



# Isolation and characterization of fluorescence-enhancing RNA tags

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## ABSTRACT

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  $\mu$ m and 60  $\mu$ m diameter polystyrene beads bearing a non-cleavable linker. The RNA-derivatized beads were incubated with the well-established FIAsH 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 FIAsH 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 FIAsH 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.<sup>1</sup> 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.<sup>2,3</sup> 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.<sup>4,5</sup> 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.<sup>6,7</sup> 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.<sup>8</sup> 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.<sup>9–11</sup> A similar approach selected a 25-mer DNA aptamer that binds to a modified conventional oligonucleotide-staining dye (Hoechst) thereby restoring its fluorescence.<sup>12</sup> Shortly thereafter Sando et al. could also demonstrate this concept for an RNA aptamer.<sup>13</sup> 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

**Abbreviations:** ACN, acetonitrile; BAL, 2,3-dimercaptopropanol; CPG, controlled pore glass; DCM, dichloromethane; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; DMT, dimethoxytrityl; EDT<sub>2</sub>, (1,2-ethanedithiol)<sub>2</sub>; EtOH, ethanol; h, hours; HOBt, hydroxybenzotriazole; min, minutes; SB, screening buffer (PBS incl. 50 mM MgCl<sub>2</sub> 1 mM CaCl<sub>2</sub> pH 7); TCA, trichloroacetic acid; TBTU, O-benzotriazole-1-yl-N,N,N',N'-tetramethyluronium tetrafluoroborate; TEA, triethylamine.

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the photoinduced electron transfer between fluorophore and quencher, thereby inducing fluorescence enhancement.<sup>14</sup> Thus, relatively short RNA sequences are able to efficiently modulate the fluorescence properties of exogenous dyes.<sup>15,16</sup> These sequences were, however, all isolated from gigantic synthetic combinatorial libraries (up to  $10^{15}$  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.<sup>17</sup> 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.

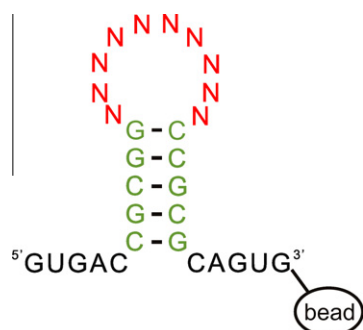
Conventional screening of combinatorial libraries offers (compared to SELEX) the advantage that many more properties can be evaluated and exploited than just binding. In particular, one-bead-one-compound (OBOC) peptide libraries of moderate complexities have yielded interesting candidates in a variety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

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 FIAsh (fluorescein arsenical helix binder) dye.<sup>20</sup>

## 2. Results

### 2.1. 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,<sup>21</sup> 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



**Figure 1.** Design of the RNA library: 10 conserved nucleotides at the 3' and 5' end (black/green) serve as primer binding sites for subsequent sequence determination. The green colored nucleotides are responsible for stem formation. Red colored nucleotides indicate the randomized loop.

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  $\mu\text{m}$  proved to smoothly pass a 90  $\mu\text{m}$  nozzle without clogging. The polystyrene resin is non-swellable and has been reported to be compatible with both FACS and automated oligonucleotide synthesis.<sup>22</sup> 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.<sup>23</sup> 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  $\sim 67$  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.

### 2.2. 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.<sup>24</sup> 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 FIAsh as a candidate dye. It is widespread in live-cell protein imaging and binds strongly to tetracysteine-motifs in certain peptides,<sup>20</sup> 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.

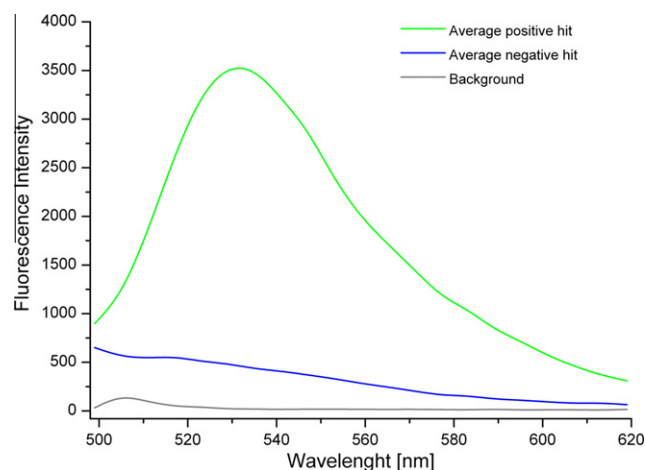
### 2.3. On-bead screening

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

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<sup>25</sup> (**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  $\mu$ M FIAsh. Additionally, the solid support without any substrate was analyzed for unspecific sticking of the FIAsh dye to the hydrophobic polystyrene surface. Compared to the negative controls, the bead-bound peptide showed a 7.5-fold fluorescence enhancement after FIAsh 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 FIAsh 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 FIAsh dye.

The RNA library was incubated with 25  $\mu$ M FIAsh, subsequently washed and suspended in screening buffer (PBS, pH 7) including 50 mM MgCl<sub>2</sub> 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)



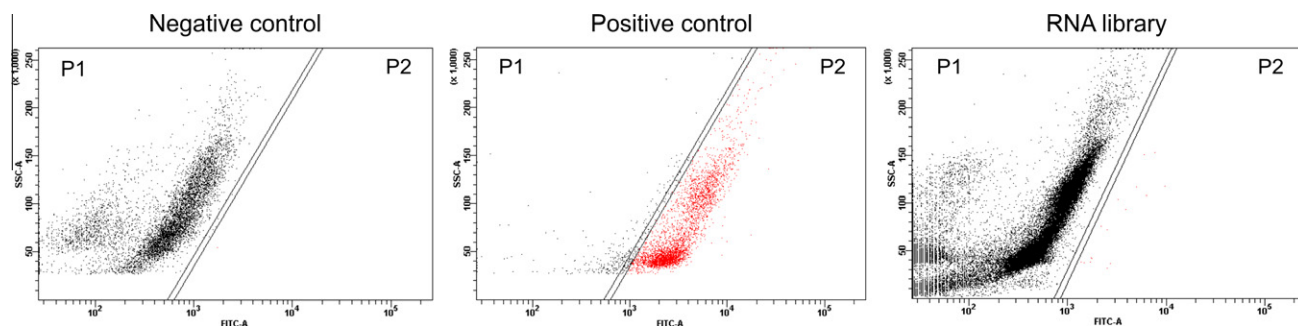
**Figure 3.** Average emission spectra of positive and negative hit beads from the on-bead RNA library isolated by FACS in screening buffer (PBS, 50 mM MgCl<sub>2</sub>, pH 7), 6 nM FIAsh, with excitation at 488 nm. Positive hits (sorted in gate P2, shown as green curve) show a 6.7-fold enhancement of fluorescence compared to the negative hits (sorted in P1, shown as blue curve) and the typical emission maximum of FIAsh (530 nm). The background represents the fluorescence of FIAsh in screening buffer (gray curve).

(Fig. 3). The average positive hit showed on bead a 6.7-fold higher fluorescence emission than the negative hits, and the typical emission maximum of FIAsh at 530 nm can only be observed for the positive hits. Out of the positive hits we selected one single bead for further characterization that showed a bathochromic shift in emission and a sixfold increase in fluorescence.

The RNAs on bead isolated by manual picking under the fluorescence microscope and by FACS underwent all steps for sequence determination (RT-PCR, T/A-cloning, sequencing) utilizing the defined 5' and 3' fixed oligonucleotide primer sequences flanking the combinatorial library segment.

## 2.4. Sequence determination of RNA on bead

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



**Figure 2.** Dotplots from FACS analysis of: negative control: bead-bound RNA, positive control: bead-bound peptide, on-bead RNA library. In gate P1 (black) all negative and in P2 (red) all positive hits were sorted.

**Table 1**

Fluorescence enhancement of re-synthesized RNA sequences in solution after incubation with FIAsh

|               | Isolated RNA sequences <sup>a</sup> | Fluorescence enhancement <sup>b</sup> |
|---------------|-------------------------------------|---------------------------------------|
| <b>RNA M1</b> | GUGACCGCGGUAUUCGUAUCCGCGCAGUG       | 1.1                                   |
| <b>RNA M2</b> | GUGACCGCGGUAUACCGAGCGCGCAGUG        | 1.1                                   |
| <b>RNA M3</b> | GUGACCGCGGACACGCUUACCGCGCAGUG       | 1.1                                   |
| <b>RNA M4</b> | GUGACCGCGGCUUUCUACCGCGCAGUG         | 1.4                                   |
| <b>RNA M5</b> | GUGACCGCGGCUCAUAGUCCGCGCAGUG        | 1.3                                   |
| <b>RNA F1</b> | GUGACCGCGGAUAGGAUCCACCGCGCAGUG      | <b>3.0</b>                            |
| <b>RNA F2</b> | GUGACCGCGGAUAGGAUCCACCGCGCAGUG      | <b>3.6</b>                            |

<sup>a</sup> RNA sequences of isolated positive hits: sequences **M1–M5** isolated by manual picking under a fluorescence microscope, sequence **F1–F2** isolated by FACS.

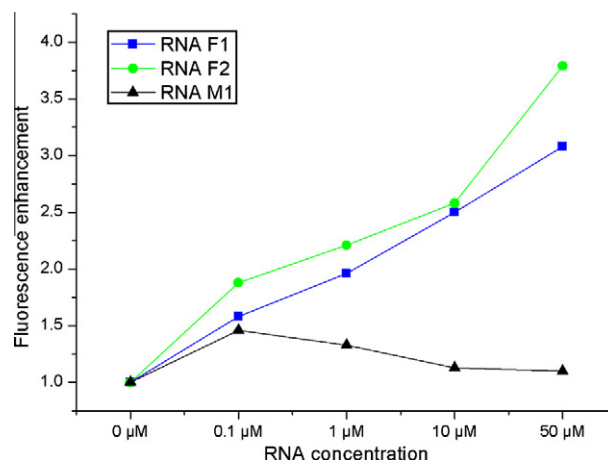
<sup>b</sup> Fluorescence enhancement (excitation 508 nm, emission 530 nm) of isolated RNA sequences (50  $\mu$ M) when incubated with FIAsh (1  $\mu$ M) in screening buffer in comparison to background (FIAsh dye 1  $\mu$ M in screening buffer) set to 1.

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  $Mg^{2+}$  ion and primer concentration, annealing temperature and number of cycles. A combination of 2 mM  $MgCl_2$ , 0.7  $\mu$ M 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 FIAsh dye in screening buffer to reproduce the changes in the fluorescence properties of FIAsh observed during on-bead screening. Disappointingly, all sequences that had been manually picked under the fluorescence microscope (**RNA M1–M5**) 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  $\mu$ M and 1  $\mu$ M FIAsh 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 FIAsh dye. Binding of fluorophores by oligonucleotides is often accompanied by a shift in the dye's excitation and emission maximum. FIAsh 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 FIAsh to 529 nm for the peptide-bound dye. The shift to 523 nm observed when incubating FIAsh 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



**Figure 4.** Fluorescence enhancement of isolated RNA sequences **RNA F1**, **RNA F2**, and **RNA M1** in increasing concentrations when incubated with FIAsh (1  $\mu$ M). Background (FIAsh dye 1  $\mu$ M in screening buffer) was set to 1.

negative controls, **RNA 1** and a ssDNA (**DNA 1**, see Section 5) show no significant shift in the emission maximum of FIAsh (**Table 2**). Furthermore the excitation maximum of FIAsh was shifted from 492 nm (unbound dye) to 510 nm when incubated with 50  $\mu$ M 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 FIAsh dye, thus apparently converting a fluorescence enhancer into a fluorescence quencher.

## 3. Discussion

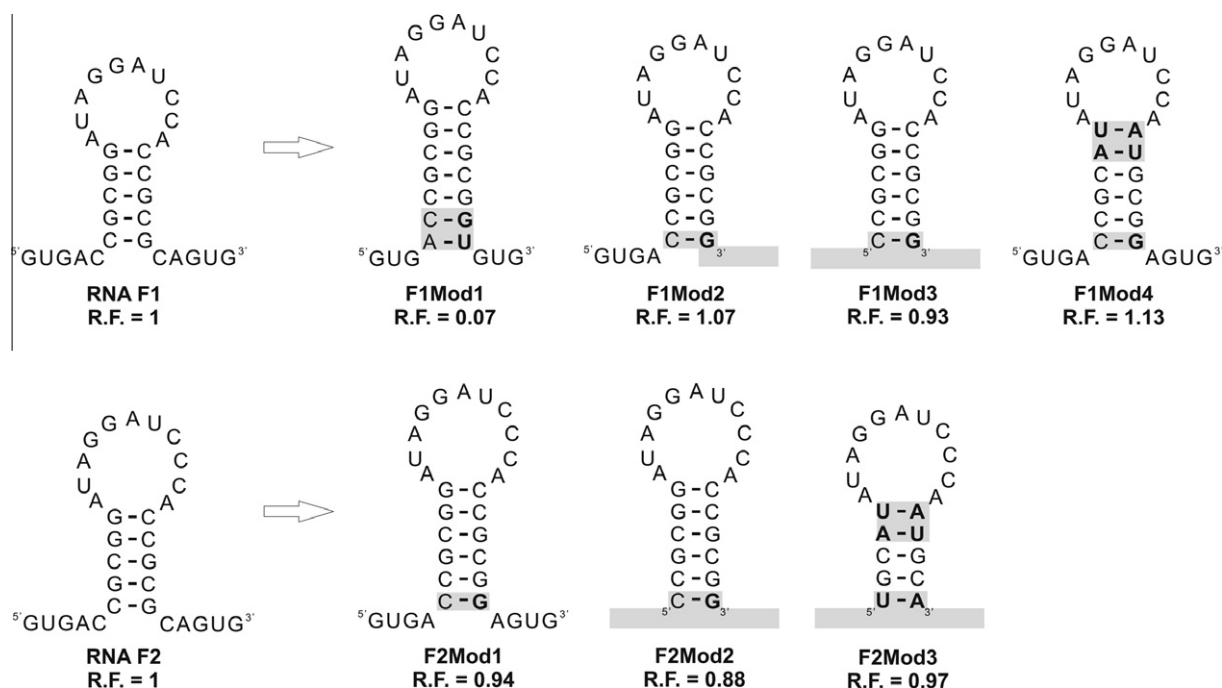
The work presented here contributes to the young field of RNA imaging. While a true nucleic acid analogue to GFP (i.e., an RNA molecule that generates a fluorophore by folding and/or rearrangement from the four genetically encoded standard nucleotides) has

**Table 2**

Emission maxima (nm) of FIAsh at a constant concentration of 1  $\mu$ M in screening buffer in the presence of oligonucleotide/peptide at increasing concentrations (1, 10, and 50  $\mu$ M): negative controls: **RNA 1**, **DNA 1**; positive control: **peptide 1**

|                  | Emission maxima (nm) of FIAsh (1 $\mu$ M) |            |            |
|------------------|---|------------|------------|
|                  | 1 $\mu$ M                                 | 10 $\mu$ M | 50 $\mu$ M |
| <b>RNA 1</b>     | 517                                       | 517        | 518        |
| <b>DNA 1</b>     | 516                                       | 516        | 516        |
| <b>RNA F1</b>    | 517                                       | 523        | 523        |
| <b>RNA F2</b>    | 518                                       | 523        | 523        |
| <b>Peptide 1</b> | 529                                       | 529        | 529        |

FIAsh concentration 1  $\mu$ M in screening buffer. A clear bathochromic shift could be observed for **RNA F1** and **F2** and the positive control. Negative controls did not change the emission maximum of FIAsh.



**Figure 5.** Modified sequences from original sequence **RNA F1: F1Mod1–4** and **RNA F2: F2Mod1–3**. Modified nucleotides are printed in bold, modified structures are highlighted in gray. R.F. = relative fluorescence of modified sequences (RNA 50  $\mu$ M, FIAsH 1  $\mu$ M in screening buffer) compared to the respective original sequences.

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  $\sim 4$  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 FIAsH 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–FIAsH interaction is under way, in particular regarding the binding mode and the three-dimensional structure. The screening system reported here is not limited to FIAsH: 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 pre-fluorophore. 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/UV<sub>254</sub> (40  $\times$  80 mm) from Macherey–Nagel. Flash chromatography was carried out on silica gel 40–63  $\mu$ m 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  $\mu$ m column (4.6  $\times$  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  $\mu$ m column (15.0  $\times$  250 mm) using a flow rate of 5 ml/min. NMR spectra were recorded on a Varian Mercury Plus 500 MHz spectrometer. FAB and EI 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-hydroxyphenylacetic acid (3-HPA) as matrix. Oligonucleotide synthesis was performed on an Expedite™ 8909 automated synthesizer using standard reagents from Sigma–Aldrich Prologo. Agarose gels were stained with

ethidium bromide and visualized by UV illumination using an AlphaImager™ 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<sub>C18</sub> 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,  $4.7 \times 10^4$  beads/mg. **Library B**:  $4 \times 1$  μmol scale on 15–20 μm non-cleavable polystyrene support (ChemGenes Corp.), loading capacity 60 μmol/g,  $6 \times 10^5$  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/EtOH 3:1 for 2 h at room temperature, followed by thoroughly washing of the beads with EtOH/ACN/H<sub>2</sub>O 3:1:1. The TBDMS protecting groups were removed by treatment with 1 M TBAF in THF for 24 h at room temperature, followed by thoroughly washing of the beads with EtOH/ACN/H<sub>2</sub>O 3:1:1. The beads were dried under vacuum.

### 5.2. Synthesis of FIAsh-EDT<sub>2</sub>

FIAsh-EDT<sub>2</sub> was synthesized as previously described.<sup>25</sup> The product was stored in a 2 mM stock solution in DMSO protected from light at –20 °C. Dilutions were done in screening buffer.

<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>, 25 °C, TMS):  $\delta$  = 3.44–3.69 (m, 8H),  $\delta$  = 6.51 (d, 2H,  $J$  = 8.7 Hz),  $\delta$  = 6.62 (d, 2H,  $J$  = 8.7 Hz),  $\delta$  = 7.20 (d, 1H,  $J$  = 7.5 Hz),  $\delta$  = 7.62 (dt, 1H,  $J$  = 1.2 and 7.5 Hz),  $\delta$  = 7.69 (dt, 1H,  $J$  = 1.4 and 7.4 Hz),  $\delta$  = 8.00 (d, 1H,  $J$  = 7.2 Hz),  $\delta$  = 9.90 (s, 2H). MS (MALDI-TOF):  $m/z$  664.6 (calcd for [(C<sub>24</sub>H<sub>19</sub>O<sub>5</sub>As<sub>2</sub>S<sub>4</sub>)<sup>+</sup> 663.8).

### 5.3. Preparation of positive and negative control

#### 5.3.1. Positive control

**Peptide 1**<sup>25</sup> (purchased from Biosyntan Berlin, Sequence Ac-WDCCPGCK-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/H<sub>2</sub>O 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.<sup>26</sup> The carboxylate-derivatized beads (300 nmol) were suspended in 0.25 ml DMF (amine-free) in an Eppendorf tube followed by addition of TBTU (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 thermoshaker 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.<sup>26</sup> 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 dithiols were reduced by treatment with 0.1 M DTT prior to incubation with FIAsh 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 FIAsh

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 FIAsh (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 FIAsh dye, since this complex is known to be unstable under certain conditions.<sup>27</sup> 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× 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 H<sub>2</sub>O, 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 H<sub>2</sub>O, 5 µl PCR buffer (Rapidozym), 2 µl MgCl<sub>2</sub> (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'-ATAGGATCCCACTGCGCGG-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 FIAsh. Background fluorescence was measured with FIAsh 1 µM in SB. All solutions were incubated for 1 h at room temperature prior to fluo-

rescence 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'-CACTGCGCGGCTCGGTACTACCGCGGTCACTATAGTGAGTCGTA TTA-3'.

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