Using tRNA-linked molecular beacons to image cytoplasmic mRNAs in live cells

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Imaging products of gene expression in live cells will provide unique insights into the biology of cells. Molecular beacons make attractive probes for imaging mRNA in live cells as they can report the presence of an RNA target by turning on the fluorescence of a quenched fluorophore. However, when oligonucleotide probes are introduced into cells, they are rapidly sequestered in the nucleus, making the detection of cytoplasmic mRNAs difficult. We have shown that if a molecular beacon is linked to a tRNA, it stays in the cytoplasm and permits detection of cytoplasmic mRNAs. Here we describe two methods of linking molecular beacons to tRNA and show how the joint molecules can be used for imaging an mRNA that is normally present in the cytoplasm in live cultured cells. This protocol should take a total of 4 d to complete.

INTRODUCTION

Detecting and imaging of specific RNAs in living cells requires probes that show changes in fluorescence upon hybridization. Among the different probes that have been considered over the years are competitive hybridization probes[1,2], binary hybridization probes[3,4], quenched autoligation probes[5,6] and light-up probes[7,8]. Adding to this toolbox of probes, we have developed hairpin-shaped oligonucleotide probes called molecular beacons that become fluorescent upon hybridization with a complementary nucleic acid[9–11]. Molecular beacons consist of a probe sequence, capable of hybridizing to the cellular target, flanked on either side by complementary sequences that create a stem; the stem brings attached fluorophore and quencher dyes into close proximity, thereby quenching the fluorophore. When the target nucleic acid binds to the probe sequence in the loop of the molecular beacon, the hairpin stem is disrupted and the fluorescence of the fluorophore is restored, revealing the presence of the target[9,10] (**Fig. 1**). Molecular beacons have been used to successfully detect specific mRNAs in live cells[11–14].

To image the distribution of RNAs in live cells with oligonucleotide probes, it is important that the probes themselves be distributed homogenously throughout the cell. If the probes are concentrated in one cellular compartment and are depleted from others, the targets will become visible only in the first compartment even when mRNAs are present in all compartments. However, we have found that molecular beacons become sequestered in the nucleus soon after their introduction into cells[12,13]. Other oligonucleotide probes have similarly been found to sequester in the nucleus[2,4]. Their sequestration in the nucleus occurs so fast, and to such a great extent, that probes are not able to bind to cytoplasmic mRNAs. Nuclear sequestration is a general property of oligonucleotides, as it has also been observed with antisense agents and is thought to be responsible for a reduction in their efficacy[15]. Therefore, preventing nuclear sequestration of oligonucleotides will not only be useful for imaging of cytoplasmic RNAs through a variety of different probe types but may also help to improve the efficacy of antisense agents.

We have developed two different approaches to prevent the nuclear sequestration of molecular beacons. In our first approach we linked a protein (streptavidin) to the probes[12]. This protein is impeded from passing through the nuclear pores and thus stays in the cytoplasm along with the attached molecular beacon. Streptavidin can be easily introduced at the end of the molecular beacon by first incorporating a biotin moiety at one end during automated synthesis. However, the tetrameric nature of biotin binding sites in streptavidin and the sequestration of cellular biotin by excess streptavidin are negative features of this approach. In our second approach we linked a tRNA to the probes[13]. tRNAs are actively exported from the nucleus to the cytoplasm, where they remain. Molecular beacons linked to tRNA accordingly stay in the cytoplasm[13]. Advantages of using tRNA tags for probes include their relatively small size, which does not significantly influence probe

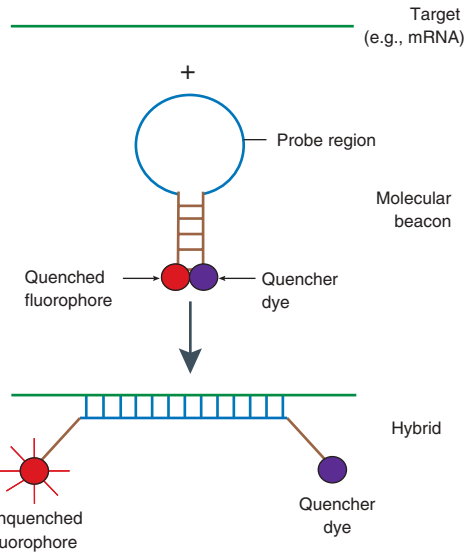

Figure 1 | Principle of operation of molecular beacons. The probe sequence in the loop binds spontaneously to the target RNA at physiological temperatures, separates a terminally linked pair of fluorophore and quencher, and restores the fluorescence of the quenched fluorophore.

characteristics, and the fact that they are nucleic acids, which allows simple base pairing–based strategies to be used to link them to molecular beacons. As tRNAs are highly abundant cellular constituents, tRNA nucleocytoplasmic export and cytoplasmic retention machineries are able to cope with high concentrations of the tRNA-probe complex[13]. Although both streptavidin and tRNA tags are successful in keeping the probes in the cytoplasm for long enough to allow them to find and bind to their cytoplasmic target mRNAs and to permit the imaging of their distribution[12,13], we describe only the tRNA approach here.

There are two simple ways in which *in vitro*–synthesized tRNA transcripts can be linked to a molecular beacon. In the first method, the probe and tRNA molecules are linked covalently; in the second method they are joined via complementary cohesive ends. For the first method, a tRNA transcript containing a sulfhydryl group at its 5′ end is first synthesized (**Fig. 2**). This transcript is then linked with a molecular beacon containing an amino group at one of its ends via a coupling reaction with a heterobifunctional reagent. Our reagent of choice is mal-sac-HNSA, which contains a maleimide moiety for coupling to a sulfhydryl group and a succinimidyl moiety for coupling to an amino group[16] (**Fig. 3a**). In the second method, a 15-nt G/C-rich 'splint' sequence is introduced at the 3′ end of the tRNA and its complement is introduced at the 3′ end of the molecular beacon. The two molecules are then linked to each other via these cohesive ends in a simple mixing step (**Fig. 3b**). Both of these methods yield similar results[13].

The DNA template from which tRNA can be transcribed is constructed from two long oligonucleotides, tRNA Forward and tRNA Reverse. The oligonucleotides are annealed to each other and their 3′ ends are extended to generate a double-stranded DNA. A promoter for T7 RNA polymerase is introduced to this DNA by using it as a template in a polymerase chain reaction (PCR) with primers T7 tRNA Forward and tRNA Reverse Short.

Although described for molecular beacons, this protocol can be used with other oligonucleotide probes for imaging the cytoplasmic distribution and dynamics of mRNAs. Furthermore, this protocol may help in targeting cytoplasmic mRNAs for repression by antisense agents.

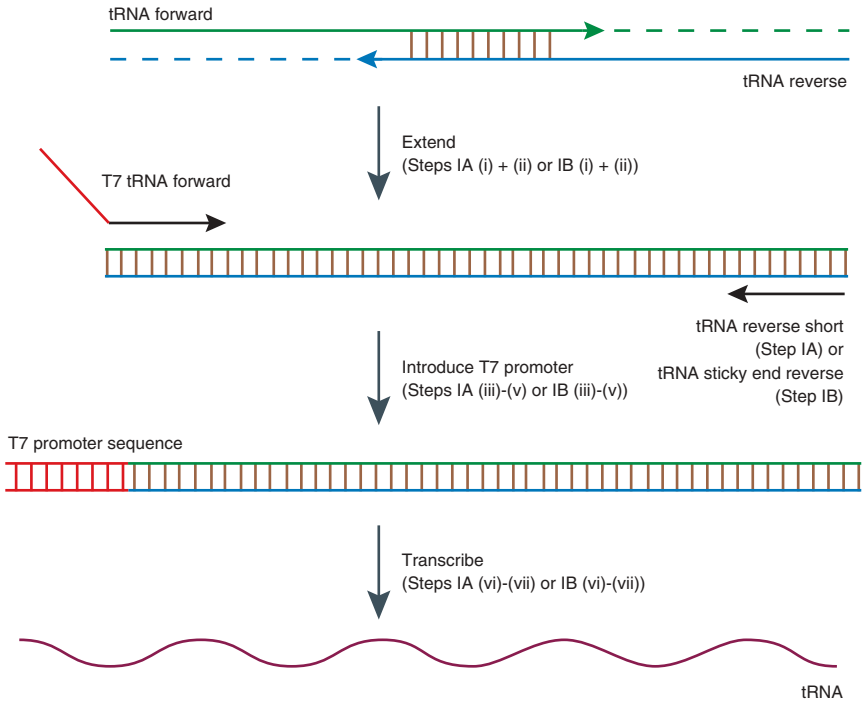

Figure 2 | Scheme for generation of tRNA transcripts. Two oligodeoxynucleotides that together correspond to the sequence of a tRNA are annealed with each other via sticky ends. Their 3′ ends are extended by *Taq* DNA polymerase to generate a fully double-stranded template. This template is used in a PCR with tailed primers to introduce a T7 promoter. The product of the PCR is used to make tRNA transcript in a transcription reaction catalyzed by T7 RNA polymerase. When a tRNA transcript with cohesive 3′ ends is needed, a reverse primer with appropriate tail sequence is used in the PCR (not shown in the diagram).

MATERIALS

REAGENTS

- 100% ethanol
- 3 M sodium acetate buffer, pH 5.6
- Molecular beacon buffer: 1 mM $MgCl_2$ and 20 mM Tris-HCl, pH 8.0
- Phosphate buffers: 0.1 M potassium phosphate, pH 6.0 and pH 7.5
- TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0
- GE buffer: 400 nM NaCl, 20 mM Tris-HCl, pH 7.5
- AmpliTaq Gold DNA polymerase (Applied Biosystems)
- 25 mM $MgCl_2$ (Applied Biosystems)
- 100 mM deoxyribonucleotide triphosphates (dNTPs) stocks (Applied Biosystems)
- 10× PCR buffer (Applied Biosystems)
- *In vitro* RNA synthesis kit containing T7 RNA polymerase, ribonucleotide triphosphates (rNTPs), RNase-free water, RNase inhibitor (RNasin), 5× transcription buffer and dithiothreitol (Promega)
- Mal-sac-HNSA (Bachem)
- Sephadex G-25, NAP-5 Column (Amersham Pharmacia Biotech)
- Thiolated 5′-guanosine monophosphate (5′-thio-GMP; Biolog)
- Molecular beacon (see REAGENT SETUP)
- Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen)
- 10% (vol/vol) FBS (Invitrogen)
- 10 units ml^{-1} penicillin-based antibiotic (Invitrogen)
- 10 units ml^{-1} streptomycin (Invitrogen)
- Opti-MEM1 (Invitrogen)
- Leibovitz's medium (Invitrogen)
- Oligofectamine (Invitrogen)
- Chicken embryonic fibroblasts (Charles River Laboratories)
- Gelatin (Sigma)
- tRNA Forward oligonucleotide (bold residues indicate the regions through which the Forward and Reverse oligonucleotides anneal to each other): 5′-GTTTCCGT AGTGTAGTGGTTATCACGTT**CGCCTAACACGCGAAAGGTC**-3′,
- tRNA Reverse oligonucleotide (bold residues as above): 5′-TGGTGTTTCCG CCCGGTTTCGAACCGGG**GACCTTTCGCGTGTTAGGCG**-3′
- tRNA Reverse Short oligonucleotide: 5′-TGGTGTTTCCGCCCGGTTTC-3′
- T7 tRNA Forward oligonucleotide (bold residues include T7 promoter sequence): 5′-**GCATAATACGACTCACTATAGGGAGA**GTTTCCGTAGTGT AGTGG-3′

- tRNA Sticky-End Reverse oligonucleotide (bold residues indicate the region through which tRNA will bind to the molecular beacon): 5′-**GTCGCTGCG GGCGGG**TGGTGTTTCCGCCCGGTTTC-3′
- Reagents and solutions for 8% polyacrylamide gel electrophoresis (PAGE), including native gels and denaturing preparative gels

EQUIPMENT

- Thermal cycler (Applied Biosystems)
- Tabletop centrifuge (Eppendorf)
- Mini PAGE apparatus (Owl Separation Systems)
- QuantaMaster spectrofluorometer (Photon Technology International)
- Axiovert 200M inverted epifluorescence microscope equipped with high numerical aperture 100× objective (Zeiss)
- Highly sensitive cooled digital camera such as Photometrics Coolsnap HQ (Roper Scientific)
- Openlab 3.2 and Volocity 2.0 software (Improvision)
- Eppendorf Femtojet/Injectman apparatus and micromanipulator, and Femtotips Sterile Injection Needles (Eppendorf)
- Delta-T cell temperature controller system consisting of microscope stage, feedback controllers, objective heater and culture dishes (Bioptech)

REAGENT SETUP

Molecular beacons A molecular beacon should be synthesized complete with its fluorophore and quencher and also containing either a 3′ or 5′ amino group, or containing a 3′-overhang sequence complementary to the overhang sequence in tRNA in addition to the fluorophore and quencher. For a comprehensive list of companies licensed by the Public Health Research Institute to make molecular beacons for research, with addresses and links, see http://www.molecular-beacons.org/PA_oligo.html. In designing molecular beacons, first identify the probe-accessible sites in the target mRNA. To predict these sites, fold the full-length RNA sequence using the RNA folding program available at http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi. Download the 'ss-count' file from the output, which contains the frequency of each nucleotide position in a single-stranded state amongst all the suboptimal folds. Open this file in a spreadsheet program and identify long stretches of sequences (at least 20 nucleotides (nt)) that tend to be single stranded. From these regions, select several regions and design 15–20-nt probes against them.

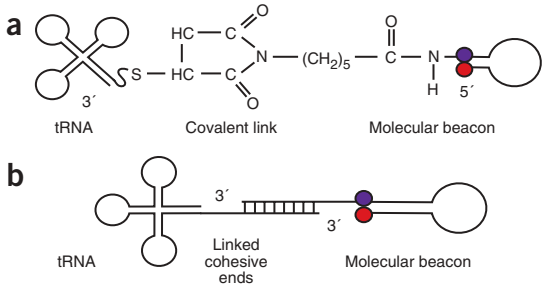

Figure 3 | Schematic diagrams of two different links between tRNA and molecular beacon. (**a**) A tRNA covalently linked to a molecular beacon. The linker shown is introduced when heterobifunctional linking agent mal-suc-HNSA is used to couple a sulfhydryl group at the 5′ end of the tRNA transcript with a primary amino group at the 3′ end of the molecular beacon. (**b**) The two molecules linked via cohesive ends.

Append a 5–6-nt G/C-rich sequence at the 5′ end of each probe sequence and its reverse complement at the 3′ end; this will form the stem. In the case that the molecular beacon will have a nuclease-resistant 2′-*O*-methylribonucleotide backbone, only a 4-nt stem sequence is necessary. The most likely folding structure of these sequences can be obtained and the melting temperature of the stem can be estimated using the DNA folding program available at http://www.bioinfo.rpi.edu/applications/mfold/dna/. A more detailed description of molecular beacon design principles is available at http://www.molecular-beacons.org/PA_design.html. Choose a fluorophore based on the requirements of the imaging setup and a non-fluorescent quencher that would efficiently quench the selected fluorophore based on estimates of quenching efficiencies provided by Marras *et al.*[17]. Test the accessibility of target sequences in the RNA using appropriately purified molecular beacons and full-length mRNAs[11].

PROCEDURE

Preparation of tRNA transcript and linking it to the molecular beacon

1| Molecular beacons can be linked to tRNAs via covalent bonds (A) or cohesive ends (B).

Option A: Linking tRNA and molecular beacons via covalent bonds

(i) Prepare the following 50-µl reaction mixture on ice in a tube suitable for your PCR apparatus.

Component	Final concentration
tRNA Forward	1 µM
tRNA Reverse	1 µM
$MgCl_2$	2 mM
dNTP mix	0.5 mM
Taq DNA polymerase	500 units

(ii) Transfer the tubes to the PCR machine and incubate the mixture at 95 °C for 10 min to activate the *Taq* DNA polymerase, 55 °C for 1 min to anneal the complementary ends of single-stranded DNAs, and 72 °C for 3 min to extend the ends and make fully double-stranded DNA.

(iii) Assemble a 50-µl PCR mixture on ice in a tube suitable for your PCR apparatus containing the components listed in the table below. This PCR will add a T7 promoter to the template, enabling transcription of the tRNA in subsequent steps.

Component	Final concentration
T7 tRNA Forward primer	0.5 µM
tRNA Reverse Short primer	0.5 µM
DNA template (from previous step)	2 µl of a 10,000-fold dilution
$MgCl_2$	2 mM
dNTP mix	0.5 mM
PCR buffer	1×
Taq DNA polymerase	500 units

(iv) Transfer the tube to the PCR machine and carry out PCR using the following thermal cycling conditions.

Cycle number	Denature	Anneal	Extend
1	95 °C, 10 min	–	–
2–41	95 °C, 30 s	58 °C, 45 s	72 °C, 30 s

(v) Analyze 5 µl of the reaction products by performing electrophoresis on an 8% polyacrylamide gel using the Mini PAGE apparatus, to confirm that a clearly visible 102-nt amplicon is produced.

(vi) Assemble a 100-µl transcription reaction mixture on ice in a 1.5-ml microcentrifuge tube containing the components listed in the table below. To synthesize a tRNA transcript with a sulfhydryl group at its 5′ end, 5′-thio-GMP is included in the transcription mixture. T7 RNA polymerase incorporates GMP into the transcript as the first nucleotide of the transcript[18].

Component	Final concentration
Transcription buffer	1×
DTT	10 mM
ATP, UTP and CTP	0.5 mM each
GTP	0.05 mM
5′-thio-GMP	0.45 mM
RNasin	30,000 units
T7 RNA polymerase	20,000 units
Reaction mixture (from previous step)	10 µl

(vii) Incubate at 37 °C for 2 h and confirm the production of a 76-nt RNA by Mini PAGE gel electrophoresis using an 8% polyacrylamide gel. Estimate the yield by comparing with quantitative standards. A reasonable yield is 10–100 µg.
? TROUBLESHOOTING

(viii) Remove proteins using a standard phenol extraction method[19].

(ix) Remove excess nucleotides by passing the supernatant through a gel exclusion column (NAP-5) equilibrated with phosphate buffer (pH 6.0) as described by the kit instructions. Collect the RNA in 500 µl of phosphate buffer. 10–100 µg RNA will be sufficient for the subsequent steps.
▲ **CRITICAL STEP** The volume of fraction collected should be adhered to carefully to avoid excessive dilution.

(x) To couple mal-suc-HNSA to molecular beacon, mix the molecular beacon synthesized with a terminal amino group (10–100 µg) and mal-suc-HNSA (10 mg) in phosphate buffer (pH 7.4) and incubate the reaction mixture (500 µl) at room temperature (20–30 °C) for 1 h. In a successful reaction, the reaction mixture will turn yellow.

(xi) Remove excess coupling reagent and side products by passing the reaction mixture through a NAP-5 column equilibrated with phosphate buffer (pH 6.0). Elute molecular beacon coupled to mal-sac-HNSA in 500 µl of phosphate buffer. The fraction to be collected will have the color of the molecular beacon, which is dependent on the dyes present.
▲ **CRITICAL STEP** The volume of fraction collected should be adhered to carefully to avoid excessive dilution.

(xii) To link the activated molecular beacon with tRNA, mix the reaction mixtures from Step (ix) (tRNA, 500 µl) and Step (xi) (molecular beacon, 500 µl) and incubate at room temperature overnight. Equimolar amounts of the two substrates should be mixed.

(xiii) Pass the reaction mixture through a NAP-5 column equilibrated with TE buffer to remove phosphate that would interfere in the precipitation in the next step, collecting 0.5–1 ml.
▲ **CRITICAL STEP** The volume of fraction collected should be adhered to carefully to avoid excessive dilution.

(xiv) Precipitate the conjugated material using a standard alcohol precipitation method[19]. Resuspend the pellet in 50 µl TE.
? TROUBLESHOOTING

(xv) Purify the tRNA–molecular beacon conjugate by electrophoresis through a preparative denaturing 8% polyacrylamide gel using the Mini PAGE apparatus. Use unlinked molecular beacon and tRNA as size controls. Cut the band corresponding to tRNA–molecular beacon from the gel and elute the conjugate by shaking it in 500 µl GE buffer overnight.
? TROUBLESHOOTING

(xvi) Precipitate the conjugate by adding 1,250 µl of 100% ethanol. Air dry the resulting pellet[19]. Resuspend the pellet in 50 µl TE.

(xvii) Determine the signal-to-background ratio of the tRNA–molecular beacon conjugate by measuring its fluorescence in 1-µM solutions (in molecular beacon buffer) in the presence and absence of the target oligonucleotide (2 µM) using a spectrofluorometer. The signal-to-background ratio should be greater than 25 for effective imaging.
■ **PAUSE POINT** Store at −20 °C in several 10-µl aliquots until needed. Avoid repeated thawing and freezing.

Option B: Linking molecular beacons to tRNA via cohesive ends

(i) Prepare the following 50-µl reaction mixture on ice in a tube suitable for your PCR apparatus.

Component	Final concentration
tRNA Forward	1 μM
tRNA Reverse	1 μM
$MgCl_2$	2 mM
dNTP mix	0.5 mM
Taq DNA polymerase	500 units

(ii) Incubate the mixture at 95 °C for 10 min to activate the *Taq* DNA polymerase, at 55 °C for 1 min to anneal the complementary ends of single-stranded DNAs, and then at 72 °C for 3 min to extend the ends and make fully double-stranded DNA.

(iii) To synthesize a template for tRNA with a 3′ cohesive end, assemble a 50-μl PCR mixture containing the components listed in the table below. This PCR will add a T7 promoter to the template, enabling transcription of the tRNA in subsequent steps and create a cohesive end for linking to the molecular beacon.

Component	Final concentration
T7 tRNA Forward primer	0.5 μM
tRNA Sticky-End Reverse primer	0.5 μM
DNA template (from previous step)	2 μl from a 10,000-fold dilution
$MgCl_2$	2 mM
dNTP mix	0.5 mM
PCR buffer	1×
Taq DNA polymerase	500 units

(iv) Carry out PCR using the following thermal cycling conditions.

Cycle number	Denature	Anneal	Extend
1	95 °C, 10 min	–	–
2–41	95 °C, 30 s	58 °C, 45 s	72 °C, 30 s

(v) Analyze 5 μl of the PCR product by performing Mini PAGE electrophoresis on an 8% polyacrylamide gel to confirm that a clearly visible 118-nt amplicon is produced.

(vi) To synthesize the tRNA transcript with a cohesive 3′ end, assemble a 100-μl transcription reaction containing the following.

Component	Final concentration
Transcription buffer	1×
DTT	10 mM
rNTP mix	0.5 mM
RNasin	30,000 units
T7 RNA polymerase	20,000 units
Reaction mixture (from previous step)	10 μl

(vii) Incubate at 37 °C for 2 h and confirm that a 92-nt RNA is produced by gel electrophoresis using an 8% polyacrylamide gel. Estimate the yield by comparing with standards. Likely yield is 10–100 μg.
? TROUBLESHOOTING

(viii) Based on the yield of tRNA transcript, add an equimolar amount of molecular beacon with 3′ extension. The reaction volume can be up to 200 μl at this stage.

(ix) Anneal the sticky ends by heating the mixture to 70 °C for 2 min and then letting it return to room temperature slowly by placing it on the bench. Wait at least 10 min.

(x) Purify the tRNA–molecular beacon complex by native 8% PAGE using the Mini PAGE apparatus. The linked species will migrate more slowly than the free tRNA transcript and the molecular beacon. The band corresponding to the linked species will have the same color and fluorescence as free molecular beacon and can be cut from the gel. Elute the linked species by shaking the gel slice in 500 μl GE buffer overnight at room temperature.

(xi) Precipitate the conjugate by adding 1,250 μl 100% ethanol to the eluent. Air dry the resulting pellet[19].

(xii) Dissolve the pellet in 50 μl water or TE buffer. To avoid separating the tRNA from the molecular beacon, always keep the conjugate at temperatures of 25 °C or below.

(xiii) Determine the signal-to-background ratio of the tRNA–molecular beacon conjugate by measuring its fluorescence in 1-μM solutions (in molecular beacon buffer) in the presence and absence of the target oligonucleotide (2 μM) using a spectrofluorometer. The signal-to-background ratio should be greater than 25 for good imaging.
■ **PAUSE POINT** Store at −20 °C in 10-μl aliquots until needed.

Introducing tRNA–molecular beacon conjugates into cultured cells and imaging a cytoplasmic mRNA

2| Coat T4 culture dishes with a thin layer of gelatin by adding 1 ml of sterilized 0.2% (wt/vol) gelatin to each dish, incubating for 2 min, removing the gelatin solution and letting the dishes dry completely. Culture chicken embryonic fibroblasts in DMEM, supplemented with 10% FBS (if other cell types are needed, modify the culture conditions appropriately), on these dishes. T4 dishes have thin cover glass (0.17 mm) with a coating of conductive material at their bottom to permit controlled heating. Their temperature is maintained at 37 °C by a feedback controller. When an oil-immersion objective is used, the temperature of the objective is also maintained at 37 °C using a collar heater controlled by a second feedback controller.

3| Replace the culture medium in the dishes with Leibovitz's medium, which does not contain phenol red and therefore generates less background fluorescence.

4| Introduce the molecular beacons to cells either by microinjection (A) or by Oligofectamine-mediated transfection (B). Although Oligofectamine-mediated transfection is effective in delivering the probes to a large number of cells at the same time, it results in internalization of large clusters of probe and Oligofectamine that are visible in many cells as fluorescent spots. The microinjection procedure, by contrast, does not create such artifacts.

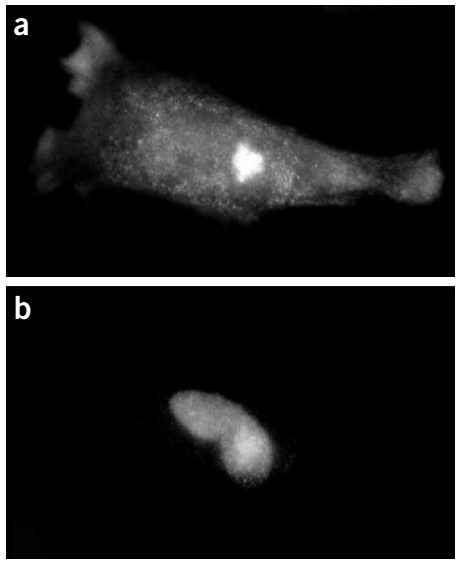

Figure 4 | Detection of β-actin mRNA in chicken embryonic fibroblasts using three tRNA-linked molecular beacons complementary to its coding sequence[13]. A mixture of tRNA-linked molecular beacon labeled with Texas red and a free molecular beacon labeled with tetramethylrhodamine (TMR) was injected into the same cell. The molecular beacons were specific for β-actin mRNA. (**a**) Texas red fluorescence. (**b**) TMR fluorescence. The lamellipodia are visible in **a** but not in **b**, whereas the nuclear sequestration of molecular beacons is prominent in **b** but not in **a**.

Option A: Microinjection

(i) Using a Femtojet microinjection apparatus, microinject a tRNA–molecular beacon conjugate (2 ng μl^{-1}). Place the cells in the cell incubator for 30 min at 37 °C.

Option B: Oligofectamine-mediated transfection

(i) Culture the cells to 70% confluency in T4 culture dishes as in Step 2.
(ii) Wash the cells with serum-free Opti-MEM1 and incubate at 37 °C in cell incubator for 1 h.
(iii) Separately dilute Oligofectamine (1 μl reagent and 9 μl Opti-MEM1) and molecular beacon–tRNA conjugate (30 ng conjugate in 10 μl Opti-MEM1) and incubate for 5 min at room temperature.
(iv) Mix the two solutions and incubate for 25 min at room temperature to form complexes.
(v) Dilute the mixture in 1 ml Opti-MEM1 and add to the cells after removing the previous medium. Place the cells in the incubator for 4 h at 37 °C.
(vi) Add 1 ml of normal medium with FBS and antibiotic and incubate overnight at 37 °C in cell incubator.

5| Replace the medium with Leibovitz's medium.

6| Image using the filter set appropriate for the molecular beacon fluorophore. Because of the limited number of target mRNA molecules in the cell, the fluorescence signals are usually very faint. To optimize the signals, we use photostable fluorophores such as tetramethylrhodamine (TMR) or Cy3; image using a highly cooled CCD camera, with the CCD detector at the base port of the microscope; and place the cells in phenol red–free medium. In order to identify the injected cells, a fluorescently labeled dextran can be co-injected.

? TROUBLESHOOTING

● TIMING

It takes 2–3 d to prepare tRNA-linked molecular beacons and 1 d to perform the imaging experiments.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Solution
Step 1A(vii)	Low yield of RNA	Increase the amount of DNA template in the transcription reaction.
Step 1A(xiv)	Very large precipitate	Pass the products through another NAP-5 column equilibrated with TE to remove the excess phosphate.
Step 1A(xv)	No coupling between tRNA and molecular beacon	Ensure that the amino and sulfhydryl groups are present on the molecular beacon and tRNA, respectively. Their presence can be confirmed by coupling with an amino-reactive and a sulfhydryl-reactive dye, respectively. If the groups are absent, resynthesize the molecular beacon and tRNA.
Step 1A(xv)	Poor coupling between tRNA and molecular beacon	Ensure that the 5′-thio-GMP is present at high concentration and GTP is present at low concentration in the transcription mixture. Remove the coupling agent more efficiently in Step 1A(x). Use fresh coupling agent in Step 1A(ix). Ensure the pH of the two phosphate buffers used in Steps 1A(ix) and 1A(x) is accurate.
Step 1B(vii)	Poor RNA yield	Increase the amount of DNA template in the transcription reaction.
Step 6	High background signals	Decrease the probe concentration.

ANTICIPATED RESULTS

A typical result obtained using a molecular beacon specific for β-actin mRNA is shown in **Figure 4a**. β-actin mRNA is localized in the lamellipodia of these motile cells. The probes conjugated to tRNA were able to illuminate the RNA in these regions of cytoplasm. If the tRNA is not linked to the molecular beacon, most of the fluorescence emanates from the nucleus (**Fig. 4b**).

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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