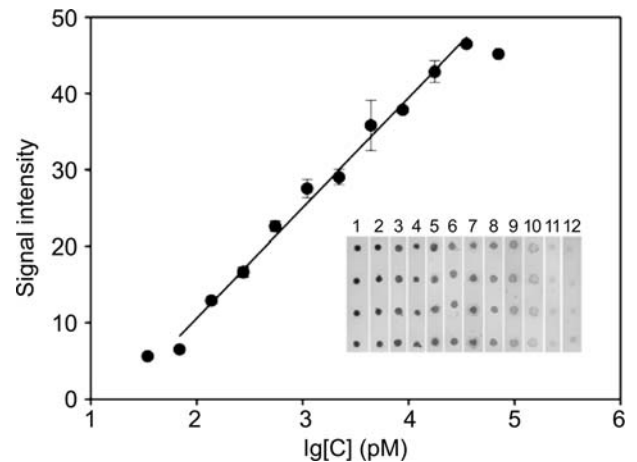
Results and Discussion

Figure 3 shows the feasibility of enzyme-linked detection method application in miRNA microarray detection. The inset image in **Fig. 3** was obtained by the CCD camera as described in "Materials and Methods". The image shows
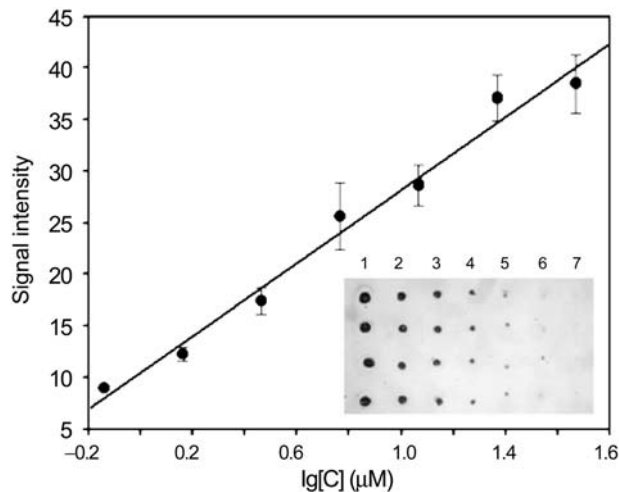
Figure 3 Correlation of the signal intensities and the concentrations of probes immobilized on the microarray The inset: the enzyme-linked detection image of different concentrations of probes immobilized on a microarray. The probe concentrations of spots from columns 1 to 7 were 47, 23.5, 11.7, 5.9, 2.9, 1.5 and 0.7 μM, respectively. The concentration of the model miRNA used for hybridization was 100 nM. Enzyme reaction temperature was at 37°C and the reaction time was 1 h. The data in the plot were obtained from the inset image by averaging the mean signal intensities of quadruplicate spots as described in part of data analysis of microarray.

Figure 4 Correlation of the signal intensities and different model miRNA concentrations in enzyme-linked detection method The inset: the detection images of different concentrations of biotin-labeled model miRNAs obtained by enzyme-linked detection method. The concentrations of model microRNA from lane 1 (70 nM) to 12 (34 pM) were 2-fold serial diluted. The probe concentration printed on the slides was 24 μM. The temperature and time of enzyme reaction were at 37°C and 1 h, respectively. The data in the plot were obtained from the inset images by averaging the mean signal intensities of four replicate spots.

that the concentration of model miRNA probes spotted on the slides can be well detected by our method as expected in schematic principle of the enzyme-linked detection method. The lowest detected concentration is about 0.7 μM. It was found that the signal intensities in **Fig. 3** correlated well with the probe concentrations. The signal intensities were linear to the logarithm of the concentrations of probes immobilized on the microarray in the range of 0.7−47 μM, suggesting that both the capture ability of the probes for miRNA and the amount of insoluble precipitate produced by enzyme-linked reaction be concentration dependent. This indicates that the current method can be used for quantitative miRNA analysis. We also found that the spot size strongly correlates with the probe concentration: higher concentration probe resulted in larger spot. Considering the signal intensity and spot size, the suitable probe concentration in miRNA microarray should be around 12−24 μM. This concentration range was mostly used in the fabrication of miRNA microarray in the current work.

Figure 4 shows the results obtained by enzyme-linked method to detect the different concentrations of model miRNA. The inset shows a set of images of various concentration miRNA from 34 pM to 70 nM. The image indicated that as low as 34 pM of miRNA could be detected by the enzyme-linked detection method, which means the detection limitation in this method was actually about 0.4 fmol miRNA considering the printed volume of each

spot was about 1 nl. This detection sensitivity is very close to that in fluorescence methods. It can be found in the image that the signal intensities from lane 1 to 12 decreased gradually, showing an obvious dependence on concentration of the model miRNA. The plot in **Fig. 4** indicated more clearly that the signal intensities were linear to the logarithm of concentrations of model microRNA in the range of 70 pM to 35 nM. This suggests that the linear detection dynamic range in enzyme-linked method was about 2.5 orders of magnitude. Such a detection sensitivity and broad dynamic range can meet the needs for most miRNA microarray detection, suggesting that the enzyme-linked detection method could be used to detect miRNA microarray.

As known, the signal intensity of enzyme reaction product is strictly time dependent, the choice of the appropriate reaction time in enzyme-linked detection method is very important. The optimum reaction time in the microarray detection should satisfy two conditions. First, the reaction time must be within the period when the enzyme reaction product is linearly proportional to time. Second, the product amount produced within the reaction time should give good signal to meet the detection requirement. **Figure 5** indicated that the signal intensities were linear in the starting period of about 100 min for 0.5, 5, and 50 nM biotin-labeled model miRNA. The signal intensities reached a plateau after 100 min. This phenomenon was even more obvious for higher concentration of miRNA. Considering the signal intensity and the time needed for whole enzyme-linked detection, we believe 40−60 min is the optimum time for enzyme reaction.
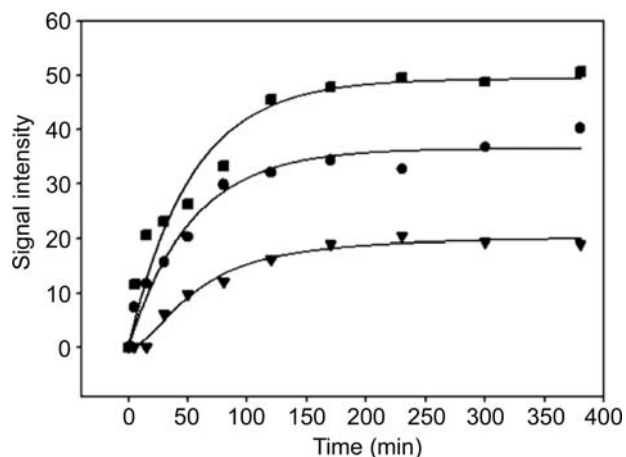
Figure 5 Time dependence of the signal intensities of enzyme reaction product for detection of three different concentrations of model miRNA The concentrations of biotin-labeled model microRNA detected with enzyme-linked methods were 0.5 nM (black triangle), 5 nM (black circle), and 50 nM (black square), respectively. In these detections the concentrations of the probe spotted on the microarray slides were all 24 μM and four replicate spots form a sub-array. The temperature for enzyme reaction was 37°C. The data in the plot were obtained by averaging the mean gray levels of four replicate spots.

Figure 6 shows the results of detection and analysis of the model miRNA microarray for eight miRNAs in rat obtained by enzyme-linked detection method. The microarray contained four sub-arrays and the printing pattern was

also shown in **Fig. 6**. As shown in **Fig. 6(A)**, the spots corresponding to eight miRNAs displayed different signal intensities, while the negative control was indistinguishable from the background. Such signal pattern was similar to that of the image detected with the fluorescence method shown in **Fig. 6(B)**. The quantitative analysis of the results from the two methods further indicated that the average correlation coefficient of each miRNA measured by both methods was about 0.93 [**Fig. 6(D)**].
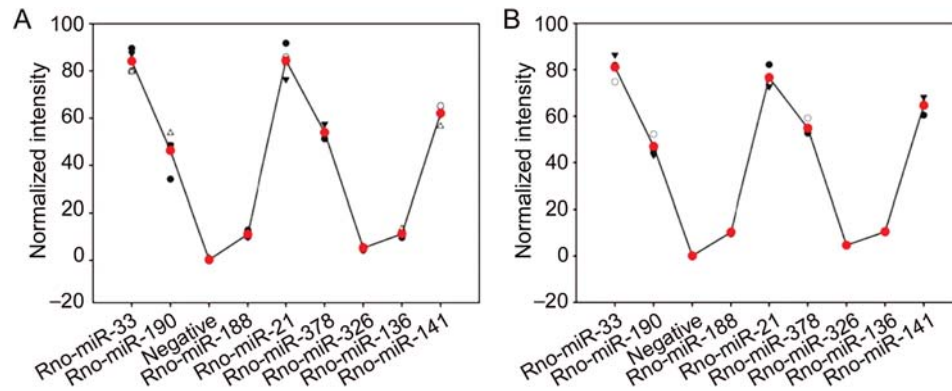
The reproducibility of the enzyme-linked detection method was shown in **Fig. 7**. The data in **Fig. 7(A)** indicated that the error of each miRNA signal in different sub-array was small. The correlation coefficients for these data in **Fig. 7(A)** were higher than 0.96, showing a good reproducibility between four sub-arrays in a microarray slides. The similar reproducibility between different slides was shown in **Fig. 7(B)**. The correlation coefficients were 0.95, very close to that in the fluorescence methods (about 0.97) in the same miRNA microarray (data not shown). All these indicated that the enzyme-linked method is of high reproducibility and reliable.

Taken together, all the results mentioned above indicated that the enzyme-linked detection method we established could provide high detection sensitivity, large linear dynamic range and high reproducibility in practical detection of miRNA microarray. More importantly, this method has minimum instrumentation requirement, only a
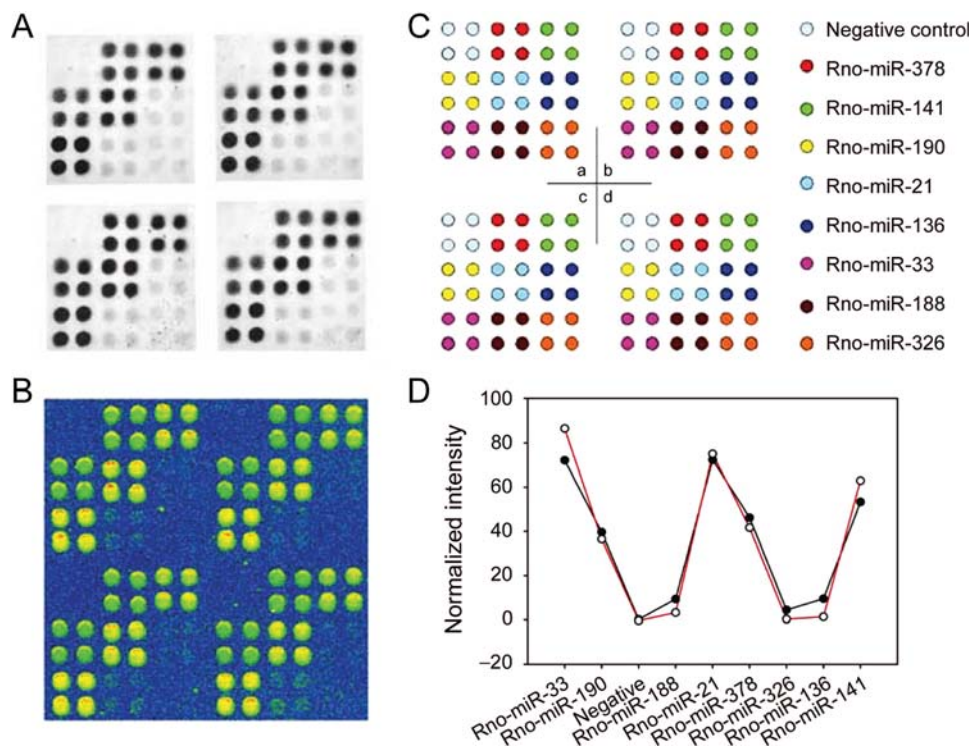
Figure 6 Image of the model miRNA microarray detected by enzyme-linked and fluorescence method (A,B) The detected images of miRNA microarray obtained by enzyme-linked method (A) and fluorescence methods (B). (C) The pattern of the model miRNA microarray for eight rat miRNAs. (D) Comparison of the signals for eight miRNAs in the microarray detected with the enzyme-linked and fluorescent methods. Solid circles represent the averaged signal intensities in the enzyme-linked method and hollow circles represent the averaged signal intensities in the fluorescent method.

Figure 7 Reproducibility of enzyme-linked method in detection of miRNA microarray (A) Mean normalized signal intensities of each miRNA in four sub-arrays (solid triangle, hollow triangle, solid circle, and hollow circle) in the microarray shown in **Fig. 6(A)**. The red solid circle is the mean value of the four mean normalized signal intensities. (B) Mean normalized intensities of each miRNA in three independent microarrays (solid triangle, solid circle and hollow circle). The red solid circle is the mean value of the three mean normalized signal intensities.

commercial digital CCD camera and a microscope were required. This method can reduce the threshold of miRNA microarray experiment and is of potential applicability in miRNA microarray and clinical diagnosis.

Funding

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