Isolation and characterization of fluorescence-enhancing RNA tags

Anna Wiesmayr, Andres Jäschke *

Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Im Neuenheimer Feld 364, Heidelberg 69120, Germany

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A B S T R A C T

Methods for the visualization of RNAs are urgently needed for studying a wide variety of cellular processes. Here we report on-bead screening of RNA libraries and its application to the isolation of specific fluorescence-enhancing RNA sequences. A one-bead-one-compound combinatorial RNA library with over one million different sequences was synthesized using the split-and-mix method. Solid-phase synthesis of 30 mer RNAs was performed on 15 μm and 60 μm diameter polystyrene beads bearing a non-cleavable linker. The RNA-derivatized beads were incubated with the well-established FlAsH pre-fluorophore and then screened for fluorescence enhancement, either by manually picking the brightest beads under a fluorescence microscope or by sorting with a FACS instrument. A protocol was established for sequence determination from single beads. While numerous RNA sequences showed increased fluorescence when immobilized, only few of them influenced the fluorescence properties of the FlAsH dye when detached from the beads. One of these sequences was found to induce a bathochromic shift in the excitation (from 492 to 510 nm) and emission (from 512 to 523 nm) maxima. This shift was accompanied by a 3.6-fold fluorescence enhancement of FlAsH fluorescence intensity. Mutation studies on the sequence revealed a rather robust structural motif.

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1. Introduction

Over the past decade it became evident that RNA molecules play a much wider role in cellular biology than previously anticipated.1 The ability to visualize the synthesis, processing, transport, interaction, and degradation of RNA in a living cell would provide a deeper understanding of RNAs roles and the underlying structural principles and thereby contribute to fields like molecular biology, disease pathophysiology or diagnostics.2,3 While the successful development of real-time imaging techniques for proteins has provided insight into various cellular processes, it remains challenging to develop similar tools for RNA imaging.4,5 One approach to RNA imaging utilizes green fluorescent protein (GFP) by fusion to an RNA-binding protein: Multiple repetitions of MS2 coat protein-binding hairpins are introduced in the 3'-untranslated region (3'-UTR) of the target RNA, which are then able to bind a MS2 coat protein-GFP fusion, thereby creating a strong fluorescence signal.5,7 However, the large size of the GFP tag that may influence the endogenous behavior of the target RNA and the high background fluorescence are strong limitations to this technique. Another approach uses hybridization of fluorogenic probes, whose fluorescence properties change upon binding to the respective RNA. Molecular beacons, for example, allow sequence-specific detection of RNA using a fluorophore-quencher pair.8 This technique is hampered by the problem of delivery of the probe and by potential accessibility problems due to stable secondary structures or RNA-binding proteins.

There is still a high demand for new approaches in the field of RNA imaging. Our aim is the development of a real-time imaging technique that enables the tracking of any RNA of interest from transcription to degradation. This could be achieved by in vivo transcription of the target RNA as a fusion to a specific short RNA tag, which would not interfere with its natural behavior. This genetically encoded tag should bind an externally provided, small pre-fluorophore and thereby enhance its fluorescence. RNA motifs that enhance the fluorescence of a dye have been described in the literature, for example, a 38-nucleotide malachite green aptamer that produces an over 2000-fold increase in fluorescence upon binding.9–11 A similar approach selected a 25-mer DNA aptamer that binds to a modified conventional oligonucleotide-staining dye (Hoechst) thereby restoring its fluorescence.12 Shortly thereafter Sando et al. could also demonstrate this concept for an RNA aptamer.13 High background fluorescence of unbound probes could be prevented by the introduction of a quencher in close proximity to the fluorophore, and selection of an RNA aptamer that suppresses...
the photoinduced electron transfer between fluorophore and quencher, thereby inducing fluorescence enhancement.\textsuperscript{14} Thus, relatively short RNA sequences are able to efficiently modulate the fluorescence properties of exogenous dyes.\textsuperscript{15,16} These sequences were, however, all isolated from gigantic synthetic combinatorial libraries (up to $10^{19}$ different sequences) using the iterative in vitro selection or SELEX process. In short, the RNA library was incubated with the immobilized dye, unbound RNA removed by washing, bound RNA was enzymatically amplified, and this process was repeated until a significant fraction bound tightly to the immobilized dye.\textsuperscript{17} Thus, selection is for binding strength and not for fluorescence enhancement, and there is currently no general proof that tight binding correlates with strong fluorescence enhancement.

Conventional screening of combinatorial libraries offers (compared to SELEX) the advantage that many more properties can be evaluated and exploited than just binding. In particular, one-bead-one-compound (OBOC) peptide libraries of moderate complexities have yielded interesting candidates in a variety of screening projects.\textsuperscript{18} In oligonucleotide chemistry, however, they have been used very rarely.\textsuperscript{19}

Here we describe a direct method for the identification of RNA sequences that enhance the fluorescence of a fluorophore of choice. Our approach utilizes on-bead screening of a randomized OBOC RNA library for fluorescence intensity. We have exploited this general concept for screening of unmodified RNA libraries for sequences with the ability to enhance the fluorescence of the well-known FlAsH (fluorescein arsenical helix binder) dye.\textsuperscript{20}

\section{Results}

\subsection{Design and synthesis of the one-bead-one-compound RNA library}

Combinatorial oligonucleotide libraries usually consist not only of randomized but also of conserved regions: indeed, constant primer binding sites are required for sequence determination by enzymatic amplification, cloning and sequencing. We took advantage of the need for fixed sequences within the library to introduce a defined secondary structure. The formation of secondary and tertiary structures is a common requirement for the interaction of aptamers with their respective targets,\textsuperscript{21} or for the interaction of oligonucleotides with dyes leading to fluorescence enhancement. To facilitate the formation of complex structures, the one-bead-one-compound RNA library was designed to form a hairpin structure, with the randomized part being (formally) single-stranded. According to these considerations, the design of the library in detail was the following (Fig. 1): a 30 mer hairpin consists of a 10 mer randomized region which is flanked by 10 conserved nucleotides at the 5’ and 3’ end that serve as primer binding sites and additionally ensure the formation of a stem, whereas the loop exposes the randomized nucleotides for interaction with the fluorophore. The fixed primer regions were chosen to be relatively short, in comparison to commonly used primers, to obtain a randomized region as large as possible while keeping the overall size in a range that can still be synthesized in a good yield.

The choice of an appropriate solid support is crucial for the successful synthesis and screening of the RNA library. After testing several commercial resins, a highly cross-linked polystyrene support was chosen that bears a non-cleavable hexaethyleneglycol linker to ensure that RNA sequences stay attached to the beads during deprotection. The individual beads of this resin are homogenous in size and show a high mechanical stability. Additionally, the beads should have an adequate diameter for screening with a fluorescence-activated cell sorter (FACS), and a size of 15–20 $\mu$m proved to smoothly pass a 90 $\mu$m nozzle without clogging. The polystyrene resin is non-swellable and has been reported to be compatible with both FACS and automated oligonucleotide synthesis.\textsuperscript{22} The conserved parts of the RNA sequences were synthesized on the solid support by standard automated phosphoramidite chemistry, whereas the combinatorial part of the library was prepared via the split–and–mix method.\textsuperscript{23} This ensures that every bead carries multiple copies of a single RNA sequence. The complexity of the OBOC library can be calculated to be $4^{10}$, hence over 1 million different sequences are present on the $\sim$40 million beads that correspond to $\sim$ 87 mg of resin. As a result every sequence is represented on multiple beads. The synthesis yield of the oligonucleotide library is critical since it is important to obtain sufficient amounts of full-length product to reach sufficient signal intensity in the screening assay, and to be able to carry out subsequent sequence determination. Therefore, the synthesis protocol had to be modified: due to the small size of the beads the flow resistance increased, which had to be compensated by increasing the duration of all delivery steps and the volume of reagents. It turned out to be particularly important to increase the volume of solvent used during the washing steps to ensure complete removal of reagents. With these optimizations, we obtained coupling efficiencies of $\sim$99% per cycle, which gave a total yield of about 75 fmol of RNA per bead.

\subsection{Choice of fluorophores}

Our strategy aims at discovering an RNA motif that can interact with a pre-fluorophore, whereupon the dye becomes fluorescent. The fluorescence of a dye depends on the electronic configuration of the dye and its vicinity and can in principle be influenced by site-specific recognition of an RNA motif, for example via the interaction of positively charged dyes with the negatively charged backbone of the RNA, by stacking of the dye into the structure or by restriction of the free rotation or vibration of constituents of the fluorophore upon binding to a defined RNA structure.\textsuperscript{24} Since our final goal is live-cell imaging, we only included non-charged, cell-permeable, and non-cytotoxic dyes in our considerations. Additionally, the dye should exhibit a low fluorescence in its unbound state to ensure low background fluorescence. For these reasons we chose the fluorescein derivative FlAsH as a candidate dye. It is widespread in live-cell protein imaging and binds strongly to tetracysteine-motifs in certain peptides,\textsuperscript{20} where the free rotation is hindered upon binding and the dye becomes fluorescent. We assumed that a binding-mediated fluorescence event may also occur when the binder is a specific RNA structure.

\subsection{On-bead screening}

During on-bead screening of the RNA library we need to separate RNAs on bead that show only low fluorescence enhancement of FlAsH (negative hit) from those exhibiting the desired high

![diagram](image.png)
fluorescence enhancement (positive hit). To facilitate the discrimination between positive and negative hits, suitable controls had to be prepared. As a positive control we coupled a tetracysteine-containing peptide\(^2\) (peptide 1, see Section 5) to the polystyrene resin. As a negative control we synthesized on the support a 30-mer RNA sequence (RNA 1, see Section 5) that carries the conserved regions and a randomly chosen sequence for the loop. For both samples fluorescence enhancement and emission spectrum were measured after incubation with 25 \(\mu\text{M FlAsH}\). Additionally, the solid support without any substrate was analyzed for unspecific sticking of the FlAsH dye to the hydrophobic polystyrene surface. Compared to the negative controls, the bead-bound peptide showed a 7.5-fold fluorescence enhancement after FlAsH incubation (180-fold compared to background), which permits a clear discrimination between positive and negative hits. The underivatized solid support proved to be only weakly fluorescent itself but did show a stronger fluorescence enhancement after incubation with FlAsH than the average RNA-functionalized beads (1.3-fold), which points to a non-specific sticking of the dye to the polystyrene surface. Taking this into account we had to carefully develop a washing protocol to remove the excess of the FlAsH dye.

The RNA library was incubated with 25 \(\mu\text{M FlAsH}\), subsequently washed and suspended in screening buffer (PBS, pH 7) including 50 mM MgCl\(_2\) to ensure proper RNA folding. A first on-bead screening of a small sub-library (10,000 beads) was performed by visual inspection under a fluorescence microscope with excitation at 514 nm. The five brightest beads of the library were picked manually under the microscope with a pipette with the assistance of a software algorithm that categorizes objects in look-up tables according to their fluorescence intensity. It turned out that inspection of the whole library by eye is impractical due to its size and to the difficulties associated with the manual picking of the selected beads. Therefore we opted for a different screening procedure utilizing FACS for the isolation of positive hits. Preliminary sorting experiments using the positive and negative control beads allowed the proper adjustment of the sorting parameters (Fig. 2). The positive control showed a 4.7-fold enhancement of fluorescence for all hits sorted in gate P2 compared to the negative hits sorted in gate P1 (excitation wavelength 488 nm instead of 514 nm during microscope screening). After this preliminary test we performed two rounds of sorting of the on-bead RNA library and isolated positive hits (gate P2) representing 0.001% of the original library. These beads showed an average fluorescence enhancement of 6.7-fold compared to the negative hits sorted in P1.

To verify the difference in fluorescence enhancement observed during FACS screening between beads sorted in P1 or P2, we performed spectral analysis of single beads with a fluorescence microscope. For this purpose we randomly picked 10 beads of the positive hits sorted in gate P2 and 10 beads of the negative hits sorted in gate P1 (all isolated from the on-bead RNA library) and 50 mM MgCl\(_2\) after FlAsH incubation. A first on-bead screening (Fig. 2). The positive control showed a 4.7-fold enhancement of fluorescence for all hits sorted in gate P2 compared to the negative hits sorted in gate P1 (excitation wavelength 488 nm instead of 514 nm during microscope screening). After this preliminary test we performed two rounds of sorting of the on-bead RNA library and isolated positive hits (gate P2) representing 0.001% of the original library. These beads showed an average fluorescence enhancement of 6.7-fold compared to the negative hits sorted in P1.

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For sequence determination, reverse transcription on bead and subsequent PCR amplification are necessary. Several issues make these steps difficult: the presence of the hydrophobic polystyrene support may hinder the polymerase, and strong RNA secondary structures, high melting temperatures due to GC-rich regions of the library and low amounts of RNA on bead further complicate the task. For these reasons optimization of RT and PCR had to be done. Several temperatures were tested for the RT reaction; the best results were obtained at 50 °C. After RT, the bead carrying the cDNA/RNA hybrid was taken out of the reaction solution and transferred into the PCR reaction vial, since it turned out to give
higher yields for the amplification (data not shown). The utilized primer sequences for the PCR reaction are relatively short (10 mer) and partially self-complementary as a result of the library design. Therefore the amplification reaction was optimized with respect to Mg²⁺ ion and primer concentration, annealing temperature and number of cycles. A combination of 2 mM MgCl₂, 0.7 µM of each primer and 5% DMSO using 65.7 °C for the annealing step led to a sharp product band after 30 cycles monitored by a high resolution agarose gel. The PCR product was cloned via T/A-cloning (Invitrogen) and sequenced. The reliability of this protocol for sequence determination of RNA on bead was confirmed by applying it to a known RNA sequence on bead. Table 1 shows the obtained sequences from the RNAs on bead isolated by manual picking under a fluorescence microscope or by FACS. For one positive hit we obtained two different sequences (RNA F1 and F2) within a similar number of clones. Therefore we decided to do further analysis with both sequences.

2.5. Fluorescence measurements with isolated RNA

After sequence determination all seven RNA sequences were re-synthesized either by T7 transcription of the respective DNA templates or by automated RNA synthesis. We measured re-synthesized RNA in solution at different concentrations with FlAsH dye in screening buffer to reproduce the changes in the fluorescence properties of FlAsH observed during on-bead screening. Disappointingly, all sequences that had been manually picked under the fluorescence microscope (RNA M1–5) showed only a 1.1–1.4-fold enhancement in fluorescence compared to the background. In contrast, the sequences RNA F1 and F2 isolated from the FACS screening showed a 3–3.6-fold enhancement of fluorescence at RNA concentrations of 50 µM and 1 µM FlAsH dye (Table 1).

Increasing the concentration of RNA F1 and F2 at constant dye concentration (Fig. 4) leads to a consistent increase in fluorescence enhancement, in contrast RNA M1 shows decreasing fluorescence intensities with increasing concentrations. A clear difference between sequences RNA F1, F2, and RNA M1 can be observed at equimolar concentrations of RNA and FlAsH dye. Binding of fluorophores by oligonucleotides is often accompanied by a shift in the dye’s excitation and emission maximum. FlAsH itself is weakly fluorescent but when bound to four cysteine residues in defined peptides, free rotation of the substituents is hindered and a bright fluorescence signal is observed. In our system, the emission maximum is shifted from 512 nm for unbound FlAsH to 529 nm for the peptide-bound dye. The shift to 523 nm observed when incubating FlAsH with sequences RNA F1 and F2 suggests that a similar restriction of rotation may be occurring, even in the absence of any thiol. This conclusion is supported by the observation that both negative controls, RNA 1 and a ssDNA (DNA 1, see Section 5) show no significant shift in the emission maximum of FlAsH (Table 2). Furthermore the excitation maximum of FlAsH was shifted from 492 nm (unbound dye) to 510 nm when incubated with 50 µM of RNA F2.

2.6. Rational mutations of isolated RNA sequences F1 and F2

To investigate the structural requirements for fluorescence enhancement, several mutations were introduced into the conserved parts of RNA F1 and F2. These mutations included changes in the length and sequence of the hairpin stem, and the (partial) removal of the dangling ends (Fig. 5). With one exception, the effects turned out to be rather small, suggesting that the interaction between RNA and dye takes place inside the randomized loop and does not involve other parts of the nucleic acid. Remarkably, even the exchange of the base pair right next to the randomized loop (upper closing base pair) from G–C to U–A is tolerated. Unexpectedly, the addition of an A–U base pair at the remote end of the stem abolishes fluorescence even below the value of the unbound FlAsH dye, thus apparently converting a fluorescence enhancer into a fluorescence quencher.

3. Discussion

The work presented here contributes to the young field of RNA imaging. While a true nucleic acid analogue to GFP (i.e., an RNA molecule that generates a fluorophore by folding and/or rearrangement from the four genetically encoded standard nucleotides) has
not been discovered yet, a number of aptamers were described that modulate the fluorescence of exogenous dyes. Here we evaluated an alternative strategy to SELEX to isolate RNA sequences with fluorescence-enhancing properties. Whereas SELEX is based solely on binding between an aptamer and a target, the method described here allows to screen directly for fluorescence enhancement. A further advantage of the on-bead technique is the possibility to recycle the library after screening, simply by thorough washing.

The identification and isolation of fluorescence-enhancing RNAs on bead with a FACS instrument was superior to the visual inspection under a fluorescence microscope: firstly, only the RNA sequence isolated via FACS showed the ability for fluorescence enhancement not only while being immobilized on a bead but also free in solution. Secondly, FACS is more practical in terms of fast screening of millions of beads and easy isolation of positive hits, in contrast to manual picking, which demonstrates its applicability as a high-throughput technique. The automated screening of ~4 million beads led to much more potent fluorescence-enhancing tags than the manual screening of 10,000 beads (see Table 1).

We demonstrated that the FlAsH dye, well-known from the field of protein imaging, is also able to interact with a defined RNA structure which triggers a change of its fluorescence properties even without any thiol present. Further characterization of the RNA–FlAsH interaction is under way, in particular regarding the binding mode and the three-dimensional structure. The screening system reported here is not limited to FlAsH: it is applicable to virtually any pre-fluorophore that is suitable for live-cell imaging. A promising class are solvatochromes, dyes that change their fluorescence depending on their environment and are rendered highly fluorescent in non-polar solvents. Such a non-polar environment could be provided by a defined folding of a short RNA tag.

The hairpin design of the library is only one of several conceivable architectures. In a very similar fashion, constant sequence elements may be utilized for the construction of libraries based on pseudoknots, asymmetric bulges, or three-way junctions, thereby presenting the randomized sequence part in different structural frameworks.

4. Conclusion

By combination of combinatorial solid phase synthesis, fluorescence-activated cell sorting, and molecular biology methods we developed a technique for on-bead screening of RNA libraries for their ability to enhance the fluorescence of an exogenous prefluorophore. Although the measured fluorescence enhancement of 3.6-fold remained moderate, we could demonstrate the general applicability of this method. This is the first time, to our knowledge, that on-bead screening of combinatorial RNA libraries for fluorescence enhancement of pre-fluorophores has been reported. This technique could provide a powerful tool for the development of small RNA tags for live-cell imaging.

5. Experimental

All reagents were purchased from Acros or Sigma-Aldrich and used without further purification. TLC was carried out on silica gel plates Polygram Sil G/UV254 (40 × 80 mm) from Macherey-Nagel. Flash chromatography was carried out on silica gel 40–63 μm from J. T. Baker. Reversed-phase HPLC analysis was performed on an Agilent 1100 Series HPLC system equipped with a diode array detector using a Phenomenex Luna C18 5 μm column (4.6 × 250 mm) and eluting with a gradient of 100 mM triethylammonium acetate pH 7.0 (buffer A) and 100 mM triethylammonium acetate in 80% acetonitrile (buffer B) at a flow rate of 1 ml/min. HPLC purification was performed with a semi-preparative Phenomenex Luna C18 5 μm column (15.0 × 250 mm) using a flow rate of 5 ml/min. NMR spectra were recorded on a Varian Mercury Plus 500 MHz spectrometer. FAB and El mass spectra were recorded on a JEOL JMS-700 sector field mass spectrometer. MALDI-TOF mass spectra were recorded on a Bruker Biflex III using either dihydroxybenzoic acid (DHB) or 3-hydroxypicolinic acid (3-HPA) as matrix. Oligonucleotide synthesis was performed on an Expedite™ 8909 automated synthesizer using standard reagents from Sigma-Aldrich Proligo. Agarose gels were stained with...
ethidium bromide and visualized by UV illumination using an Alphalager™ 2200. Denaturing polyacrylamide gels were stained with SYBR gold and visualized by a Typhoon 9400 imager. Analysis was carried out with Image Quant software (Version 5.2). For desalting oligonucleotides, Zip tip® pipette tips purchased from Millipore were used.

5.1. Synthesis of the RNA library

Standard phosphoramidite chemistry was used. Two libraries were prepared on a non-cleavable polystyrene support carrying a DMT-protected aliphatic hydroxyl function: Library A: 1 μmol scale on 60–70 μm non-cleavable polystyrene support (ChemGenes Corp.), 300 Å, loading capacity 60 μmol/g, 4.7 × 10^6 beads/mg. Library B: 4 x 1 μmol scale on 15–20 μm non-cleavable polystyrene support (ChemGenes Corp.), loading capacity 60 μmol/g, 6 × 10^6 beads/mg. Library A with larger beads was screened by visual inspection under a fluorescence microscope. Library B was screened with a FACS instrument.

The conserved 20 nucleotides were synthesized in a standard fashion, the 10 randomized positions via the split-and-mix technique. All washing steps during the synthesis were performed with a doubled volume of dry ACN and a 1.5-fold duration compared to standard protocols. After the oxidation and deblocking step the volume of dry ACN was increased to threefold. The volume of deblocking solution (3% TCA in DCM) was increased by 25%. For coupling of the monomer three steps instead of two were used, with a 1.4-fold duration compared to standard protocols. For the split-and-mix synthesis the resin was dried under vacuum, divided in four equal portions, each of which was packed into one column. Then each column was attached to the synthesizer and coupled with one of the four nucleoside phosphoramidites. After completion of the synthesis cycle the resin was again dried under vacuum, each column was opened and the resin combined. These steps were repeated 10 times to obtain a 10 mer randomized region. The average coupling yield was >99% as determined by the dimethoxytrityl cation assay. The deprotection of the RNA library was carried out in ammonia/ethanol 3:1 for 2 h at room temperature, followed by thoroughly washing of the beads with EtOH/ACN/H₂O 3:1:1. The TBDMS protecting groups were removed by treatment with TBAF in THF for 24 h at room temperature, followed by thoroughly washing of the beads with EtOH/ACN/H₂O 3:1:1. The beads were dried under vacuum.

5.2. Synthesis of FlAsH-EDT₂

FlAsH-EDT₂ was synthesized as previously described. The product was stored in a 2 mM stock solution in DMSO protected from light at −20 °C. Dilutions were done in screening buffer.

5.3. Preparation of positive and negative control

5.3.1. Positive control

Peptide 1 was purchased from Biosyntan Berlin, Sequence Ac-WDCCPGCCK-amide) was attached to the polystyrene beads (10–15 μm, same as used for RNA library) via a succinate linker: The beads were first detritylated with 3% TCA in DCM to obtain the free hydroxyl group. After washing with EtOH/ACN/H₂O 3:1:1 and drying under vacuum the beads (1 μmol, 16.66 mg) were suspended in a flask in 1.5 ml pyridine. DMAP (0.2 equiv) and succinic anhydride (10 equiv) were added and the suspension was stirred for 12 h at room temperature. The beads were washed with DMF and pyridine and dried under vacuum. A qualitative colorimetric test for carboxylic acids was done with malachite green as described. The carboxylate-derivatized beads (300 nmol) were suspended in 0.25 ml DMF (amine-free) in an Eppendorf tube followed by addition of TBDU (4.9 equiv) and HOBT (4.9 equiv). After addition of DIPEA (10 equiv) a solution of peptide 1 (500 nmol) in 0.25 ml DMF was immediately added. The suspension was shaken on a thermostaker at room temperature and 500 rpm for 2.5 h followed by several washing steps with DMF, afterward the beads were dried under vacuum. Quantification of the peptide immobilized on bead was performed using Ellmann’s reagent. The yield was calculated from a calibration curve (peptide 1 0–100 μM with 3.75 mM Ellmann’s reagent) to be 15 μmol/g. It has to be considered that thiols are prone to formation of disulfide bonds, which will not react with Ellmann’s reagent. Potentially present thiols were reduced by treatment with 0.1 M DTT prior to incubation with FlAsH and fluorescence measurements.

5.3.2. Negative control

A 30 mer hairpin construct that carries the conserved regions and a randomly chosen sequence for the loop was synthesized in a standard fashion on the same polystyrene support that was used for the RNA library using the same modifications to the synthesis cycle. RNA 1: 5'-GUGACCGCGGAUCGACUUCACCGCGCAGUG-3'.

5.4. Incubation of the RNA library and the controls with FlAsH

RNAs on bead (negative control and RNA library) were heated to 50 °C for 5 min in screening buffer and cooled down to room temperature to allow for proper folding. Peptide on bead (positive control) was treated with 0.1 M DTT in SB for 20 min at 45 °C followed by several washing steps with SB. Controls and the RNA library were incubated with 25 μM FlAsH (incl. 25 μM BAL) in SB at room temperature for 2 h followed by thoroughly washing with BAL 250 μM in SB, SB, DMSO 20% in SB, SB, BAL was used to restore the dithiol complex formed with the arsenic moiety of the FlAsH dye, since this complex is known to be unstable under certain conditions. All washing steps were carried out in a centrifugal filter (VWR). All samples were resuspended in SB and subjected to screening by FACS or fluorescence microscopy.

5.5. On-bead screening of the RNA library

5.5.1. Fluorescence microscopy

Visual inspection of over 10,000 beads (60–70 μm) was done under a fluorescence microscope Nikon AZ100 (Nikon Imaging Center, Bioquant, Heidelberg) using a GFP fluorescence filter and 2 x objective. Manually picking was done with an Abimed 2 μl pipette.

Spectral analysis of single beads was done with a Nikon A1R (Nikon Imaging Center, Bioquant, Heidelberg) using a 514 nm laser line and 32 channels for imaging.

5.5.2. FACS

A BD FACSARia instrument was used for screening of over 4 million of beads (15–20 μm), hence ~10% of the original library. Excitation was done with an Octagon laser (488 nm blue laser), emission signals were collected through a FITC filter (530/30 nm). Samples were acquired on three parameters: FSC (forward scatter for size), SSC (side scatter), and FITC signal.

5.6. Sequence determination

5.6.1. Reverse transcription

A selected single bead was mixed with following components: 11.4 μl H₂O, 1 μl dNTP (10 mM) and 0.5 μl primer 1 (10 μM). The reaction mixture was heated to 65 °C for 5 min followed by a quick
chill on ice. Addition of 4 μl first strand buffer (Invitrogen) and 2 μl DTT (100 mM) was followed by heating to 42 °C for 2 min. After the reaction was cooled down to room temperature 1 μl of Super Script II RT (Invitrogen) was added. The RT reaction was carried out in a PTC 100 (Biozym) cycler at 50 °C for 55 min. The enzyme was heat inactivated by heating to 70 °C for 15 min.

5.6.2. PCR

The single bead was transferred from the RT reaction (by manually picking under a Nikon SMZ 1500 microscope) into a microcentrifuge tube and the following PCR components were added: 31 μl H2O, 5 μl PCR buffer (Rapidozym), 2 μl MgCl2 (50 mM), 1 μl dNTP (10 mM), 3 μl primer 1 + 2 (10 μM), 2.5 μl DMSO, 0.5 μl Taq DNA polymerase (Rapidozym). The PCR was run on a PTC 100 cycler (Biozym) using following program: 95 °C for 3 min (one cycle); 95 °C for 1 min, 65.7 °C for 1 min, 73 °C for 1 min (30 cycles); 73 °C for 7 min (one cycle). The PCR reaction was analyzed on a high resolution agarose gel (Invitrogen).

5.6.3. TA cloning

The unpurified PCR product was cloned using the T/A cloning procedure (Invitrogen).

5.6.4. Sequencing

Sequencing was done by SeqLab (Göttingen) using the M13 forward and reverse primer binding sites present in the vector.

5.6.5. Primer sequences

Primer 1: 5'-ATAGGATCCACTGCGCGG-3';
Primer 2: 5'-ACTGAATTCGTGACCGCGG-3'.

5.7. Re-synthesis of determined RNA sequences

Determined RNA sequences and modified RNA sequences were either synthesized by automated oligonucleotide synthesis on CPG in a standard fashion or by T7 transcription of the respective DNA templates (IBA, Göttingen). All products were purified by semi-preparative reverse-phase HPLC or by 15% denaturing PAGE. Analysis was done by reverse-phase HPLC and mass spectrometry (MALDI).

5.8. Fluorescence measurements

Fluorescence spectra were obtained at 25 °C using a JASCO FP-6500 spectrofluorometer. RNA was diluted with SB to concentrations from 0.1–50 μM followed by addition of 1 μM FlAsH. Background fluorescence was measured with FlAsH 1 μM in SB. All solutions were incubated for 1 h at room temperature prior to fluorescence measurements. The solutions were excited at 488 or 508 nm and the emission was monitored in the 498/518–620 nm wavelength range. The enhancement of fluorescence was determined by comparing the intensity of fluorescence emissions at 530 nm.

5.8.1. Sequence of negative control DNA 1

5'-CAGCTGCCGGCCTCGGTACTACCGCGGTCTACATTAGTGATGCATTA-3'.

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References and notes