Detection of microRNAs Expression Dynamics Using LNA/DNA Nanobiosensor

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Abstract:

The investigation of complex biological processes requires effective tools for probing the spatiotemporal dynamics of individual cells. Single cell gene expression analysis, such as RNA in situ hybridization and single cell PCR, has been demonstrated in various biological applications [1-3]. However existing techniques require cell lysis or fixation. The dynamic information and spatiotemporal regulation of the biological process cannot be obtained with these methods. Real-time gene expression analysis in living cells remains an outstanding challenge in the field. Here, we described a single cell gene expression analysis method in living mammalian cells using a locked nucleic acid/DNA (LNA/DNA) nanobiosensor. This LNA/DNA nanobiosensor consists of a fluorophore –labeled detecting strand and a quenching strand. The fluorophore-labeled LNA probe is designed to hybridize with the target microRNA (miRNA) specifically and displace from the quenching strand, allowing the fluorophore to fluorescence. Large-scale single cell dynamic gene expression monitoring can be performed using time-lapse microscopy to study spatiotemporal distribution and heterogeneity in gene expression. Multiplex detection of miRNAs can be achieved using different fluorophores labeled LNA/DNA nanobiosensor. This LNA/DNA protocol is fast, generally applicable and easily accessible.

Keywords: microRNA, LNA/DNA, nanobiosensor, molecular probe

Introduction

microRNAs (miRNAs) are small, non-coding RNA molecules with the length of 21-23 nucleotides that have been shown to have critical roles in various dynamic biological processes, including proliferation, differentiation, apoptosis, and development. It has been demonstrated that miRNAs regulate biological activities by mediating translational repression through targeting message RNA (mRNA). [4-9] In the last several decades, increasing evidence has shown that miRNAs are involved and regulate gene expressions in the development of human diseases, including angiogenesis,[10-12] cancer,[13, 14] cardiovascular diseases, [15] and infectious diseases.[16] Recent studies have shown that several miRNAs were directly functioning as oncogenes or tumor suppressors in human cancers, including breast cancer, lung, brain, liver, colon cancer and leukemia. [17-19] Aberrantly expressed miRNAs could modulate the epigenetic status of the genome, leading to the changes of cancer-associated genes that consequently affect on initiation, progression, metastasis of human cancers.[20] Thus, it is essential to understand miRNAs regulated pathways to identify potential new biomarkers for anticancer therapy. Since miRNAs can target different mRNAs to repress gene expression, it is critical to detect and visualize miRNAs expression dynamics in various biological processes. The current challenge in the field of miRNA biology is to obtain spatiotemporal miRNA expression information under pathophysiological conditions, which are required to identify multimodal miRNAs, characterize expression variability, and enable accurate investigation of cancer. However, current miRNAs detection techniques such as microarrays, Northern blot and RT-PCR are limited for dynamic miRNAs expression and visualization due to the requirements of fixation or cell lysis. Thus, the miRNAs expression dynamics at the single cell level are

limited. In recent years, we have developed various molecular probes to detect mRNA, miRNA and protein in mammalian cells [21-27]. A double-stranded locked nucleic acid (LNA) / DNA nanobiosensor has been demonstrated to detect spatiotemporal dynamics of miRNAs during collective cell migration, microvascular self-organization, *in vivo* tissue morphogenesis and angiogenesis, and stem cell differentiation.

Here, we discussed the basic principles and design strategies of LNA/DNA nanobiosensor, including working mechanisms, and optimization. We next introduced the characterization of the LNA/DNA probe by comparing it with a random negative control. Taking miR-155 and miR-21 as examples, we described multiplex detection of miRNAs in mammalian cells by using two different miRNA LNA/DNA probes. Lastly, we discussed how to detect exogenously and endogenously expressed miRNAs in MDA-MB-231 breast cancer cells. Together with advancements in design strategies and performance, these LNA/DNA nanobiosensors will provide opportunities for the application of discovering potential biomarkers for therapeutic strategies.

2 Materials

2.1 Materials

Cell Culture Material

1. MDA-MB-231 cells (ATCC)
2. Fetal Bovine Serum (FBS) (ThermoFisher Scientific, Gibco™, Cat no. 26140079)
3. Penicillin-Streptomycin-Glutamine (100X) (ThermoFisher Scientific, Gibco™, Cat no. 10378016)

4. RPMI 1640 Medium (ThermoFisher Scientific, Gibco™, Cat no. 11875119)
5. Gentamycin solution (Sigma Aldrich, 50 mg/mL, Cat no. G1397)
6. Trypsin-EDTA (0.25%), phenol red (ThermoFisher Scientific, Gibco™, Cat no. 25200056)

Transfection

1. Lipofectamine™ 2000 Transfection Reagent (Invitrogen™, Cat no. 11668019)
2. optiMEM I Reduced Serum Media (Gibco™, Cat no. 31985062)
3. Lipofectamine RNAiMax transfection reagent (Invitrogen™, Cat no. 13778030)
4. miRNA-155 mimics 5′-UUAAU GCUAA UUGUG AUAG GGGU-3′ (Assay ID. MC28440;Cat no. 4464066, Thermo Fisher Scientific)
5. miRNA-21 mimics: 5′-UAGCU UAUC AGACU GAUG UUGA-3′ (Assay ID. MC10206; Cat no. 4464066, Thermo Fisher Scientific)
6. Negative control mimics (mirVana™ miRNA Mimic, Negative Control #1, Cat no. 4464058, Thermo Fisher Scientific)
7. Negative control inhibitor (mirVana™ miRNA Inhibitor, Negative Control #1, Cat no. 4464079, Thermo Fisher Scientific)
8. miRNA-155 inhibitor, mirVana® miRNA inhibitor (Assay ID. MC28440; Cat no. 4464084, Thermo Fisher Scientific)
9. miR-21 inhibitor, mirVana® miRNA inhibitor (Assay ID. MC10206; Cat no. 4464084, Thermo Fisher Scientific)

Reverse transcription and RT-PCR

1. TaqMan MicroRNA Cells-to-CT Kit (Aplied Biosystems™, Cat no. 4391848)

2. TaqMan MicroRNA Reverse Transcription Kit (Aplied Biosystems™, Cat no. 4366596)
3. TaqMan MiRNA Assays (Applied Biosystems™, Cat no. 4440888)

2. 2 Equipment list

1. Real-time PCR instruments (BioRad Real Time PCR system)
2. T100 Thermal Cycler (BioRad)
3. Fluorescence Microplate Reader (BioTek, Synergy 2)

3. Methods

3.1 Design of LNA/DNA probe for miRNA detection

The LNA/DNA molecular probe for miRNA detection can be designed following the procedure described below. Briefly, the LNA/DNA molecular probe is a double-stranded LNA/DNA oligonucleotides, consisting of a detecting strand (Strand A, or donor) and a quenching strand (strand B, or quencher). Strand A is typically 20-30 nucleotides long, which strand B is normally half the length of strand A. Strand A and strand B will bind to each other due to hybridization. A fluorophore is labeled to the 5′ or 3′ of strand A, while a corresponding quenching fluorophore will be labeled to 3′ or 5′ of strand B. Upon hybridization, the fluorophore will be quenched. Then the LNA/DNA probe are transfected into mammalian cells using transfection reagents. In the absence of target miRNA sequence, the double-stranded LNA/DNA probe will not separate and no fluorescence signal will be detected. In the presence of target miRNA sequence, the strand B (or quencher) will be displaced by target miRNA sequence, thus activating the fluorophore on strand A to fluorescence. Thus this LNA/DNA probe can be utilized to detect and monitor miRNA dynamics in living cells.

Design procedure:

1. Identify the target of interest of miRNAs and find the relative sequences from miRBase (https://www.mirbase.org/). Here, we will take miR-21 and miR-155 as examples. We need to find the miR-21 and miR-155 sequences from miRBase first. It is noted that different species have different sequences for the same miRNAs. Thus, it is important to identify the species first. For miRNA detection in human mammalian cells, we need to find miRNA sequences for homo sapiens (has), has- miR-155 and has-miR-21. 5p-arm or 3p-arm mature miRNAs could be chosen. Here we choose 5p-arm mature miRNAs for miR-21 and miR-155 detection.

- has-miR-21-5p (5′-3′): UAGCUUAUCAGACUGAUGUUGA
- hsa-miR-155-5p (5′-3′): UUAAUGCUAAUCGUGAUAGGGGUU

2. Write down the relative DNA sequences for miR-21 and miR-155.

- has-miR-21-5p (5′-3′): TAGCTTATCAGACTGATGTTGA
- hsa-miR-155-5p (5′-3′): TTAAT GCTAA TCGTG ATAGG GGTT

3. Design detecting strands for miR-21 and miR-155. The detection regions are DNA sequences that are complementary to miRNA sequences.

- Detection strand of miR-21 (5′-3′): TCAAC ATCAG TCTGA TAAGC TA
- Detection strand of miR-155 (5′-3′): AACCC CTATC ACGAT TAGCA TTAA

4. Design LNA/DNA sequence for strand A.

For both miR-21 and miR-155 detection, we use alternating LNA/DNA monomers. The following are strand A sequences for miR-21 and miR-155 probe, respectively, "+" indicate LNA monomers.

- Strand A for miR-21 (5′-3′): +TC+AA+C A+TC+AG+TC+TG+A T+AA+GC+TA
- Strand A for miR-155 (5′-3′): +AA+CC+CC+TA+TC+AC+GA+TT+AG+CA+TT+AA

The next step is adding fluorophore to each detecting strand. There is no limitation for choosing different fluorophores, here we choose FAM for miR-21 detection, and TexasRed for miR-155 detection. (*See* **Note 1**.)

- Strand A for miR-21 (5′-3′): FAM-+TC+AA+CA+TC+AG+TC+TG+AT+AA+GC+TA
- Strand A for miR-155 (5′-3′): TexasRed-+AA+CC+CC+TA+TC+AC+GA+TT+AG+CA+TT+AA

These customized two strands can be synthesized by IDT.

5. Design strand B for miR-21 and miR-155

The quenching sequences can thus be designed based on the detecting strand (strand A). Strand B is half-length of strand A.

- Strand B for miR-21 (5′-3′): +TA+GC+TT+AT+CA+GA
- Strand B for miR-155 (5′-3′): +TT+AA+TG+CT+AA+TC

Add Iowa Black dark quenchers modifications for strand B sequences. Iowa Black FQ is used with fluorescein and other fluorescent dyes that emit in the green to pink spectral range. Iowa Black RQ is ideal for use with Texas Red and other dyes that emit in the red spectral range.

- Modified strand B for miR-21 (5′-3′): +TA+GC+TT+AT+CA+GA-FQ
- Modified strand B for miR-155 (5′-3′): +TT+AA+TG+CT+AA+TC-RQ

These customized two strands can be synthesized by IDT.

6. Design random LNA/DNA probe for negative control

A random probe with completely random sequence is designed for negative control. The random sequence could be generated using IDT or other web servers. Here we used IDT web server to generate a random sequence.

- Random sequence (5′-3′): +AG+AG+GG+CG+CT+TA+AA+AT+TG+GG+AG

The LNA/DNA probe for random control can be designed.

- Strand A (5′-3′): FAM-+AG+AG+GG+CG+CT+TA+AA+AT+TG+GG+AG
- Strand B (5′-3′): +CT+CC+CA+AT+TT+T – FQ

These customized two strands can be synthesized by IDT.

3.2 Preparation of LNA/DNA probe for miRNA detection

First, prepare the strand A and Strand B in 1x Trisethylenediaminetetra acetic acid (EDTA) buffer (pH 8.0) at a concentration of 100 nM. Next, mix strand A and strand B at the ratio of 1:2 and incubate at 95 ℃ for 5 minutes in a pre-heated heat block, then slowly cool down to room temperature over a course of 2-3 hours, **Figure** 1. This hybridization step is to make sure the fluorescence signal on strand A is fully quenched before transfecting into cells. (*See* **Note 2**).

It is noted that each LNA/DNA probe needs to be characterized before use. *Ex vivo* characterization were performed to acquire the optimal mixing ratio of strand A and strand B,

Figure 2. The strand B concentrations were adjusted and binding efficiency were evaluated by measured the fluorescence intensity of the mixture solution. The strand A concentration was set to 100 nM. As shown in **Figure 2A**, the fluorescence intensity decreased as the quencher-to-donor ratio increases. It is noted that for different LNA/DNA nanobiosensors, the optimal quencher-to-donor ratio may be slightly different due to the different length of detecting strand. The detectable range of target miRNAs were further characterized by setting the LNA/DNA probe concentration at 100 nM. As showing in **Figure 2B**, this LNA/DNA probe is capable of detecting a large dynamic range for quantifying target concentrations ranging from 1 nM to 10 mM.

3.3. LNA/DNA probe transfection

The hybridized LNA/DNA probe can be transfected into mammalian cells using Lipofectamine 2000 transfection reagent (Life Technologies). Here we took 12-well plates as an example.

1. Cells were seeded at the concentration of 1 x 10^5 cells per well with 800 μL culture medium.
2. Once the cells reached 70-80% confluency, transfections will be performed. For each well, the following procedures are followed. For multiple wells of cells, multiply the amount of solution by the number of wells.
    a. Use a 1.5 mL centrifuge tube, dilute LNA/DNA probe in 150 μL Opti-MEM I reduced serum media at a concentration of 25 nM.
    b. Use another 1.5 mL centrifuge tube, dilute 1 μL of lipofectamine 2000 in 150 μ Opti-MEM I reduced serum media.

c. Incubate for 5 minutes at room temperature.

d. Mix DNA samples from step (a) and diluted lipofectamine and incubate for 15 minutes at room temperature.

e. Add the mixed solution to the cells.

f. Incubate for 6-12 hours, replace transfection solution with fresh medium.

For miRNA mimics or inhibitors, transfections were performed using Lipofectamine RNAiMax transfection reagent according to manufacturer's protocol. The concentrations of miRNA mimics, inhibitors, and negative control are set to 25 nM.

3.4 Reverse Transcription and RT-PCR

For miRNA quantification, the TaqMan MicroRNA RT Kit and TaqMan MiRNA Assays (Life Technologies) were used to generate cDNA and to quantitatively detect mature miRNAs, respectively. In addition, the TaqMan MicroRNA Cells-to-CT Kit (Life Technologies) was used to quantify the levels of mature miRNAs in cells. Cells were cultured in 96-well plates with a concentration of 5000 cells per well.

1. Cell lysis

    a. The cells were rinsed three times with cold 1 x phosphate-buffered saline (PBS) and aspirated.

        i. Estimate the number of cells, make sure the cell number is about 1000-10000 cells per well.

        ii. Aspirate and discard the culture medium.

        iii. Add 50 μL cold 1x PBS to each well

iv. Aspirate PBS from the well. Remove as much PBS as possible.

b. Add 50 μL of lysis solution and mix 5 times.

i. Add 50 μL of lysis solution to each sample.

ii. Mix the lysis reaction by pipetting up and down 5 times.

c. Incubate 8 minutes at room temperature. (19-25°C). During this incubation, cells were lysed, and RNA was released into a lysis solution containing reagents to inactivate endogenous RNases.

d. Add 5 μL stop solution and mix 5 times. In this step, a stop solution is used to inactivate the lysis reagents to avoid inhibition of reverse transcription.

e. Incubate for 2 minutes at room temperature.

2. Reverse Transcription

a. Program thermal cycle for reverse transcription.

b. Assemble RT master mix and distribute to reaction, **Table 1**.

c. Mix Master mix solution gently or centrifuge briefly.

d. Distribute RT master mix to PCR tubes.

e. Add 3 μL 5x RT primers.

f. Add 5 μL cell lysate soluton.

g. Run the RT thermal cycle program.

3. Real-Time PCR

a. Program the real-time PCR.

b. Assemble PCR Cocktail and aliquot into reaction.

i. PCR cocktail setup: 10 μL of Taqman Master Mix (2x), 1 μL of Taqman microRNA assay (20x), and 7.67 μL of water. Total 18.67 μL.

ii. Distribute PCR cocktail into PCR tubes.

c. Add RT product. The reverse transcription product (1.33 μL) were added to PCR cocktail mix.

d. Run the PCRs in real-time PCR instruments (BioRad Real Time PCR system). In this step, the RT product was amplified by real-time PCR using TaqMan Universal PCR Master Mix and TaqMan MicroRNA Assay.

f. The data was collected and analyzed after the quantitative PCR was done.

4. Data analysis

a. C_T values for all samples were obtained in order to compare relative concentration.

b. ΔC_T is a relative gene expression level which was calculated by subtracting the C_T value of miR-155 from the C_T value of the same sample.

c. For comparing fold change, this number was converted using $\log(2^{-(\Delta CT)})$.

3.5 Detection of endogenously and exogenously expressed miRNAs

TheLNA/DNA nanobiosensor is capable of detecting endogenously and exogenously expressed miRNA in mammalian cells. For detecting of endogenously expressed miRNAs, a negative random control probe was used for comparison.

a. Cells were seeded in 24-well plates at a density of 200 cells per mm^2 with a volume of 400 μL. Three groups of experiments were designed, Control, miR-21, and miR-155.
b. Once the cells reach 70-80% confluency, cells were transfected with negative control, miR-21, miR-155 LNA/DNA probes, respectively.
c. After transfection, cells are ready for imaging. (*See* **Note 3**)
d. The mean intensity of each sample was then acquired and compared, **Figure 3B**.

For exogenously expressed miRNAs detection, miRNA expression levels were first modulated using miRNA mimics or inhibitors.

a. The miRNA expression level in mammalian cells (MDA-MB-231) were first analyzed using RT-PCR in the presence of miRNA mimics or inhibitors. This step is to make sure the mimics or inhibitors can change the expression of miRNAs.
b. Once confirmed, the cells (MDA-MB-231) were transected with negative control, miR-21, and miR-155 LNA/DNA probes, respectively.
c. After transfection, images were taken and the fluorescent intensity of each cell can be acquired.
d. The mean intensity of each sample can thus be compared.

3.6 Detection of miRNAs expression dynamics in living cells

The LNA/DNA probes has the advantage that it is capable of detecting and tracking the dynamic expression profile of miRNAs in mammalian cells, allowing researchers to study the dynamic process of biological questions. For example, for dynamics tracking miR-21 expression

profile, cells were transfected with miRNA-21 LNA/DNA probe. The miR-21 expression can be quantified by measuring the fluorescence intensity of each cell over a course of 7 days. (*See* **Note 4**)

3.7 Multiplex detection

The LNA/DNA molecular probes can be designed for multiplex detection of miRNAs, mRNAs, and proteins in mammalian cells. (*See* **Note 5**) Theoretically, there is no limitation of the numbers of genes this LNA/DNA probes can detect. However, due to the limitation of fluorophores that optical microscope can detect, the excitation/emission wavelength of different fluorophores should not be too close to avoid bleed through. Thus, typically, this LNA/DNA probes can detect up to three genes. The following steps introduced the procedure of designing multiplex LNA/DNA probes for miR-21 and miR-155 detection.

1. The miR-21 and miR-155 LNA/DNA probes were first designed following the procedure mentioned in *Section* 3.1. A random probe was designed as a negative control. It is important that different probes should have fluorophores that have different excitation/emission wavelengths that could be separated.
2. Characterize miR-21 and miR-155 probes by measuring exogenously expressed miR-21 and miR-155 in cells. First, miR-21 and miR-155 expression levels can be modulated using miR-21/miR-155 mimics or inhibitors. The green fluorescence intensity (indicate miR-21 expression) and red fluorescence intensity (indicate miR-155) expression were measured and analyzed by comparing mean fluorescence intensity. It is expected that in the presence of miR-21 mimics, miR-21 expression is increased while there is no change

for miR-155 expression. With the presence of miR-155 mimics, miR-155 expression is increased while miR-21 expression has no change. With miRNA inhibitors, the respective miRNA expression is decreased, with decreased fluorescence intensity. (*See* **Note 6**).

3. For the random control probe, the fluorescence intensity has no change in the presence of miR-21/miR-155 mimics or inhibitors.
4. After optimization, both miR-155 and miR-21 LNA/DNA probes can be utilized for multiplex detection of miR-155 and miR-21 in mammalian cells.

4. Note

1. For single miRNA detection, there is no limitation to choose from different fluorophores that can be used to label LNA/DNA probes. For multiplex detection, it is critical to make sure the different fluorophores have different excitation/emission wavelengths that could be separated.
2. It is important to make sure the fluorescence signal of fluorophore was fully quenched by quencher. This can be validated using a fluorescence microplate reader (BioTek, Synergy 2). There should be minimum fluorescence signal after hybridization, if not, the ratio of strand A and stand B should be adjusted.
3. To quantify miRNA expression levels in mammalian cells, the mean fluorescence intensity of each cell was measured. For comparing miRNAs expression in a group of cells, mean fluorescence intensity of each cell was measured and compared. The

miRNAs expression can be calculated as: (mean fluorescence intensity of miRNA probe – mean fluorescence intensity of random probe).

4. For tracking miRNA expression dynamics, the fluorescence intensity of miR-21 or miR-155 LNA/DNA probes can be detected as early as 4 hrs after transfection. The signal is stable for up to 10 days.
5. This LNA/DNA probes can be designed for multiplex detection of miRNA, mRNA, and protein expression in mammalian cells.[21, 24, 25, 28] For mRNA detection, the LNA probe was designed to be complementary to a loop region of the target mRNA structure. The secondary structures, binding affinity, and specificity were optimized using the mFold server and NCBI Basic Local Alignment Search Tool (BLAST) database. [22, 23, 29, 30] For protein expression, an aptamer LNA/DNA probe will be utilized. The aptamers are nucleic acid sequences identified using an iterative enrichment technique. Oligos with high affinity and specificity to the target protein or cell are isolated from a large random sequence pool with multiple rounds of selection. The aptamer sequences for protein detection can be acquired from literature.
6. If there are some non-specific binding during multiplex detection, the miRNA probes can be optimized by adding 2-3 random oligonucleotides on the 5′ or on the 3′. The optimal length for miRNA detection is 20~30 nts. By adding several nucleotides, the LNA/DNA probe will avoid non-specific binding and will enhance the stability and specificity of miRNA probes.

5. References

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Figure Captions

Figure 1. Illustration of working principle of LNA/DNA nanobiosensor.

Figure 2. Characterization of LNA/DNA nanobiosensor.

Table Captions

Table 1. Assembly of RT master mix solution

Running Head: LNA/DNA Nanobiosensor for microRNA detection

Tables

Table 1. Assembly of RT master mix solution

10X RT buffer	1.5 μL
dNTP mix	0.15 μL
RNase Inhibitor	0.19 μL
MultiScribe TM RT	1 μL
Water	4.16 μL
Total (Master mix solution)	7 μL