Chapter 9

Tiny Molecular Beacons for in vivo mRNA Detection

Diana P. Bratu, Irina E. Catrina, and Salvatore A.E. Marras

Abstract

The molecular beacon technology is an established approach for visualizing native mRNAs in living cells. These probes need to efficiently hybridize to accessible RNA regions in order to spatially and temporally resolve the dynamic steps of the RNA life cycle. A refined method using two computer algorithms, mfold and RNAstructure, is described for choosing shorter, more abundant target regions for molecular beacon binding. The probes are redesigned as small hairpins and are synthesized from 2‘-O-methyl RNA/LNA chimeric nucleic acids. These tiny molecular beacons are stable in the cellular environment and have a high affinity for binding to target RNAs. The user-friendly synthesis protocol and ability to couple to a variety of fluorophores make tiny molecular beacons the optimal technology to detect less abundant, highly structured RNAs, as well as small RNAs, such as microRNAs. As an example, tiny chimeric molecular beacons were designed to target regions of oskar mRNA, microinjected into living Drosophila melanogaster oocytes and imaged via spinning disc confocal microscopy.

Key words: Molecular beacons, 2‘-O-methyl RNA, LNA, Fluorescence live cell imaging, mRNA localization, mRNA transport

1. Introduction

Significant advances have been made over the last decade for visualizing and tracking of individual mRNAs within distinct mRNP complexes in subcellular space in real time (1, 2). Such approaches have provided a live glimpse of specific biological processes that have remained opaque thus far (3). Among them is the molecular beacon technology, which involves probes that fluoresce only upon hybridizing to specific complementary mRNA sequences. Introduced as an innovative and general approach, molecular beacons have been used in a variety of cell types, detecting mRNA at various levels of expression (4). Coupled with fast 3D imaging over time, molecular beacon technology has enabled highly time-resolved studies of RNA–protein interactions in vivo.
providing details of the dynamically orchestrated relationship between an mRNA and various proteins involved in its transport.

Molecular beacons are internally quenched hairpin-shaped oligonucleotide probes that fluoresce upon hybridization with their target sequence (Fig. 1a) (5, 6). Target-bound probes fluoresce as much as 100 times more intensely than background levels of unbound probes, enabling highly sensitive detection (Fig. 3b). Molecular beacons are designed using the *mfold* RNA-folding software (7, 8). The most stable predicted structure must reflect a hairpin, ensuring that the fluorophore and quencher are within minimum distance from each other (9). Due to their stem, the recognition of targets by molecular beacons is so specific that if the target differs even by a single nucleotide, the probe does not bind to it. Molecular beacons can be synthesized from modified nucleic acids (i.e., 2'-O-methyl-ribonucleic acids, LNA – locked nucleic acids) (Fig. 1b) to ensure greater stability of the probe and the probe–target hybrid, and be labeled with a wide variety of fluorophores and respective quenchers (Tables 1 and 2), enabling detection of multiple targets simultaneously (6).

For live cell imaging, molecular beacons have been synthesized from 2'-O-methyl ribonucleotides, thus assuring resistance to cellular nucleases, and hybrid stability with RNA, thus evading degradation by Ribonuclease H (9).

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**Fig. 1.** (a) Scheme of molecular beacon operation. In the absence of a complementary target, these molecules are nonfluorescent, because the stem hybrid keeps the fluorophore in close proximity to the quencher. In the presence of target, the probe sequence in the loop hybridizes to the target, forming a rigid double helix inducing a conformational reorganization that separates the quencher from the fluorophore leading to an increase in fluorescence. (b) Molecular structures of LNA vs. 2'-O-methyl RNA. Locked Nucleic Acid (LNA) is a nucleic-acid modification where the 2' oxygen and the 4' carbon atoms in the furanose ring are bridged via a methylene moiety.
Table 1
Fluorophore labels for molecular beacon probes

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Alternative fluorophore</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMR</td>
<td>Alexa 546&lt;sup&gt;a&lt;/sup&gt;, Cy3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>555</td>
<td>575</td>
</tr>
<tr>
<td>Texas Red</td>
<td>Alexa 594&lt;sup&gt;a&lt;/sup&gt;</td>
<td>585</td>
<td>605</td>
</tr>
<tr>
<td>Cy5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Alexa 647&lt;sup&gt;a&lt;/sup&gt;</td>
<td>650</td>
<td>670</td>
</tr>
</tbody>
</table>

<sup>a</sup>Alexa fluorophores are available from Invitrogen
<sup>b</sup>Cyanine dyes are available from Amersham Biosciences

Table 2
Quencher labels for molecular beacon probes

<table>
<thead>
<tr>
<th>Quencher</th>
<th>Absorption maximum (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deep Dark Quencher 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>430</td>
</tr>
<tr>
<td>Dabcyl</td>
<td>475</td>
</tr>
<tr>
<td>Eclipse&lt;sup&gt;b&lt;/sup&gt;</td>
<td>530</td>
</tr>
<tr>
<td>Iowa Black FQ&lt;sup&gt;c&lt;/sup&gt;</td>
<td>532</td>
</tr>
<tr>
<td>Black Hole Quencher 1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>534</td>
</tr>
<tr>
<td>Black Hole Quencher 2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>580</td>
</tr>
</tbody>
</table>

<sup>a</sup>Deep Dark Quenchers are available from Eurogentec
<sup>b</sup>Eclipse quenchers are available from Epoch Biosciences
<sup>c</sup>Iowa quenchers are available from Integrated DNA Technologies
<sup>d</sup>Black Hole Quenchers are available from Biosearch Technologies

Incorporation of LNA bases into oligonucleotides leads to exceptionally high-affinity binding to complementary sequences. Their negatively charged backbone confers good solubility, making these RNA derivatives easily synthesized using standard DNA/RNA synthesis methods (10).

Here, we show that introducing LNAs into 2′-O-methyl RNA oligonucleotides enables the design of smaller hairpins that match the stabilities of longer 2′-O-methyl RNA variants when bound to target. We describe how to design and synthesize these tiny LNA/2′-O-methyl RNA chimeras as well as introduce a refined approach for target region selection. We demonstrate that these tiny molecular beacons are stable, bright, highly specific, and effective in binding multiple mRNA target sites. The implementation of these changes in molecular beacon design will make this technology more practical for targeting highly structured mRNAs, thus enabling the simultaneous visualization of several mRNAs. Moreover, their application can extend to the detection of small RNA targets, such as microRNAs or piRNAs in living cells.
2. Materials

2.1. Target mRNA Selection and Design of Tiny Molecular Beacons


3. RNAstructure program: Free download from the Mathews Lab at the University of Rochester Medical Center: http://rna.urmc.rochester.edu/rnastructure.html.

2.2. Tiny Molecular Beacon Synthesis

1. 394 DNA/RNA Synthesizer (Applied Biosystems, Foster City, CA).

2. Locked Nucleic Acid (LNA) Phosphoramidites (Exiqon, Woburn, MA).

3. 2′-O-methyl RNA Phosphoramidites (Glen Research, Sterling, VA).

4. Dabcyl-linked controlled-pore glass synthesis column (Biosearch Technologies, Novato, CA).

5. 5′-thiol modifier C6 (Glen Research) (see Note 1).

6. High-pressure liquid chromatograph equipped with C18 reverse-phase column and dual wavelength detector: System Gold (Beckman Coulter, Brea, CA).

7. HPLC Buffer A: 0.1 M Triethylammonium acetate at pH 6.5, filtered and degassed.

8. HPLC Buffer B: 0.1 M Triethylammonium acetate in 75% (v/v) Acetonitrile at pH 6.5, filtered and degassed.


10. 3 M Sodium acetate at pH 5.2.

11. 0.15 M Dithiothreitol (DTT).

12. 0.15 M Silver nitrate.

13. Tetramethylrhodamine-5-iodoacetamide (Invitrogen, Carlsbad, CA) (see Note 1).

14. Texas Red C5 bromoacetamide (Invitrogen).

15. N,N-Dimethylformamide (DMF).

16. 0.2 M Sodium bicarbonate at pH 9.0.

17. TE buffer: 1 mM EDTA and 10 mM Tris–HCl at pH 8.0.

18. Nuclease-free water (Ambion Inc., Austin, TX).

2.3. In Vitro Characterization of Tiny Molecular Beacons

1. Hybridization buffer: 10 mM Tris–HCl at pH 8.0, 50 mM KCl, and 1 mM MgCl₂.

2. Molecular beacon. Dissolve molecular beacons for stock solutions in TE buffer and store at −20°C. Dilute working
solutions in nuclease-free water, keep protected from light, and store at −20°C up to 1 month.

3. Oligonucleotide target complementary to the probe sequence of the molecular beacon. Dissolve in TE and store at −20°C.


5. Thermal cycler with a capacity to monitor fluorescence in real time: iCycler iQ5. (Bio-Rad, Hercules, CA).

Theoretically, any sequence within a target RNA can be chosen as a site for molecular beacon binding. The endless possibilities give one the confidence that such regions are easily identified. However, the extent of target accessibility is primarily a consequence of complex secondary and tertiary intramolecular structures, which are not easy to predict and can mask many of these regions.

2.4. In Vivo mRNA Imaging with Tiny Molecular Beacons

3. Methods

3.1. Selection of mRNA Target Regions and Design of Tiny Molecular Beacons

3.1.1. Selection of RNA Target Regions

1. Fold mRNA sequence using RNA folding program mfold (7, 8).

2. Using default settings at 37°C, 1 M NaCl, and no divalent ions, obtain an immediate output for RNA sequences <800 nt in length. A “batch job” is otherwise submitted, which gets completed in 15 min on average, depending on the server’s availability. One can also receive an email notification that the job is complete and a link to the result posted on the web server.
3. Select to view the first MFE structure (e.g., jpg file), that represents the most thermodynamically stable secondary structure predicted. Use default parameter settings for suboptimal structures, which are sufficient for this analysis.

4. Analyze the entire ensemble of structures using two parameters: ss-count and P-num. ss-count defines the probability of a nucleotide to be single-stranded. P-num denotes the total number of different base pairs that can be formed by a particular base within the full set of structure results. These values are assigned to each nucleotide in the mRNA sequence and can be represented via a color-coded map. To view the folded RNA structure representing ss-count values, choose the “ss-count” annotation, and click on the image to redraw the structure. Each nucleotide will become colored with warm or cold colors depending on the respective ss-count values. Warm colors represent high values, indicating single-stranded regions. Change the annotation to “P-num”, and the colors will now reflect the values that indicate the stability of structures formed (warm colors represent well determined base pairings). Evaluate the ss-count and P-num color-annotated secondary structures (see Note 2).

5. To winnow down the number of candidate sites, employ a second algorithm (11). Input the RNA sequence (in CAPS) in the RNAstructure program as “New Sequence” and select to “Fold as RNA.” The file will be saved as a “Sequence File” (.seq), and a “CT File” (.ct) will automatically be generated. Use the default Suboptimal Structure Parameters. Start fold. The calculation will take a few minutes, depending on the length of the RNA (approx. 30 min for 3 kb using 8GB RAM at 1066 MHz) and if the suboptimal structure parameters are changed.

6. Select RNA OligoWalk module. This scans the folded RNA sequence for regions to which various-length oligonucleotides are capable of binding. The “Input File” is the “.ct” file saved above. This file contains the sequence with base pair information. Use the default mode, “Break Local Structure”, for calculating the free energy of intramolecular structure of the oligonucleotide and of the local RNA structure predicted. In this mode, the target structure breaks wherever the oligonucleotide binds \( \Delta G_{\text{break target}} \), oligonucleotides lose pairs in self-structures \( \Delta G_{\text{oligo-oligo}} \) and \( \Delta G_{\text{oligo-self}} \) and gain pairs in oligonucleotide-target binding \( \Delta G_{\text{duplex}} \). This algorithm calculates the equilibrium affinity of a set-length complementary oligonucleotide to the RNA target and predicts its overall free energy of binding while taking into account stability of the newly formed duplex, local secondary structure within the target RNA, as well as intermolecular and intramolecular secondary structures formed by the oligonucleotide (12).
7. Run OligoWalk calculation for an oligonucleotide concentration of 100 ng/µL, ranging in length from 10 to 15 nucleotides, including suboptimal target structures. The calculation will take just a few minutes, depending on the length of the oligonucleotide and target RNA.

8. The ΔG bar graph generated will facilitate the “walk” of the oligonucleotide on the RNA sequence, one nucleotide at a time, thus obtaining information about each oligonucleotide/RNA hybrid formed.

9. Choose a target region that generates the most negative (−) kcal/mol values for ΔG_{duplex} and ΔG_{overall}, with a difference between them of ≤ −10 kcal/mol (see Note 3).

10. Correlate these potential target regions with the ss-count and P-num annotation structures and make an educated “guess” for an RNA target region.

3.1.2. Design of Tiny Molecular Beacons

Molecular beacons synthesized from 2′-O-methyl-RNA are normally designed to have 4–5 nt stems (with a low G/C rich content) and 18–25 nt in the loop/probe region of the hairpin which ensures >10°C difference between melting temperature of the probe–target hybrid and the target detection temperature (25 or 37°C) (http://www.molecular-beacons.org).

With the incorporation of LNA bases in the loop/probe region of the hairpin, the stability of the probe/target hybrid increases (see Note 4), thus enabling the design of probes with shorter hairpin loops and stems. Tiny molecular beacons are chimeras with 2′-O-methyl RNA/LNA loops and 2′-O-methyl RNA stems. They have 3–4 nt length stems (with GC variations only) with 9–12 nt in the hairpin loop (see Note 5–7).

3.2. Synthesis of Tiny Molecular Beacons

Molecular beacon probes possessing a backbone chemistry containing a combination of LNA and 2′-O-methyl RNA nucleotides can be synthesized using standard automatic DNA chemistry using quencher labeled solid supports, a 5′ fluorophore or 5′ terminal modifier phosphoramidites, LNA, and 2′-O-methyl RNA phosphoramidites (13). The 5′ terminal modifier phosphoramidite is utilized if a fluorophore reporter is required for which no phosphoramidite is available (see Note 1). For the synthesis of the molecular beacon probes described in this chapter, we utilized a controlled-pore glass column to introduce dabcyl at the 3′ end of the oligonucleotide during the automated synthesis. Compared to standard automated DNA synthesis protocols, and as recommended by the phosphoramidite manufacturers, the coupling step and oxidation step in each synthesis cycle was doubled. At the 5′ end of the oligonucleotide, a trityl-protected sulfhydryl modifier was introduced for a subsequent manual coupling of Texas Red C5 bromoacetamide or Tetramethylrhodamine-5-iodoacetamide. Standard post-synthesis protocols were followed.
after the oligonucleotide synthesis was completed. Before the conjugation of the fluorophore, the oligonucleotide was first purified by HPLC to remove non-full-length oligonucleotides that do not contain a 5′ trityl-protected sulfhydryl group. The protective trityl moiety was then removed from the 5′-sulfhydryl group and a fluorophore was introduced in its place using an iodoacetamide or bromoacetamide derivative. This conjugation was followed by a second HPLC purification to remove loose fluorophores and unconjugated oligonucleotides.

1. After the automated synthesis and post-synthesis steps, dissolve the oligonucleotides in 500 µL of HPLC Buffer A.

2. Purify the oligonucleotides on a C-18 reverse phase HPLC column, utilizing a linear elution gradient of 20–70% HPLC Buffer B in HPLC Buffer A and run for 25 min at a flow rate of 1 mL/min. Monitor the absorption of the elution stream at 260 nm (absorption of nucleotides) and 491 nm (absorption of dabcyl). Fig. 2a shows a typical HPLC chromatogram of the purification of an oligonucleotide labeled with a trityl-protected sulfhydryl group, a mixture of LNA and 2′-O-methyl RNA nucleotides, and a 3′ dabcyl group. Collect peak B that absorbs in both wavelengths. Due to the increased hydrophobicity of this trityl containing oligonucleotide, its HPLC retention time is longer than non-full-length oligonucleotides.

3. Precipitate the collected material with ethanol and sodium acetate for at least 2 h at −20°C and spin in a centrifuge for 10 min at 8,000 × g. Discard the supernatant, dry the pellet, and dissolve the oligonucleotide in 250 µL of HPLC Buffer A.

4. In order to remove the trityl moiety, add 10 µL of 0.15 M silver nitrate and incubate for 30 min. Add 15 µL of 0.15 M DTT to this mixture and incubate for 5 min. Spin for 2 min at 8,000 × g and transfer the supernatant to a new tube. Dip the point of a pipette tip in the bottle containing the fluorophore and dissolve a small amount of tetramethylrhodamine-5-io-doacetamide or Texas Red C5 bromoacetamide in 10 µL of DMF (see Note 8). Add the fluorophore solution to 250 µL of 0.2 M sodium bicarbonate at pH 9.0. Incubate the mixture for 120 min. Each of the above mentioned solutions should be prepared just before use.

5. Precipitate the fluorophore–oligonucleotide mixture with ethanol and sodium acetate for at least 2 h at −20°C and spin in a centrifuge for 10 min at 8,000 × g. Discard the supernatant, dry the pellet, and dissolve the oligonucleotide in 500 µL of HPLC Buffer A.
6. Purify the fluorophore labeled oligonucleotides on a C-18 reverse phase HPLC column, utilizing a linear elution gradient of 20–70% HPLC Buffer B in HPLC Buffer A and run for 25 min at a flow rate of 1 mL/min. Monitor the absorption of the elution stream at 260 nm (absorption of nucleotides) and 555 nm (absorption of Tetramethylrhodamine) or 584 nm (absorption of Texas Red). Fig. 2b shows a typical HPLC chromatogram of the purification of an oligonucleotide labeled with a Texas Red bromoacetamide group, a mixture of LNA and 2'-O-methyl RNA nucleotides, and a 3' dabcyl group. Collect peaks C and D that absorbs in both wavelengths.
The Texas Red bromoacetamide derivative is a mixture of two isomeric sulfonamides, which results in a slightly different HPLC retention time for each isomer, and hence two peaks are observed. The tetramethylrhodamine-5-iodoacetamide derivative is a single isomer product and only one HPLC peak will be observed.

7. Precipitate the collected material with ethanol and sodium acetate for at least 2 h at −20°C and spin in a centrifuge for 10 min at 8,000 × g. Discard the supernatant, dry the pellet, and dissolve the molecular beacon in 50 μL TE buffer. Determine the absorbance at 260 nm and estimate the yield (1 OD_{260} ~ 33 μg/mL).

After the synthesis and purification, the molecular beacon is characterized in two short in vitro experiments to determine if the purity of the molecular beacon preparation is sufficient to avoid background fluorescence generation during live cell imaging and for the ability of the molecular beacon to form stable hybrids with target nucleic acids.

The thermodynamic characteristics of the molecular beacons are obtained by measuring the denaturing profile in a real-time PCR instrument. Ideally, the melting temperature of the molecular beacon probe–target nucleic acid hybrids should be at least 7–10°C higher than the detection temperature, which is usually room temperature or 37°C. This is also true for the stem hybrid melting temperature of the molecular beacon. Molecular beacons that increase their fluorescence intensity at least 20 times upon hybridizing to their target nucleic acid are considered good. When designed correctly and after a successful HPLC purification, most molecular beacon preparations increase their fluorescence intensity ranging from 30 to 100 times. Fig. 3 represents an example of a denaturation profile and signal-to-background ratio determination. The molecular beacon contains a combination of LNA and 2′-O-methyl RNA nucleotides, and was labeled with 5′ Texas Red and 3′ dabcyl. Fig. 3a shows the denaturation profile of this molecular beacon in presence of a complementary nucleic acid target containing a backbone chemistry of deoxyribonucleotides (DNA, dashed line) and 2′-O-methyl ribonucleotides (2′-O-methyl RNA, solid line), or no nucleic acid target (dotted line). This example shows that although a DNA target could be used (probe – target melting temperature is about 35°C), the molecular beacon forms a much more stable hybrid with the 2′-O-methyl RNA target (probe – target melting temperature is about 70°C), which is closely related to RNA in structural properties. The melting temperature of the stem hybrid is 80°C. Fig. 3b shows the result of the signal-to-background ratio experiment of the molecular beacon determined with the DNA target (open circles) and 2′-O-methyl RNA target (closed circles). The signal-to-background ratio
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Determined with the DNA target is about 20 and with the 2′-O-methyl RNA target is about 30 (see Note 9). Both results are sufficient, however, the plots also show that the kinetics of hybridization between the molecular beacon and its DNA target are slower than the kinetics of hybridization between the molecular beacon and its 2′-O-methyl RNA target. The results in this figure shows that, if possible, it will benefit to utilize a 2′-O-methyl RNA target in order to obtain a better characterization profile of the molecular beacon used in live cell mRNA imaging.

1. Signal-to-background ratios of molecular beacons are determined in a spectrofluorometer using the optimal excitation and emission wavelength for the reporter fluorophore.
2. Determine the fluorescence \((F_{\text{buffer}})\) of 150 \(\mu\)L of hybridization buffer using (as in above mentioned example) 584 nm as the excitation wavelength and 603 nm as the emission wavelength.

3. Add 10 \(\mu\)L of 1 \(\mu\)M molecular beacon to this solution and record the new level of fluorescence \((F_{\text{closed}})\).

4. Add a twofold molar excess of the oligonucleotide target and monitor the rise in fluorescence until it reaches a stable level \((F_{\text{open}})\).

5. Calculate signal-to-background ratio as \((F_{\text{open}} - F_{\text{buffer}})/(F_{\text{closed}} - F_{\text{buffer}})\).

6. Thermal denaturation profiles of molecular beacons are determined in a thermal cycler with a capacity to monitor fluorescence in real time using the wavelength of excitation and emission specific for the fluorophore.

7. Prepare two tubes containing 25 \(\mu\)L of 200 nM molecular beacon dissolved in hybridization buffer and add the oligonucleotide target to one of the tubes at a final concentration of 400 nM.

8. Determine the fluorescence of each solution as a function of temperature. Decrease the temperature of these tubes from 95 to 25°C in 1°C steps, with each hold lasting 30 s, while monitoring the fluorescence during each hold.

3.4. In Vivo Imaging of oskar mRNA

We have previously used 2'-O-methyl RNA molecular beacons to directly visualize the endogenous expression of the maternal gene oskar in the Drosophila melanogaster oocytes (4). Following our report, others have confirmed the target specificity of molecular beacons within various cellular contexts (14–16).

Here we show in vivo accessibility to previously selected oskar mRNA target regions of tiny LNA/2'-O-methyl RNA chimera molecular beacons designed and synthesized as described in Subheadings 3.1 and 3.2 (Fig. 4, see Note 10).

In Drosophila melanogaster egg chambers, mRNAs are transcribed in the nurse cell nuclei throughout oogenesis and are transported into the oocyte via connecting ring canals. Oskar mRNA localizes during mid-oogenesis at the posterior pole of

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Fig. 4. Tiny molecular beacon detects oskar mRNA in vivo. (a) Co-visualization of a tiny molecular beacon and an established 2'-O-methyl RNA probe. A solution containing 200 ng/\(\mu\)L of each molecular beacon (osk2209-2'-OMe-Cy5/BHQ2 (red) and osk1227-chimera-TxRed/dabcyl (green)) was injected in egg chambers of various stages. The top two rows show a stage 8 egg chamber. Images represent Z-projections of 7 × 1 \(\mu\)m sections at 10 and 35 min following microinjection. The bottom row represents a single section of a stage 10 egg chamber at 60 min after microinjection. (b) Simultaneous, in vivo targeting of two regions within oskar mRNA with two tiny molecular beacons labeled with the same fluorophore (TMR). A cocktail of osk1227-chimera-TMR/dabcyl and osk2213-chimera-TMR/dabcyl, containing 100 ng/\(\mu\)L each, was injected into stage 8 egg chambers (see Note 14). A DIC image of the oocyte 30 min after microinjections, followed by Z-projections of 13 × 0.5 \(\mu\)m sections at 0, 10, and 30 min. All images were acquired with a spinning disc microscope set-up, using 40× oil objective of 1.25 NA. The scale bars represent 20 \(\mu\)m.
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the oocyte, while briefly anchoring at the anterior cortex in the earlier stages.

1. Feed newly hatched wild type female flies with fresh yeast paste for 2–4 days.

2. Dissect ovaries in Halocarbon Oil 700 directly on glass coverslip and tease apart each ovariole, thus separating individual egg chambers.

3. Mount the glass coverslip onto the spinning disc microscope stage (see Note 11) and microinject the molecular beacon solution (2′-O-methyl RNA and/or chimera) into a nurse cell most proximal to the oocyte. Select egg chambers that are of stage 8 or older.

4. Begin recording immediately, selecting a Z-stack (6–14 slices of 0.5–1 μm each), while acquiring data every 30 or 60 s for 15 min, up to 1 h (see Note 12).

5. Acquire images using Volocity and export the file of interest as OpenLab liff file format (see Note 13).

6. Open liff files using ImageJ. Adjust images for brightness/contrast. The merged images are created using “Merge Channels”.

### 4. Notes

1. 5′ sulfhydryl (or thiol) modifiers are used for the conjugation of iodoacetamide, bromoacetamide, or maleimide derivatives of fluorophores. 5′ amino modifiers are used for the conjugation of succinimidyl ester derivatives of fluorophores.

2. Unlike predictions of local hairpins, long-range interactions or multibranch junctions remain poorly determined. Such predicted structures could provide insights into regions of potential structural plasticity within an RNA molecule and thus reveal potential accessible sites for antisense probes. Therefore, when considering probes for targeting an RNA sequence, it is very helpful to pay close attention to the information offered by a secondary structure fold.

3. It is possible for a longer region of the target to be chosen as favorable, not just the region comprising the length of the oligonucleotide. \( \Delta G_{\text{oligo-oligo}} \) and \( \Delta G_{\text{oligo-self}} \) are disregarded, as the molecular beacon structure is analyzed separately using mfold (see Subheading 3.1.2).

4. The effect of LNA substitutions is approximately additive when LNA nucleotides are spaced by at least one 2′-O-methyl nucleotide (17). On average, an internal LNA substitution for a 2′-O-methyl RNA makes duplex stability more favorable
by \(-1.4\) kcal/mol at 37°C (17). This corresponds to a tenfold increase in binding constant.

\[
\Delta G^{\circ}_{37} (\text{chimera/RNA}) = \Delta G^{\circ}_{37} (2'-O\text{-MeRNA/RNA}) - 1.10 n_{i\text{AL/UL}} - 1.6 n_{i\text{IGL/CL}},
\]

where \(\Delta G^{\circ}_{37} (2'-O\text{-MeRNA/RNA})\) is the free energy change at 37°C for duplex formation in the absence of any LNA nucleotides, \(n_{i\text{AL/UL}}\) and \(n_{i\text{IGL/CL}}\) are the numbers of internal LNAs in AU and GC pairs, respectively.

5. Avoid stretches of 3 or more Gs or Cs.

6. Keep the loop GC content between 30 and 60%.

7. Do not include all LNAs in loop sequence.

8. Some fluorophores are directly soluble in aqueous solutions and do not need to be dissolved in DMF. Refer to the manufacturer application note for the particular fluorophore derivative.

9. If the manual conjugation of a fluorophore to an oligonucleotide resulted in a low yield, check the pH of the buffers used in the coupling reactions and use fresh dyes. The reactive dyes should be stored at ~20°C in the presence of a desiccant.

10. Tiny molecular beacons:

   - The # indicates the range of nucleotides on mRNA sequence, followed by the fluorophore–quencher pair labels, and size and stability of the hairpin structure.

   - The sequence includes the stem forming nucleotides (underlined font) and the probe loop complementary to target region (CAPS font).

   - All nucleotides are 2'-O-methyl RNA with LNA substitutions in bold font.

   osk2209-2233 Cy5-BHQ2
   (stem = 5, loop = 25, \(\Delta G = -6.7\) kcal/mol)

   5'-gcgc AAA AGC GGA AAA GUU UGA AGA GAA Ggcg-3'

   osk2213-2224 Texas Red-dabcyl
   (stem = 4, loop = 11, \(\Delta G = -4\) kcal/mol)

   5'-cgcc AAG UUU GAA GA Gccg-3'

   osk1227-1238 Texas Red-dabcyl
   (stem = 4, loop = 11, \(\Delta G = -3.8\) kcal/mol)

   5'-gccg AAU CGU UGU AG cgcc-3'.

11. Confocal Microscope Setup: Leica DMI4000B inverted microscope mounted on a TMC isolation platform, Yokogawa CSU10 spinning disc head, Hamamatsu C9100-13 ImagEM EM-CCD camera, diode lasers - 491, 561, and 638 nm,
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Eppendorf Patchman-Femtojet microinjector, Volocity acquisition software and ImageJ processing software.

12. Though these experiments are done at 25°C, the accessibility to the target region predicted in mfold at 37°C is not affected. The tiny molecular beacons are stable on target for a range of temperatures (Fig. 3b).

13. Processing can be performed with Volocity, but it is not as flexible as ImageJ for reconstructing Z-stacks.

14. Mixture of tiny molecular beacons targeting multiple RNA regions results in an increased signal per target molecule, thus benefiting the visualization of low abundance RNAs.

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References


