Published in final edited form as:
Anal Chem. 2012 May 1; 84(9): 4153–4160. doi:10.1021/ac3004055.

Scanometric microRNA (Scano-miR) Array Profiling of Prostate Cancer Markers Using Spherical Nucleic Acid (SNA)-Gold Nanoparticle Conjugates

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Abstract

We report the development of a novel Scanometric MicroRNA (Scano-miR) platform for the detection of relatively low abundance miRNAs with high specificity and reproducibility. The Scano-miR system was able to detect 1 fM concentrations of miRNA in serum with single nucleotide mismatch specificity. Indeed, it provides increased sensitivity for miRNA targets compared to molecular fluorophore-based detection systems, where 88% of the low abundance miRNA targets could not be detected under identical conditions. The application of the Scano-miR platform to high density array formats demonstrates its utility for high throughput and multiplexed miRNA profiling from various biological samples. To assess the accuracy of the Scano-miR system, we analyzed the miRNA profiles of samples from victims of prostate cancer (CaP), the most common noncutaneous malignancy and the second leading cause of cancer death among American men. The platform exhibits 98.8% accuracy when detecting deregulated miRNAs involved in CaP, which demonstrates its potential utility in profiling and identifying clinical and research biomarkers.

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SUPPORTING INFORMATION AVAILABLE

Additional information includes the experimental details about isolation of RNA, obtaining and processing clinical samples, cell culture preparation, hypoxia mimetic treatment, miR-210 quantification using qRT-PCR, and bioinformatics analysis. Information includes a supplementary figure (S1) Functional Analysis of Deregulated miRNAs in Prostate Cancer, and six supplementary tables: (S1) Sequences of the synthetic oligonucleotide and miRNA, (S2) Latin Square experimental design, (S3) Clinical samples, (S4) Serum miRNAs detected from healthy donor, (S5) List of deregulated miRNAs involved in prostate cancer, (S6) Function-gene-miRNA network analysis.

INTRODUCTION

MicroRNAs (miRNA, miR), small noncoding RNA molecules, have emerged as new candidate biomarkers for detecting a wide variety of cancers.[1–2] miRNAs are regulatory elements believed to control between 30–90% of all protein-coding genes in the human genome by binding to the 3'-untranslated region (3'-UTR) of target mRNAs.[3–6] Importantly, miRNA expression levels have been shown to be tissue- and function-specific.[7] Furthermore, miRNAs in the bloodstream are protected from nuclease degradation and can be extracted for detection and downstream analyses.[8–9] Therefore, molecular diagnostic tools that allow one to identify miRNAs and measure their concentrations at biologically relevant levels in blood and tissue samples may become useful for studying and diagnosing disease. Individual markers rarely are unambiguously indicative of disease type and state, and miRNAs are often at very low concentrations in serum and precious tissue samples, which precludes the use of conventional diagnostic tools with low sensitivity. Although amplification strategies such as polymerase chain reaction (PCR) provide a means to amplify nucleic acid targets, the short length of the miRNA targets makes it difficult to develop multiplexed high sensitivity PCR-based quantification protocols.[10] Ideally, one needs a method that allows for high sensitivity nucleic acid detection, which does not rely on enzymatic-based target amplification, and can be used to simultaneously profile hundreds to thousands of miRNAs. Herein, we report the development of a novel Scanometric microRNA (Scano-miR) Array Profiling assay that is capable of detecting low abundance miRNAs from human serum, cultured human cells, and cancer tissue samples with a limit of detection (LOD) at 1 fM with single nucleotide polymorphism (SNP) selectivity. Moreover, this technique is applicable to high density array formats where thousands of targets can be interrogated at once. Prostate cancer cell lines and tissue samples are used to help validate the performance of the Scano-miR assay, and also to demonstrate its potential utility in profiling and identifying prostate cancer biomarkers from human prostate cancer tissue.

The current workhorse technologies for miRNA profiling are high density microarrays (miRNA-arrays), quantitative real time PCR (qRT-PCR), and deep sequencing methods;[10] however, no single technique provides the ability to study miRNAs at low cost, with high selectivity, at low abundance, in a high throughput and multiplexed fashion. In all cases, the detection of low abundance miRNAs (below 10 fM) is a major obstacle because of their short length (typically 20–25 nucleotides)[11]. For microarrays, typical detection strategies are dominated by techniques that involve direct detection with molecular fluorophores as target labels (i.e. no PCR amplification).[12–13] However, the fluorophore-based arrays are challenged with respect to LOD (typically > 1 pM) and therefore are inherently prone to false negatives.[14] qRT-PCR-based profiling assays have been shown to provide higher sensitivity (~10 fM for miR-141)[8] and are ideal for screening a small number of miRNAs,[10] but there are challenges associated with using qRT-PCR for high-throughput miRNA profiling. Specifically, miRNAs are difficult to amplify using PCR because of their short length.[15] Ligation of PCR primers at the 3' and/ or 5' ends of miRNAs is required to increase their length for successful amplification, which introduces challenges with regard to primer design.[10] Indeed, qRT-PCR assays are limited to studying miRNAs based upon the design of a combination of universal and specific primers and probes for each miRNA target, which leads to a reduction in specificity. Deep sequencing, a more sophisticated approach to miRNA detection, has emerged as an alternative to microarray- and qRT-PCR-based detection of miRNAs.[16] Deep sequencing of miRNA libraries from biological samples has been used to perform multiplexed, highly specific, and quantitative detection of both novel and previously identified miRNA sequences.[17] A limitation of deep sequencing is its lower sensitivity compared to qRT-PCR due to the need to ligate platform-specific linking sequences to the 5' and 3' ends of the miRNAs because of their short length[15, 17] with

subsequent yield-reducing purification steps. Moreover, deep sequencing assays rely on PCR amplification where the relative abundance of miRNA species in the starting materials cannot be preserved.[10] These steps increase the complexity and cost of such assays[10] and simultaneously decrease the sensitivity of deep sequencing techniques.

In 2000, we reported the development of the scanometric assay,[14] a novel nucleic acid detection method based upon the use of spherical nucleic acid-gold nanoparticle conjugates (SNA-Au NPs).[18–22] The assay utilizes a low density microarray on a glass slide to capture DNA target and then sandwiches it with the SNA-Au NP probes. The signal is then amplified by catalytic reduction of Ag^+ in the presence of hydroquinone[14] or gold enhancement with tetrachloroaurate and hydroxylamine[23–24]. After the reduction step, the slide is used as a wave guide, and scattered light is measured from the metal spots to determine target identity and concentration. The LOD of the method is 100 aM for large DNA targets and does not require PCR or related target amplification techniques.[25] Because the SNA-Au NP probes exhibit cooperative melting transitions over more narrow temperature ranges than duplexes formed from molecular fluorophore probes of the same sequence, stringency conditions can be employed to provide significantly higher target discrimination capability.[14] We rationalized that this assay might be ideal for detecting short, relatively low abundance miRNAs, without the need for enzymatic amplification steps with high selectivity and sensitivity. Therefore, we have studied a new version of this assay aimed at profiling the expression of miRNA species from human serum, cell culture, and human tissue samples. Data demonstrate that the Scano-miR assay is highly specific, sensitive, and reproducible for profiling miRNAs. Importantly, these studies not only show this modified scanometric method can be used, for the first time, with high density arrays but also demonstrate its ability to identify miRNA markers with higher sensitivity and selectivity than fluorophore based high-density array techniques.

EXPERIMENTAL SECTION

Spherical nucleic acid-gold nanoparticle (SNA-Au NP) Synthesis

MicroRNA (miRNA) Cloning Linker II and a propylthiol-modified ssDNA recognition sequence complementary to Linker II with a 10 adenosine base (A10) spacer (5'-propylthiol-$(A)_{10}$-TCCTTGGTGCCCGAGTG-3') were purchased from IDT (Coralville, Iowa). The propylthiol terminated strands at a concentration of 4 μM were chemisorbed onto 13 nm gold nanoparticles (Au NPs, 10 nM concentration) by incubating the strands with the solution of citrate stabilized Au NPs for 1 hr at room temperature.[20] Next, sodium dodecylsulphate (SDS), phosphate buffer (pH = 7.4), and sodium chloride (NaCl), were added to the solution at final concentrations of 0.01%, 10 mM, and 0.1 M, respectively, and incubated for an additional 1 hr at room temperature. Two aliquots of NaCl were added with an incubation time of one-hour between each addition to achieve a final concentration of 0.3 M. The mixture was sonicated (10 sec) between each salt addition. Following this brief salt aging process, the mixture was incubated overnight at room temperature with gentle shaking (130 rpm). For purification, three successive rounds of centrifugation (16000 × *g* for 20 min), supernatant removal, and re-suspension in phosphate buffered saline (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) were performed.

Characterization of the Scano-miR assay sensitivity and specificity

All reagents used to synthesize miRNA sequences were purchased from Glen Research. Synthetic miRNA species were synthesized on a MerMade 6 (Bioautomation) and purified using high performance reverse-phase liquid chromatography (Varian). The synthetic miRNA sequences used in this study are listed in Table S1. Synthetic miR-16 at concentrations of 1, 10, and 100 fM, were ligated to the universal miRNA cloning linker II

by using T4 RNA Ligase II (New England Biolabs) following the manufacturer's protocol. Briefly, truncated T4 RNA Ligase 2, miRNA cloning linker II, PEG 8000, and T4 RNL2 buffer, were added to 200 ng of synthetic miR-16 at a final concentration of 200 U, 900 ng, 12%, and 1X, respectively. This solution was allowed to incubate for 3 hrs at 37°C. The ligated RNA was added into 1 mL of pre-denatured serum followed by total RNA extraction (see Supplementary Information). Isolated total RNA was re-suspended in 400 μL RNase-free 2X SSC hybridization buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), denatured at 95°C for 2 min, and incubated on ice for another 2 min. The whole mixture was hybridized overnight at 52°C onto a high density complement miRNA microarray (NCode Human miRNA microarray V3, Invitrogen) displaying the complement to miR-16 as well as the appropriate mutant control with a single mismatch. Following the incubation, the array was washed with a pre-warmed (52°C) 2X SSC for 2 min, followed by a 2 min immersion in the following RNase-free solutions at room temperature: 2X SSC, phosphate buffered saline (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4), and nanopure water. The array was then exposed to a solution of 1 nM (final concentration) SNA-Au NPs in 400 μL RNase-free 2X SSC at 56°C for 1 hr. Unreacted SNA-Au NPs were washed away with pre-warmed (56°C) 2X SSC followed by a 2 min immersion in the same RNase-free solutions as described above. Gold enhancing solution [a freshly mixed 1:1 (v:v) solution of 1 mM $HAuCl_4$ and 10 mM NH_2OH] was used to deposit Au^0 onto the immobilized SNA-Au NPs in order to increase the light scattering of the Au NPs.[23] This amplification process was performed by covering the array with 1 mL of the gold enhancing solution and incubating the array for 5 min at room temperature followed by washing with nanopure water. The array was then air-dried and imaged with a scanner (LS Reloaded, Tecan, Salzburg, Austria). Three rounds of gold deposition for 5 minutes each were performed according to literature methods[23] in order to characterize the sensitivity of the Scano-miR system. The gray scale images were converted into colored ones and intensity values were calculated using GenePix Pro 6 software (Molecular Devices).

Comparative hybridization

To examine the array selectivity of this design, five synthetic miRNAs (miR-20a, −143, −143*, −205, and −210) were synthesized and ligated to the universal miRNA cloning linker II as described above (see Table S1 for sequences). Four of these synthetic miRNAs were added in 2X SSC at 10^2, 10^3, 10^4, and 10^5 fM, respectively in one pool and labeled as Array 1 (see Table S2 for mixture information). The orders of the miRNA concentrations were inverted in a Latin Square experimental design to produce a total of five different pools, labeled as Arrays 1–5 (Table S2). These five pools were hybridized to five separate miRNA-arrays and analyzed with the Scano-miR system as described above. Each miRNA-array was developed once with gold enhancing solution for 5 minutes and the image was recorded using the Tecan scanner.

To perform the direct sensitivity comparison of the Scano-miR assay to the fluorophore-based approach, a Cy5-labeld ssDNA recognition sequence complementary to Linker II with a 10 adenosine base (A10) spacer was purchased from IDT (5'-Cy5-$(A)_{10}$-TCCTTGGTGCCCGAGTG-3'). Eight synthetic miRNAs (miR-200c, −21, −210, −205, −20a, −143*, −143, and −16) were synthesized, ligated to the universal miRNA cloning linker II, and added in 2X SSC at 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10, and 1 fM, respectively (see Table S1 for sequences). The sensitivity of Cy5-labeled arrays was measured as follows. A 400 μL aliquot of the miRNA solutions was hybridized overnight at 40°C to a separate miRNA-array. Following the incubation, the array was washed with a pre-warmed (40°C) 2X SSC for two min, followed by 2 min immersions in the same RNase-free wash solutions described above. This array was labeled by adding 1 nM of the Cy5-labeled probe in 400 μL RNase-free 2X SSC at 40°C for 1 hr. Unreacted Cy5-labeled probes were washed

away with pre-warmed (40°C) 2X SSC followed by a 2 min immersion in the same RNase-free solutions as described above. Labeled arrays were scanned immediately. Another aliquot of the miRNA solutions used above (400 μL) was hybridized to a separate miRNA-array and analyzed with the Scano-miR system as described above and scanned after each round of gold deposition to achieve a total of three rounds of gold deposition, each for 5 min. Similar protocols were applied to obtain complete miRNA profiles from 0.8 μg total RNAs isolated from the prostate cancer cell line (PC-3). The signal intensity values, calculated using GenePix Pro 6 software, were generated from arrays analyzed with both the Scano-miR system and the Cy5-labeled arrays and used to perform the direct sensitivity comparison.

To investigate the ability of the Scano-miR system to detect miRNAs from human serum (see Table S3), total RNA isolated from 5 mL serum (see Supporting Information) was added to a solution of truncated T4 RNA Ligase 2, miRNA cloning linker II, PEG 8000, and T4 RNL2 at final concentrations of 200 U, 900 ng, 12%, and 1X, respectively, and incubated for 3 hrs at 37°C. Following incubation, the ligated species were analyzed with the Scano-miR system as described above. The signal intensity values, calculated using GenePix Pro 6 software, were generated from two Scano-miR arrays after miRNA profiling two replicates of 5 mL serum. Signal intensity values were used to calculate the correlation coefficient between the two biological replicates.

RESULTS AND DISCUSSION

Development and Characterization of the Scano-miR Assay

To realize a high throughput ScanomiR system, we had to create a universal probe that could be utilized to develop an array in a single set of assay steps (Scheme 1). In a typical assay, miRNA targets in a sample mixture are ligated to a universal miRNA cloning linker (see Table S1 for sequences) using truncated T4 RNA ligase II.[8] Importantly, the products of the ligation reaction are added directly to the miRNA-array (NCode Human miRNA Microarray V3, Invitrogen) without the need for purification steps, thereby avoiding the loss of low abundance miRNAs. The miRNA-array consists of a pattern of multiple DNA sequences that are complementary to miRNA sequences of interest. Target miRNA sequences that are present in the sample hybridize with appropriate complement sequences at unique spots on the array. Following hybridization, unreacted linkers, unbound miRNA strands, and ligation enzymes are then easily washed away. The universal SNA-Au NPs, which contain a DNA sequence that is complementary to the universal miRNA cloning linker (see Table S1 for the sequence), are then added to the miRNA-array. Hybridization is performed at 56 °C to prevent off-target hybridization of the SNA-Au NPs. Dehybridized miRNAs and unreacted SNA-Au NPs are washed away, and the miRNA-array is subjected to gold enhancement with tetrachloroaurate and hydroxylamine. This amplification process deposits Au^0 onto the immobilized SNA-Au NPs, and dramatically increases their light scattering properties.[23–24]

The LOD of the Scano-miR assay was determined by adding synthetic miR-16 strands into denatured human serum at known concentrations. To avoid detecting endogenous miR-16, synthetic miR-16 (see Table S1 for the sequence) was pre-ligated with the universal linker prior to the synthetic miR-16 addition step, and the endogenous miRNAs were not ligated to the universal linker. The LOD for the synthetic miR-16 miRNA was determined to be 1 fM (~600 copies/μl serum) without PCR amplification (Figure 1a). The high sensitivity of the Scano-miR system stems from the increased light scattering properties of the nanoparticles upon gold deposition.[23] In addition to high sensitivity, high selectivity is equally important if one wants to differentiate low abundance miRNAs that are closely related, including those that may only vary by a single nucleotide. To test the specificity of the Scano-miR system,

synthetic miR-16 was hybridized to a miRNA-array that displayed two types of capture probes: one that was perfectly matched to miR-16, and another one with a single nucleotide mismatch (the position of the mismatch is not disclosed by the commercial supplier of the array). The intensities of the scattered light from both the perfectly matched capture probes (Rno-miR-16) and the capture probes with a single nucleotide mismatch (mut1-Rno-miR-16), respectively, were compared. The Scano-miR assay exhibits single nucleotide mismatch specificity down to an LOD of 1 fM (Figure 1a).

The analytical performance of the Scano-miR system was investigated through a comparative hybridization of a set of five synthetic miRNAs (Table S1) in a Latin Square experimental design. This design is a five-by-five matrix wherein detection of targets within pools of miRNAs provides the opportunity to compare the quantitative array response for multiple targets, assess the quantitative capabilities of the assay as a function of varying target concentrations, and determine the variability of different arrays without the need to individually probe a large number of test samples. In a typical experiment, test samples were created by loading hybridization buffer with four different synthetic miRNAs at concentrations of 10^2, 10^3, 10^4, and 10^5 fM target, respectively, and compared to buffer without target. The concentrations of each of the targets were sequentially changed four more times, according to the original concentration profile, to complete the five-by-five matrix (see Table S2 for Latin Square Design). The five mixtures of synthetic miRNAs were hybridized to five separate miRNA-arrays and then studied via the Scano-miR system (Figure 1b). Plotting signal intensity versus miRNA concentrations provides a correlation coefficient of $r^2 = 0.97$, which confirms the profiling reliability of the Scano-miR assay and its low variability from one array to another (Figure 1b). As a result, the Scano-miR assay is able to simultaneously detect multiple miRNA targets over a range of concentrations that vary by at least four orders of magnitude with high accuracy and reproducibility across arrays.

A comparison of the conventional microarrays, which utilize fluorophore probes to detect miRNAs, with the Scano-miR methodology was directly evaluated to determine the sensitivity advantage afforded by the SNA-Au NP conjugates. Consistent with literature reports,[14, 26] the fluorophore-based approach with Cy5-labeled probes allows one to detect targets over the 10 nM to 100 pM concentration range (Figure 2a, b). One advantage of the Scano-miR system is the ability to perform sequential metal deposition steps to intensify signal in a controllable manner.[23] The Scano-miR system is able to detect miRNAs from 1 nM to 100 fM after one round of gold deposition, resulting in a dynamic range of four orders of magnitude. However, additional rounds of gold deposition improve the limit of detection down to 1 fM (Figures 2a, b). Importantly, the ability to perform sequential metal depositions allows the user to tune the dynamic range of the Scano-miR system to detect high or low abundance miRNAs over a wide overall dynamic range (~6 orders of magnitude) (Figures 2a, b) without the need to perform separate hybridization experiments which would require additional samples. To further compare the capabilities of fluorescence and Cy5-labeled probes to SNA-Au NPs of the Scano-miR approach, experiments were performed to detect miRNAs isolated from a prostate cancer cell line (PC-3). Interestingly, the Scano-miR assay identified 97% of those targets identified using the fluorophore-based approach, and detected 88% more miRNA species than the Cy5-based detection method (Figure 2c). Taken together, these data highlight the potential of the Scano-miR system to generate a more complete profile of miRNA expression, especially for low-abundance miRNAs.

After determining the Scano-miR system's performance by directly comparing it with conventional fluorophore-based technology, we used it to examine miRNAs recovered from human serum (Figure 3a) (see Table S3 for information about donor 277). A rank-order list

of the serum miRNAs detected is provided in the supporting information (Table S4). These results demonstrate that the Scano-miR system can profile endogenous miRNAs isolated from human serum in a multiplexed and high-throughput fashion. Two biological replicates of serum miRNA isolates were also used to hybridize miRNAs onto two different miRNA-arrays and detected using the Scano-miR system. Signal intensities generated from both miRNA-arrays were quantified and used to examine the extent of the Scano-miR system's reproducibility in the presence of biological background. The correlation coefficient of signal intensities between the two biological replicates was 0.95 (Figure 3b).

Profiling Research and Clinical Biomarkers

Arrays are useful for profiling miRNAs from samples that have been treated differently in order to identify miRNAs that are being regulated by a chosen intervention. Accordingly, we sought to demonstrate the capability of the Scano-miR technology for the semi-quantitative detection of known miRNAs and compare the results using qRT-PCR. We chose to compare the miRNA profiles from PC-3 cells following hypoxia inducible factor 1-alpha (HIF-1a) stabilization using cobalt chloride ($CoCl_2$) treatment.[27–29] The miRNA expression of $CoCl_2$-treated PC-3 cells was compared to that of PC-3 cells without $CoCl_2$ treatment. More specifically, miR-210 has been called a "hypoxamiR" due to its robust increase upon HIF-1a stabilization.[27] Cobalt chloride treatment results in a 3.85 fold increase in miR-210 levels (Figure 4a). This difference in magnitude is consistent with our qRT-PCR results (Figure 4b) and with literature reports.[28]

Finally, we investigated the applicability of the Scano-miR assay to tissue samples with human prostate cancer (CaP). In the US, CaP is the most frequent noncutaneous malignancy and the second leading cause of cancer-related death among men.[30] Contemporary studies have demonstrated conflicting results on the utility of prostate specific antigen (PSA) and have called into question PSA screening due to the overdiagnosis of so-called insignificant cancer and its treatment.[31–34] CaP screening includes a digital rectal exam and serum PSA testing. An elevated serum PSA value often prompts a prostate biopsy for definitive diagnosis and to provide pathological staging based on the Gleason classification system. The Gleason score (range 2–10) is determined by a pathologist after microscopic analysis of the prostate tissue. The Gleason score is based on the sum of the two predominant microscopic gland architectures (the microscopic morphology and the glandular differentiation patterns), and a higher Gleason score is associated with more aggressive disease.[35] In addition to current methods that include PSA measurements and pre-treatment Gleason score to assess risk of aggressive cancer, miRNA profiles may provide diagnostic insight into the biological potential of insignificant versus aggressive CaP prior to therapy, as has been shown for other cancers,[36] in order to optimally risk-stratify patients for treatment.

As a proof-of-concept, two RNA samples from CaP specimens were studied in order to compare the expression profiles of 706 miRNAs in high Gleason grade versus in low Gleason grade CaP samples (samples CR562458 and CR562503, details described in Table S3). The RNA (0.8 μg) from each sample was analyzed by the Scano-miR assay, and a total of 163 miRNAs were determined to be differentially expressed as defined by 1.5 fold difference in signal; 109 miRNAs were downregulated and 54 miRNAs were upregulated (Table S5). Based on the identity of these 163 miRNAs, target genes were retrieved and further analyzed by disease ontology gene enrichment analysis. This search revealed 94 CaP related target genes.[7, 37] As validation, these CaP related genes were mapped to the 163 deregulated miRNAs, and 161 miRNAs (98.8%) were obtained (Figure S1, Table S5). As a result, the Scano-miR system was able to detect deregulated miRNAs that might be important for prostatic progression of high Gleason grade CaP with 98.8% accuracy (161/163). Moreover, we performed functional analysis to construct a function-gene-miRNA

network for 35 select miRNAs and 13 target genes that are involved in 5 hallmarks of CaP like regulation of apoptosis, epigenetic changes, cell migration and differentiation, and response to hormone stimuli (Figure 5, Table S6). The miRNA expression profiling of CaP by the Scano-miR assay has revealed deregulated miRNAs that might play critical roles in the regulation of CaP tumorigenesis. While some of these miRNAs may prove to be novel biomarkers, a larger clinical study is essential as a follow-up to this proof-of-concept one.

CONCLUSIONS

In conclusion, we have developed, characterized, and demonstrated the utility of a novel Scano-miR platform for miRNA detection. miRNAs are ideal targets for recognition using the Scano-miR platform due to their short length, the inability to directly PCR amplify miRNAs, and their presence at relatively low abundance in many biological samples. This simple yet powerful Scano-miR assay design allows rapid and simultaneous quantification of all known miRNA sequences with minimal modification and purification steps, which provides a highly attractive alternative to current conventional methods. The Scano-miR system is able to detect low abundance miRNA species due to the employment of a light scattering amplification protocol, which provides a lower LOD than fluorophore-based detection systems. The narrow melting transitions associated with duplexes formed from SNA-Au NP conjugates leads to the ability to differentiate perfectly complementary miRNA targets from ones with single base mismatches, and are responsible for the high specificity of this novel high throughput assay. The Scano-miR system has the potential to allow identification of novel miRNA targets for a wide variety of biomedical targets, especially those that pertain to cancer diagnostic applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The project described was supported by Award Number U54CA151880 from the National Cancer Institute, the Nanoscale Science and Engineering Initiative of the National Science Foundation under NSF Award Number EEC-0647560, and by the Prostate Cancer Foundation. Any opinions, findings and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect those of the Sponsors. C.S.T acknowledges the Howard Hughes Medical Institute for a Physician-Scientist Early Career Award and the Northwestern Memorial Foundation Dixon Translational Research Grant Initiative Award number SP0010333. A.H.A acknowledges the King Abdullah Scholarships Program funded by the Ministry of Higher Education of Saudi Arabia for doctoral scholarship support.

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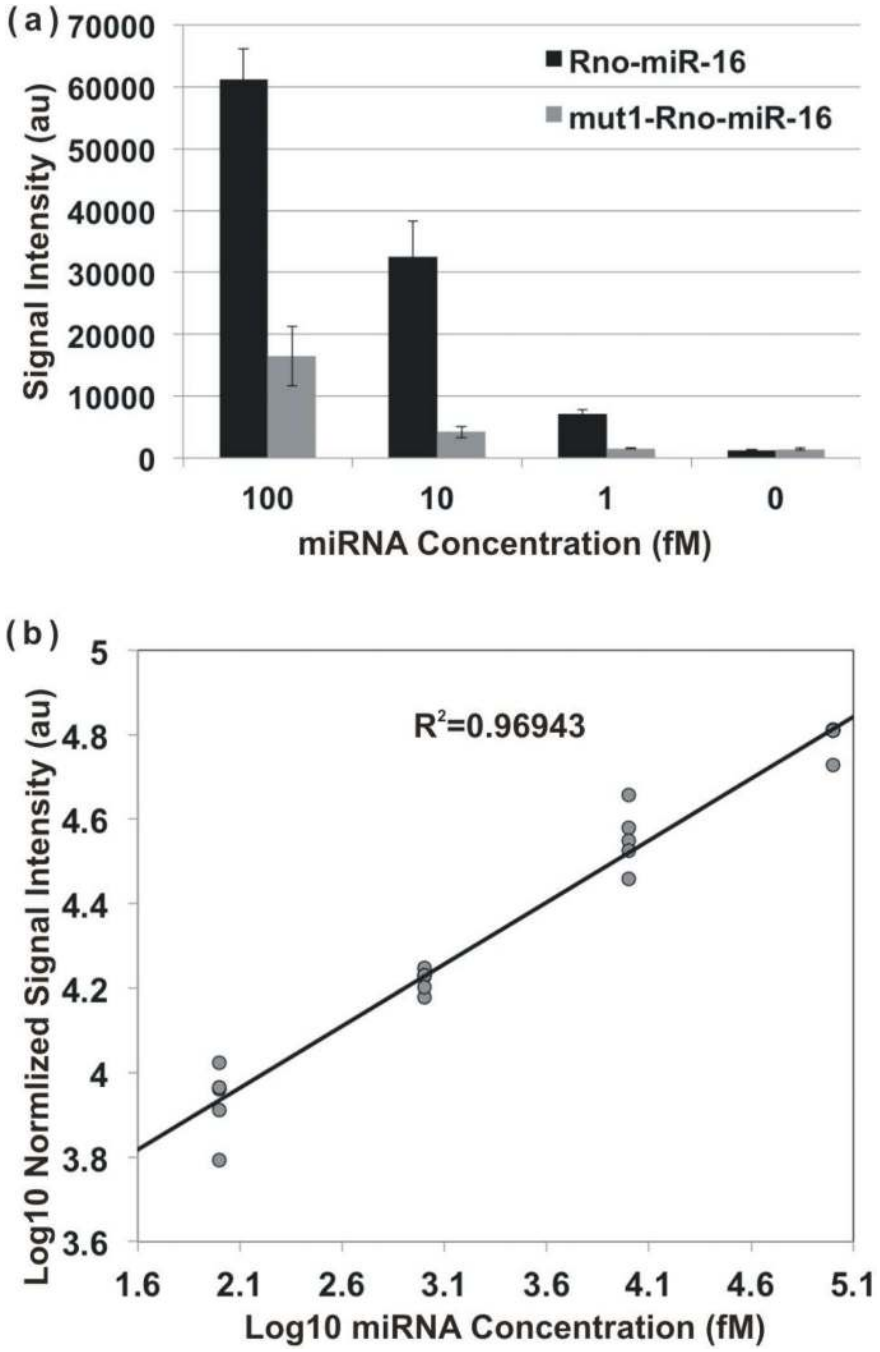


Figure 1.

(a) Synthetic miR-16 was added into denatured human serum at different concentrations and analyzed using the Scano-miR platform. Signal intensities generated from both perfectly matched capture probe sequences (Rno-miR-16) and capture probe with a single nucleotide mismatch (mut1-Rno-miR-16) were plotted. (b) Comparative hybridization of five synthetic miRNAs (miRNA-20a, −143, −143*, −205, and −210) in a Latin square design was performed to complete a five by five matrix (Table S5, see Experimental Sections for experimental details). Plotting signal intensity versus miRNA concentrations generated R^2=0.97.

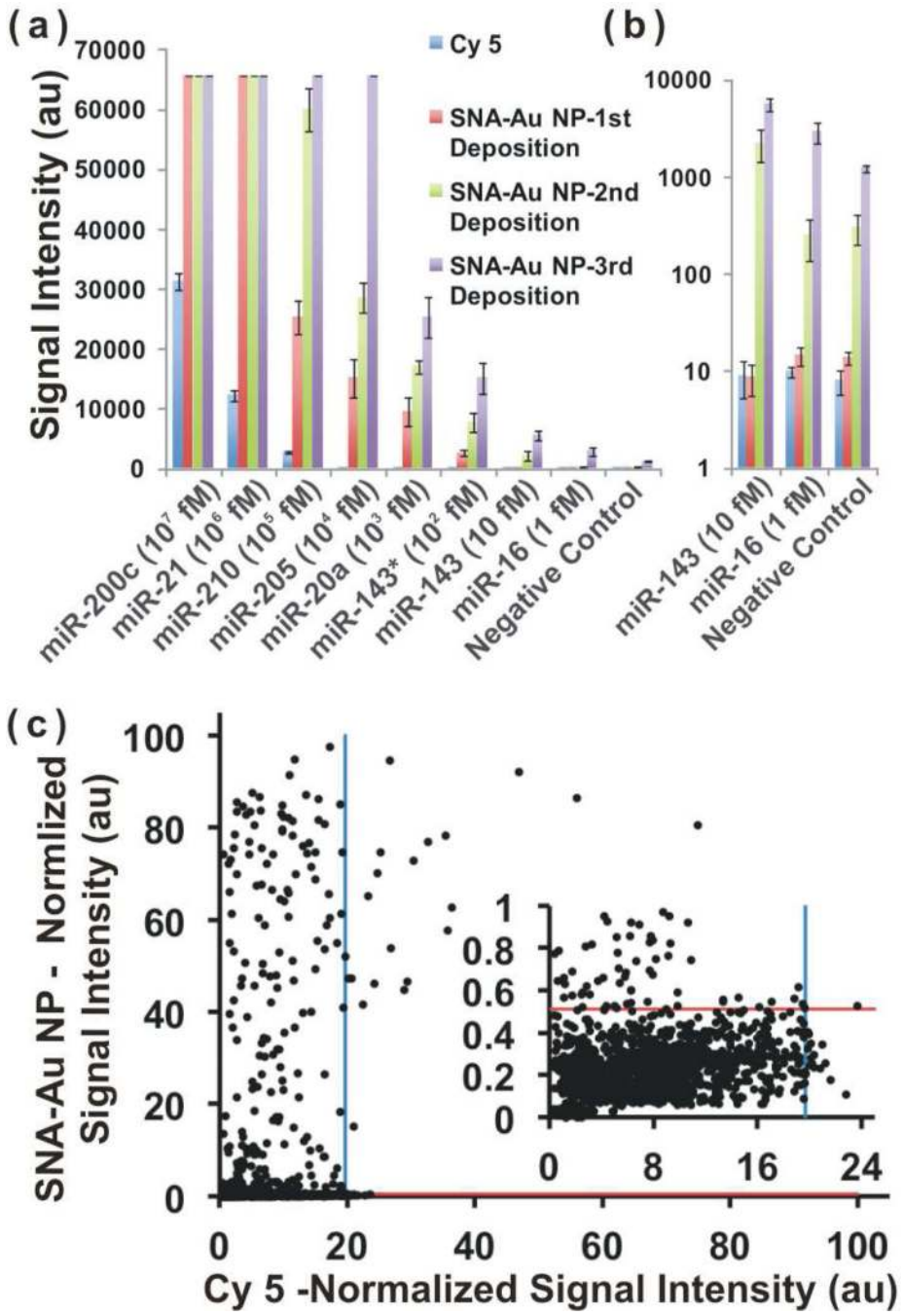


Figure 2.

(a) Profile of synthetic miRNAs at different concentrations and (c) Profile of total miRNAs from PC-3 using the Scano-miR assay was plotted and compared to conventional Cy5-based miRNA-array. (b) The low-end detection of (a) is expanded. (c) Inset: scale for the background is expanded.

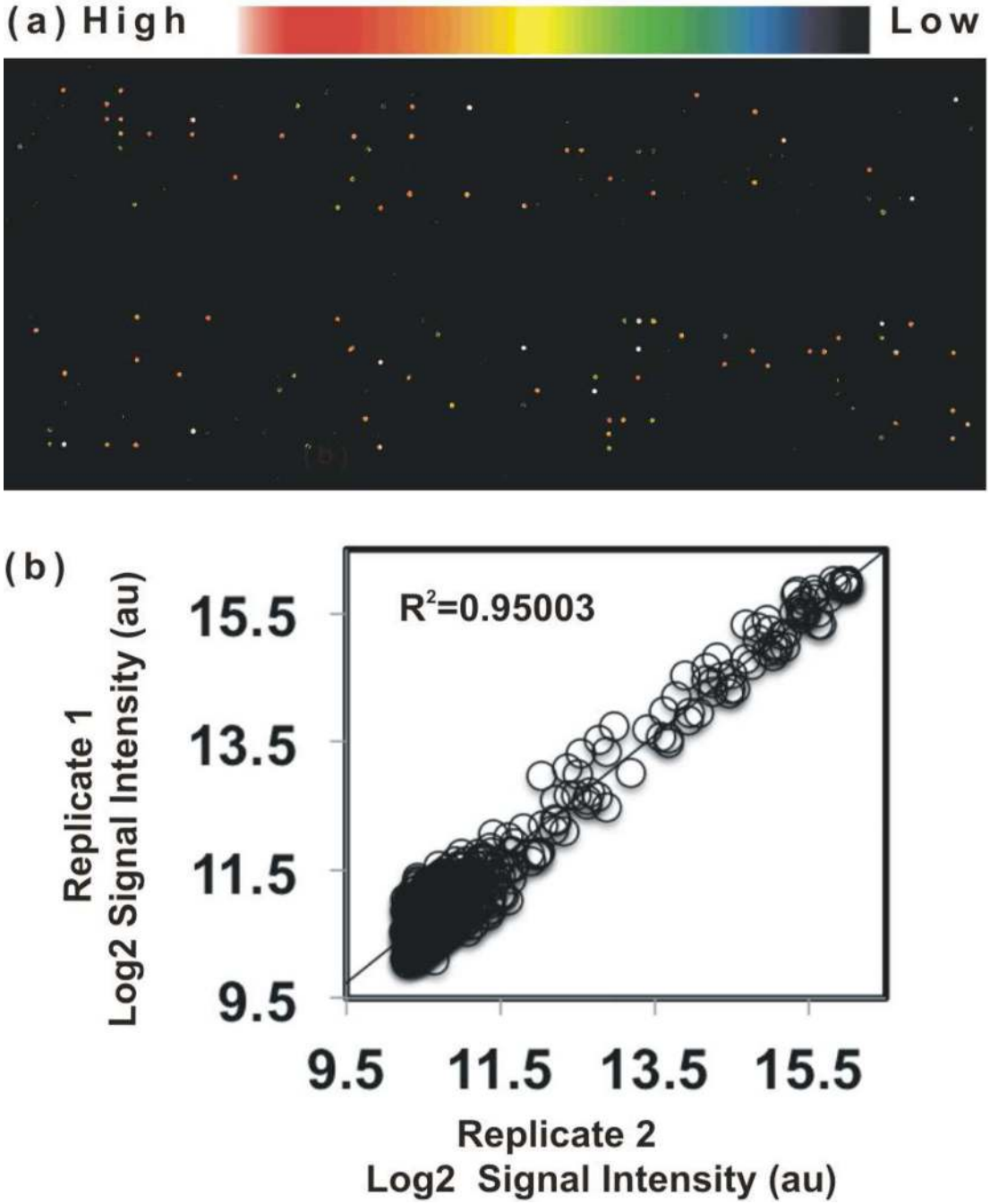


Figure 3.

(a) Detection of human serum miRNAs (from donor 277, Table S6) using the Scano-miR system was performed after hybridizing recovered miRNAs onto a high-density miRNA-array and recorded using the Tecan scanner, where the gray scale image was converted into color using GenePix Pro 6 software (Molecular Devices). Each spot corresponds to a single miRNA and the intensity of the color corresponds to the miRNA concentrations. (b) Biological replicates were analyzed at two different time points using the Scano-miR system. The signal intensity values, calculated using GenePix Pro 6 software, are plotted to calculate the correlation coefficient (R^2=0.95). The Log2 of the signal intensities are plotted.

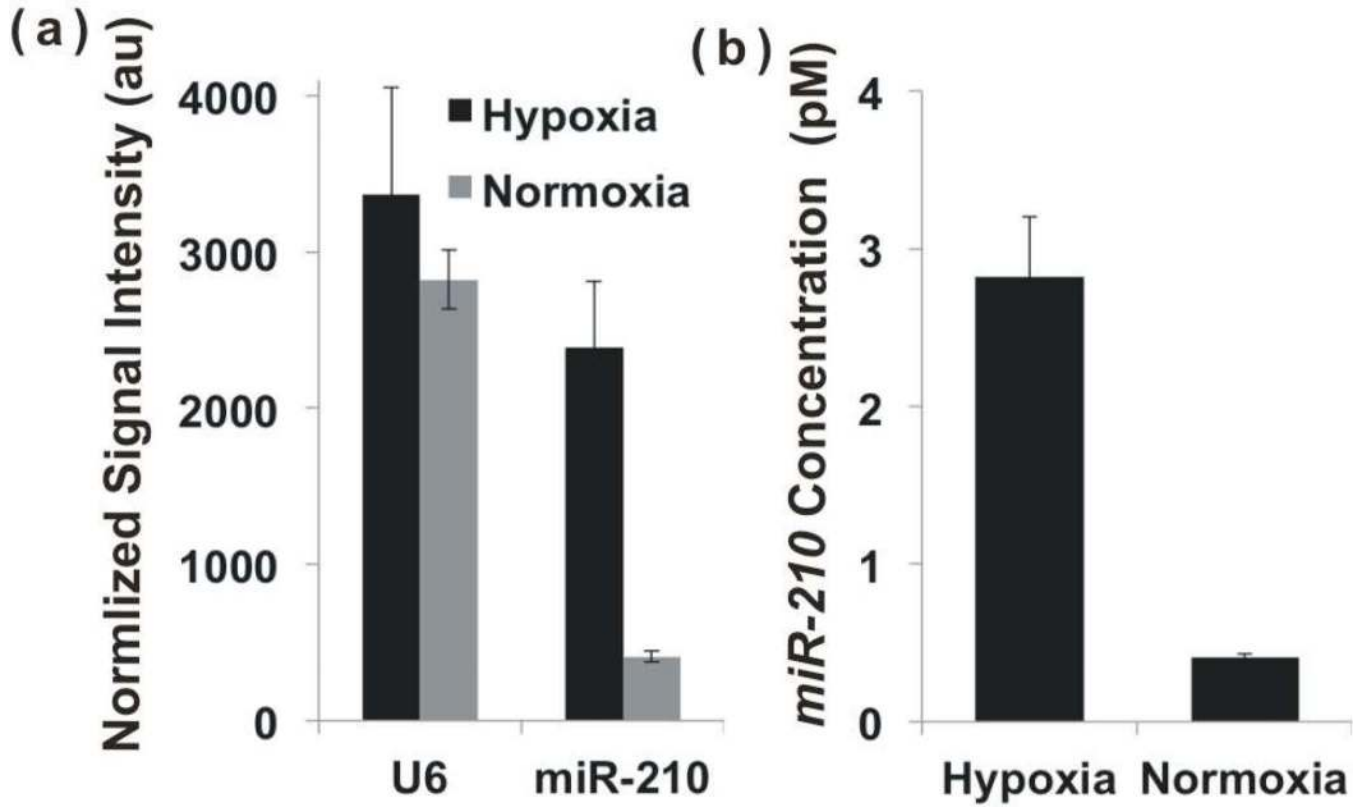


Figure 4.

Determining the change in expression level of miR-210 in PC-3 cells in normoxia and hypoxia conditions using (a) the Scano-miR assay and compare the results using (b) qRT-PCR. a) U6 serves as an endogenous loading control. b) Concentrations on y-axis were calculated based on known spiked-in miR-210 concentrations.

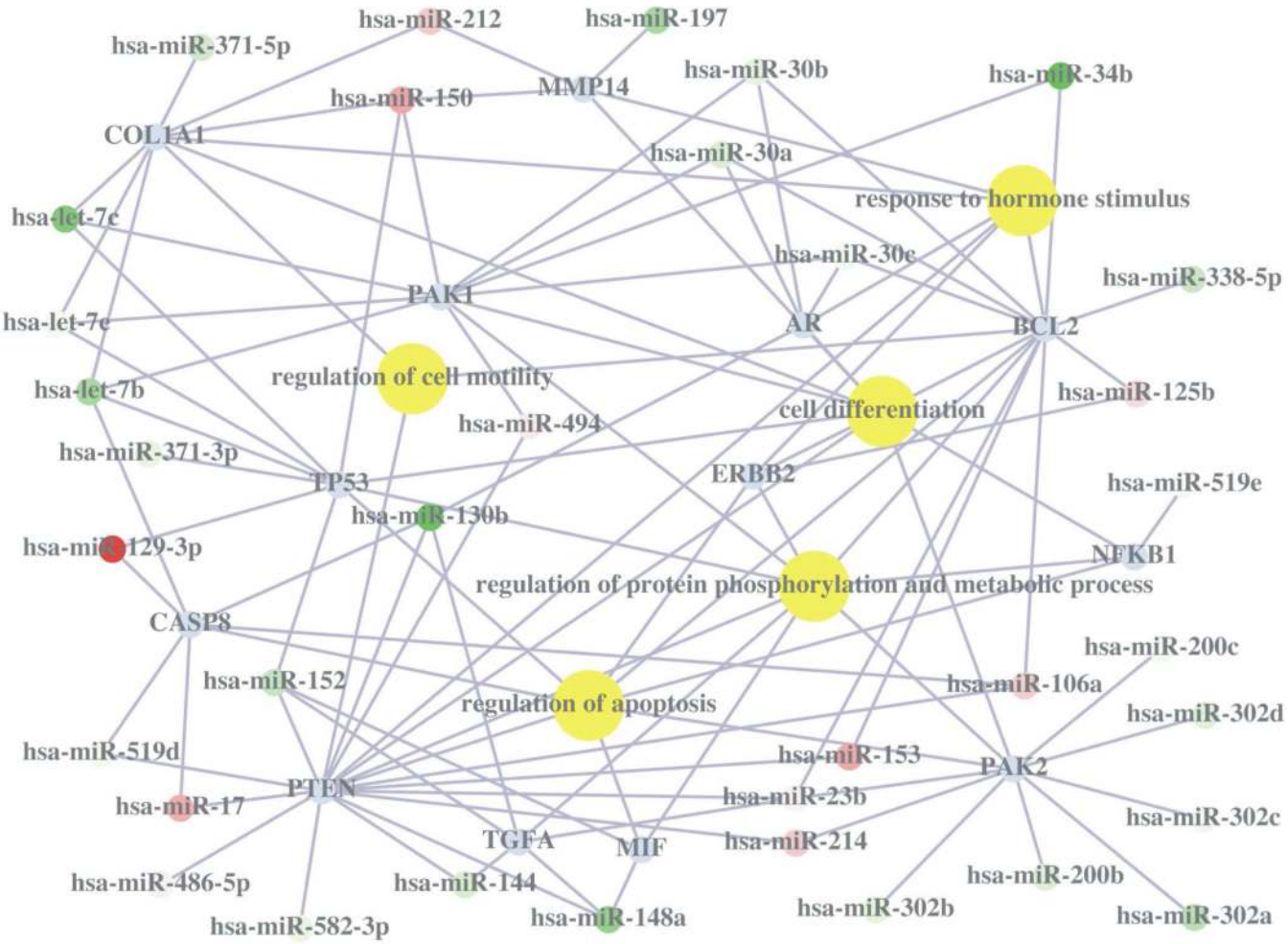
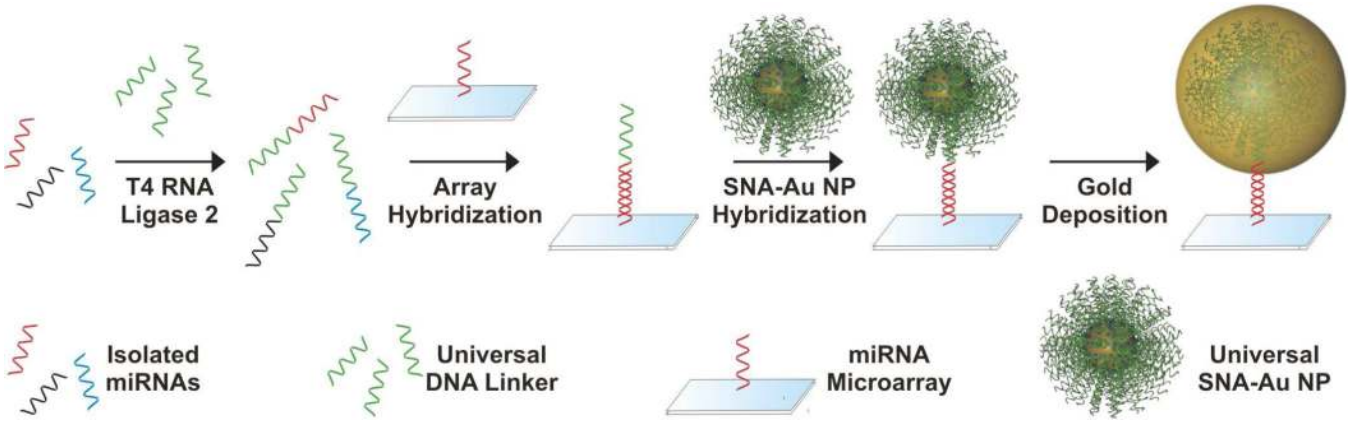


Figure 5.

A function-gene-miRNA network of 35 deregulated miRNAs was analyzed that target 13 prostate-cancer related genes involved in 5 Gene Ontology terms. A large yellow node in the network represents a single Gene Ontology term, while the small blue nodes stand for genes. Green and red nodes stand for miRNAs with negative and positive logR ratios, respectively. The saturation is proportional to the absolute value of logR ratio.



Scheme 1.

Scheme for the scanometric array-based multiplexed detection of miRNA species (Scano-miR). Isolated miRNAs are enzymatically ligated to a universal linker followed by hybridization onto miRNA microarray. After washing away unbound miRNA species, universal SNA-functionalized gold nanoparticle conjugates (SNA-Au NPs) are subsequently hybridized to detect captured miRNA targets. Next, signal intensity is amplified by depositing gold with gold enhancing solution (1:1 (v:v) mixture of 1 mM $HAuCl_4$ and 10 mM NH_2OH) for 5 min and imaged with a Scanner (LS Reloaded, Tecan, Salzburg, Austria).