Fluorophore ligand binding and complex stabilization of the RNA Mango and RNA Spinach aptamers

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ABSTRACT

The effective tracking and purification of biological RNAs and RNA protein complexes is currently challenging. One promising strategy to simultaneously address both of these problems is to develop high-affinity RNA aptamers against taggable small molecule fluorophores. RNA Mango is a 39-nucleotide, parallel-stranded G-quadruplex RNA aptamer motif that binds with nanomolar affinity to a set of thiazole orange (TO1) derivatives while simultaneously inducing a 103-fold increase in fluorescence. We find that RNA Mango has a large increase in its thermal stability upon the addition of its TO1-Biotin ligand. Consistent with this thermal stabilization, RNA Mango can effectively discriminate TO1-Biotin from a broad range of small molecule fluorophores. In contrast, RNA Spinach, which is known to have a substantially more rigid G-quadruplex structure, was found to bind to this set of fluorophores, often with higher affinity than to its native ligand, 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), and did not exhibit thermal stabilization in the presence of the TO1-Biotin fluorophore. Our data suggest that RNA Mango is likely to use a concerted ligand-binding mechanism that allows it to simultaneously bind and recognize its TO1-Biotin ligand, whereas RNA Spinach appears to lack such a mechanism. The high binding affinity and fluorescent efficiency of RNA Mango provides a compelling alternative to RNA Spinach as an RNA reporter system and paves the way for the future development of small fluorophore RNA reporter systems.

Keywords: aptamer; fluorophore; RNA Mango; RNA Spinach; concerted binding; G-quadruplex

INTRODUCTION

The in vitro selection (Ellington and Szostak 1990; Robertson and Joyce 1990; Tuerk and Gold 1990) of high-affinity RNA aptamers able to dramatically enhance the fluorescence of target fluorophores offers significant potential for the tracking and purification of cellular RNA complexes (Dolgosheina et al. 2014; Buxbaum et al. 2015; You and Jaffrey 2015). Until recently, aptamers with sufficiently high affinity (low KD) and fluorescence enhancement (the ratio of bound to unbound fluorophore fluorescence, Fb/Fu) have been lacking, but the in vitro selection of RNA Spinach (Paige et al. 2011) and RNA Mango (Dolgosheina et al. 2014) has provided much-needed tools for studying biological RNAs. Curiously, RNA G-quadruplexes formed by the Hoogsteen to Watson-Crick face pairing of guanines into planar tetrad structures (Sen and Gilbert 1988; Kim et al. 1991) are at the core of both RNA Mango and RNA Spinach. Based on the RNA aptamer’s potassium ion dependence, circular dichroism, RNase T1 protection, and core sequence motif, we inferred that RNA Mango consists of a parallel-stranded G-quadruplex when bound to TO1-Biotin (Dolgosheina et al. 2014). Crystal structures demonstrate that RNA Spinach assembles one face of its fluorophore-binding pocket out of an unusual, rigid antiparallel G-quadruplex platform and the opposite face out of a uniquely positioned RNA base triple (Paige et al. 2011; Huang et al. 2014; Warner et al. 2014). We, therefore, wondered how the artificially selected RNA Mango and RNA Spinach aptamers might differ in their ability to discriminate between their own fluorophores and a set of fluorophores known to interact with G-quadruplex structures (Fig. 1).

We find that RNA Mango is able to distinguish its intended target ligand, TO1-Biotin, from other fluorophores, whereas RNA Spinach and a set of biologically derived G-quadruplexes are unable to do so. Simultaneously, the strong thermal stabilization observed when RNA Mango binds TO1-Biotin suggests a concerted binding mechanism for this aptamer. The ability of RNA Mango to distinguish ligands combined with its unusually high binding affinity for TO1-Biotin may allow the development of orthogonally...
RESULTS

RNA Mango and RNA Spinach fluorophore binding and fluorescence enhancement

We initially wondered whether the RNA and fluorophore components of the RNA Spinach and RNA Mango systems could be orthogonal to each other. While RNA Mango did not detectably bind or enhance the fluorescence of DFHBI, RNA Spinach was able to bind to TO1-Biotin ($K_D = 830 \pm 130 \text{ nM}$) exhibiting only a twofold weaker affinity for this ligand than its DFHBI binding partner ($K_D = 420 \pm 40 \text{ nM}$) (Table 1; Supplemental Fig. S1). The finding that RNA Mango could strongly discriminate between TO1-Biotin and DFHBI, while RNA Spinach was weak in this respect, immediately suggested that the mechanisms of binding between the two aptamers were distinct despite the fact that both contain G-quadruplex motifs (Dolgosheina et al. 2014; Huang et al. 2014; Warner et al. 2014).

Surprisingly, once TO1-Biotin bound to RNA Spinach, the complex was remarkably bright. The TO1-Biotin:RNA Spinach complex exhibited an $F_E$ of $4590 \pm 770$ (Table 2; Supplemental Fig. S1). This $F_E$ is significantly higher than that observed with TO1-Biotin:RNA Mango ($F_E = 1020 \pm 20$, quantum yield 0.13) or DFHBI:RNA Spinach ($F_E = 1590 \pm 60$, quantum yield 0.72 [Paige et al. 2011]). The TO1-Biotin:RNA Spinach $F_E$ is without precedence for a TO1-based fluorophore and exceeds that observed for unmodified thiazole orange (TO1) intercalation into duplex DNA (Algar et al. 2006). The $F_E$ for TO1-Biotin:RNA Spinach corresponds to an absolute brightness of $\sim 50,000 \text{ M}^{-1} \text{ cm}^{-1}$, making this complex approximately threefold brighter than DFHBI:RNA Spinach (Brightness of 17,500 M$^{-1}$ cm$^{-1}$) and 1.4-fold brighter than eGFP (Brightness of 34,000 M$^{-1}$ cm$^{-1}$ [Shaner et al. 2005]). This finding may prove a significant advantage for RNA Spinach systems where brightness, and not binding affinity, is critical, and suggests that RNA Mango variants may be found in the future with considerably higher brightness.

TO1-Biotin was designed with modifications at the nitrogen of the benzothiazole ring as side chains at this location have previously been demonstrated to destabilize TO1 intercalation into duplex dsDNA by about sevenfold (Carreon et al. 2004; Dolgosheina et al. 2014). To explore the importance of the TO1-Biotin side chain for binding to RNA Mango, we measured the $K_D$ and $F_E$ of unmodified TO1 binding to RNA Mango as well as to a 17-bp double-stranded RNA (dsRNA) duplex. TO1-Biotin was six to seven times brighter at saturation than was TO1 when bound to RNA Mango (Fig. 2A), whereas binding affinity was slightly weaker for TO1 than for TO1-Biotin. Conversely, TO1-Biotin significantly destabilized intercalation into dsRNA, being $\sim 20$-fold less fluorescent than TO1 when measured at nearly $\mu$M
concentrations of RNA (Fig. 2B). The distinct binding and fluorescence enhancement properties for TO1 and TO1-Biotin to RNA Mango and to the dsRNA control, again, point to the ability of RNA Mango to distinguish ligands by a potentially unique mechanism.

RNA Mango and RNA Spinach are artificially selected aptamers that coincidentally adopt a G-quadruplex structure. We therefore compared these aptamers to a pair of biologically relevant parallel-stranded G-quadruplexes, and a synthetic 4-stranded tetramolecular G-quadruplex in addition to the 17-bp duplex RNA control just mentioned (see Supplemental Table S1 for sequences). These RNAs included PITX1 (PITX11901-1930), a G-quadruplex from the human PITX1 mRNA, and hTR, a quadruplex from human telomerase RNA (Meier et al. 2013; Booy et al. 2014). Based on a combination of UV/VIS spectroscopy, CD spectroscopy, small-angle X-ray scattering, and nuclear magnetic resonance spectroscopy experiments, PITX1 and hTR RNA have both previously been found to adopt a parallel-stranded G-quadruplex structure (Meier et al. 2013; Ariyo et al. 2015). Efficient staining with \( \text{N}-\text{methyl mesoporphyrin IX} \) (NMM, a parallel G-quadruplex ligand, Fig. 1), and their interaction with a protein-binding partner, RNA helicase associated with AU-rich element (RHAU), specific for parallel-stranded G-quadruplexes (Meier et al. 2013; Booy et al. 2014). SynTet (a synthetic tetrameric RNA quadruplex) is also a parallel-stranded quadruplex, and interacts with NMM (Creacy et al. 2008). Unsurprisingly, binding of NMM and Thioflavin T (ThT, Fig. 1) to PITX1, hTR, and SynTet has been demonstrated (Renaud de la Faverie et al. 2014).

All of these control quadruplex constructs were 300- to 400-fold worse than RNA Mango at binding to TO1-Biotin (Table 1; Supplemental Fig. S1), while the dsRNA control bound with a barely detectable linear increase in fluorescence enhancement (Fig. 2B, blue). The micromolar affinities exhibited by these sequences are similar to those reported for the binding of unmodified TO1 to a range of DNA G-quadruplexes (Lubitz et al. 2010), again suggesting that the binding mode of RNA Mango to TO1-Biotin is unusual. The fluorescence enhancements of these complexes at saturation were similar to that seen for RNA Mango (Table 2), implying that all of these G-quadruplex structures are ultimately able to rigidify the TO1-Biotin fluorophore at saturation. In comparison, DFHBI, the Spinach fluorophore, showed no detectable binding or fluorescence enhancement for any RNA other than RNA Spinach (Tables 1, 2; Supplemental Fig. S1). We note, however, that binding interactions 300-fold weaker than the nearly micromolar DFHBI:RNA Spinach binding interaction would have been hard to detect under our titration conditions (i.e., assuming a \( K_D \) of 100 µM at the 1 µM end point of our RNA titrations fluorescence signal would have only been 1% of that seen at full ligand binding).

**Fluorophore ligand discrimination by RNA Mango**

Since RNA Spinach bound TO1-Biotin and RNA Mango did not bind DFHBI detectably, we tested their ability to bind three other unrelated fluorophores. NMM and ThT are

### Table 1. Binding affinities (in nM) of each fluorophore:RNA combination at room temperature

| RNA        | TO1-Biotin | DFHBI | NMM    | Thioflavin T | EtBr   |
|------------|------------|-------|--------|--------------|--------|
| RNA Mango  | 3.9 ± 1.0  | >>    | 14 ± 11| 620 ± 40     | 550 ± 60|
| RNA Spinach| 830 ± 130  | 420 ± 40| 50 ± 5 | 260 ± 10     | 110 ± 30|
| PITX1      | 1490 ± 350 | >>    | 180 ± 20| >            | 1650 ± 1200|
| hTR        | 1290 ± 230 | >>    | 160 ± 50| >            | 830 ± 90|
| SynTet     | 940 ± 350  | >>    | 180 ± 160| 1410 ± 370  | 320 ± 40|
| dsRNA      | >          | >>    | >>     | >>           | 1260 ± 490|

(>>) Could not be reliably estimated. (>) Linear fluorescence enhancement observed during titration. Errors are the standard deviation of three measurements. Affinities >1000 nM are estimates from RNA titrations with end points at 1024 nM.

### Table 2. Fluorescence enhancement (FE) of each fluorophore:RNA combination at room temperature

| RNA        | TO1-Biotin | DFHBI | NMM    | Thioflavin T | EtBr   |
|------------|------------|-------|--------|--------------|--------|
| RNA Mango  | 1020 ± 20  | >>    | 11 ± 1 | 9.6 ± 0.5    | 4.7 ± 0.2|
| RNA Spinach| 4590 ± 770 | 1390 ± 60| 21 ± 1 | 57 ± 2       | 6.5 ± 0.3|
| PITX1      | 770 ± 90   | >>    | 12 ± 1 | >            | 4.4 ± 0.5|
| hTR        | 1000 ± 50  | >>    | 11 ± 1 | >            | 5.1 ± 0.4|
| SynTet     | 870 ± 130  | >>    | 19 ± 2 | 36 ± 6.7     | 6.3 ± 0.2|
| dsRNA      | >          | >>    | >>     | >>           | 8.9 ± 1.4|

Fluorescence enhancements could not be determined for pairs with unknown dissociation constants and are indicated as in Table 1 (“>>” or “>).

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previously described G-quadruplex binders (Nicoludis et al. 2012a; Renaud de la Faverie et al. 2014), while EtBr is an established double-stranded nucleic acid intercalator (Fig. 1). Strikingly, RNA Mango could discriminate between these fluorophores, binding to each of them with weaker affinity than to its selected ligand TO1-Biotin (Table 1). In distinct contrast, RNA Spinach bound NMM, ThT, and EtBr with significantly higher affinity than to DFHBI (Table 1).

Crystal structures reveal that NMM binds selectively to parallel-stranded DNA G-quadruplexes by stacking interactions between NMM and the G-tetrad face, and importantly, via its methyl group that fits into the center of the G-quadruplex (Nicoludis et al. 2012b). NMM was therefore unsurprisingly the tightest binder to parallel-stranded biological and synthetic control G-quadruplex RNAs used in this study (Table 1). Nevertheless, binding of NMM to RNA Mango and RNA Spinach was stronger than these controls, suggesting that artificial selection for fluorophore binding had also resulted in enhanced NMM binding and that the antiparallel G-quadruplex structure of RNA Spinach does not preclude NMM binding. Likewise, ThT bound worse to the control parallel-stranded quadruplexes compared to both artificially selected aptamers, with the four-stranded SynTet quadruplex showing the tightest fluorophore binding amongst the control quadruplexes (Table 1). While EtBr bound with 110 nM $K_D$ to RNA Spinach, weaker binding to EtBr was measured for all other constructs, as summarized in Table 1.

Fluorescent efficiencies induced by RNA quadruplex binding are correlated with ligand discrimination

Consistent with the ability of RNA Mango to bind preferentially to TO1-Biotin, RNA Mango showed the lowest fluorescence enhancements of all the quadruplexes tested for fluorophores other than TO1-Biotin (Table 2). In marked contrast, all fluorophores tested had the highest fluorescence enhancement when bound to RNA Spinach. This indicated to us that the rigid G-quadruplex of RNA Spinach is well suited to bind a broad range of fluorophores and enhances their quantum yields, but the aptamer is unable to discriminate between these ligands. Furthermore, a recent study (Su et al. 2016) indicates that DFHBI:RNA Spinach is strongly inhibited in 10 mM ATP, while TO1-Biotin:RNA Mango complex remains fully fluorescent in the presence of this concentration of ATP (data not shown). The ability of RNA Mango to distinguish between such ligands makes it a promising component for a system of orthogonal aptamers, each able to bind tightly and enhance the fluorescence of distinct fluorophore ligand partners.

The overall signal-to-noise ratio of a fluorescent RNA aptamer system is determined by both its fluorescence enhancement and binding affinity. Assuming that a fluorophore–aptamer system is optimally imaged with fluorophore concentration only several times higher than the $K_D$ of the complex, then the fluorescent efficiency ($E = F_0/K_D$) (Panchapakesan et al. 2015; Dolgosheina and Unrau 2016) is a good metric to compare and contrast fluorophore–aptamer pairs (i.e., the value of fluorescent efficiency represents the best combination of affinity and fluorescence enhancement of the fluorophore to overcome noise in an imaging experiment). TO1-Biotin:RNA Mango was by far the most fluorescently efficient complex ($E = 260$ nM$^{-1}$, Table 3) with TO1-Biotin:RNA Spinach being 50-fold worse, having a fluorescent efficiency of 5.5 nM$^{-1}$, which in turn was just higher than the efficiency of the DFHBI:RNA Spinach complex ($E = 3.8$ nM$^{-1}$). Notably NMM, ThT, and EtBr all have very poor fluorescent efficiencies owing mainly to the
low $F_0$ values observed upon binding. While TO1-Biotin did fluoresce strongly with a range of G-quadruplexes (Table 2), RNA Mango imaging would be expected to use TO1-Biotin concentrations considerably lower than the $K_D$ values (Table 1) required to saturate the fluorescent signal for these RNAs, giving the Mango system considerable theoretical contrast over both the naturally occurring biological G-quadruplexes and RNA Spinach. Thus, for example, in a given visual field we might expect that only when the local hTR RNA concentration is 300-fold higher than that of RNA Mango would the fluorescent signals from either the TO1-Biotin:Mango or the TO1-Biotin:hTR complexes be comparable (i.e., $260/0.78 \approx 300$ from Table 3). Improvements to RNA Mango’s binding affinity and fluorescence enhancement might reasonably enhance this contrast still further.

**Temperature-dependent binding and fluorescent properties of RNA Mango and RNA Spinach**

We decided to further characterize TO1-Biotin bound to either RNA Mango or RNA Spinach, given that these two complexes had the highest fluorescent efficiencies of any combination tested. First, we measured the effect of temperature on fluorescence. TO1-Biotin:RNA Spinach exhibited a sigmoidal-type fluorescence dependence (Fig. 3A, blue curves) as seen previously for DFHBI:Spinach2 (Filonov et al. 2014). TO1-Biotin:RNA Mango, however, displayed a nearly linear decrease in fluorescence as temperature increased (Fig. 3A, red curves). Fluorescence became undetectable for TO1-Biotin:RNA Spinach at $\sim 68^\circ C$ and at $\sim 79^\circ C$ for TO1-Biotin:RNA Mango, consistent with the higher binding affinity of the RNA Mango complex.

We next investigated the temperature dependence of the TO1-Biotin complex by measuring absorbance at 260 nm using micromolar concentrations of RNA and ligand (Supplemental Fig. S2). The derivative of absorbance as a function of temperature was most revealing for both aptamers (Fig. 3B,C). As expected, based on the rigid global fold of RNA Spinach (Huang et al. 2014; Warner et al. 2014),

|      | TO1-Biotin | DFHBI | NMM   | Thioflavin T | EtBr |
|------|------------|-------|-------|--------------|------|
| RNA  | 5.5        | 3.8   | 0.42  | 0.22         | 0.060|
| RNA Mango | 260 | <<    | 0.79  | 0.015        | 0.009|

(<<) Less than the detection limit of the assay used.

aValue determined from the linear increase in fluorescence observed during titration.

**FIGURE 3.** Temperature-dependent spectroscopy of TO1-Biotin complexed with RNA Mango or RNA Spinach. (A) Normalized fluorescence of TO1-Biotin complexed with RNA Mango (red) or RNA Spinach (blue) as a function of temperature. (B) Simple derivatives of the RNA Mango fluorescence (red) and $A_{260}$ of unbound RNA Mango (purple) and RNA Mango with an excess of TO1-Biotin (green). (C) Derivatives of fluorescence (blue) and absorbance of unbound RNA Spinach (purple) and RNA Spinach with an excess of TO1-Biotin (green). For both B and C: Left $y$-axis is the local slope of the $A_{260}$ temperature data (see Supplemental data), right $y$-axis is the local slope of the normalized fluorescence plot from panel A. Dots represent the local slope ($\Delta Y_i = Y_i - (Y_{i+1} + Y_{i-1})/2$), where $Y_i$ is either $A_{260}$ or the normalized RFU value and $T_i$ is the corresponding temperature. Curves are interpolations of the data points. The darker colors represent decreasing temperature ramps of 1°C/min, while the lighter colors represent increasing rates of 1°C/min.


To explore the fluorescence dependence below the melting temperature of the RNA Mango complex, we next measured TO1-Biotin:RNA Mango binding affinities and fluorescence enhancement as a function of temperature using 20 nM TO1-Biotin while simultaneously titrating RNA Mango concentrations (Supplemental Fig. S3). The $K_D$ was found to increase as the melting temperature of the complex was reached, moving from 3 nM at 25°C to 26 nM at 60°C (Fig. 4, black symbols). Since the temperature dependence of the fluorescence complex in Figure 3A was generated using 5 µM TO1-Biotin and 1 µM RNA Mango, such a small change in binding affinity would by itself be insufficient to explain the approximately fivefold change in fluorescence observed over this temperature range. Rather, we infer that the fluorescence of the bound complex significantly attenuates due to either a change in the quantum yield or an extinction coefficient of the complex. Consistent with this hypothesis, the $F_E$ obtained from our temperature-dependent $K_D$ titrations (Fig. 4, purple dots) agreed well with the data obtained at a much higher complex concentration, and rather precisely reproduces the observed changes in fluorescence at the 50-fold higher concentration shown in Figure 3A (reproduced in Fig. 4 by the solid red lines).

**Fluorescent lifetimes of TO1-Biotin:RNA Mango and TO1-Biotin:RNA Spinach are distinct**

Since RNA Mango and RNA Spinach both bind by distinct mechanisms to TO1-Biotin, we wondered whether the photophysics of these complexes differed in observables other than the fluorescence enhancement previously discussed. We measured the first-order fluorescence lifetimes ($\tau$) (Supplemental Fig. S4; Supplemental Table S2) and Stokes shifts (Supplemental Fig. S5; Supplemental Table S3) of TO1-Biotin bound to either RNA Mango or RNA Spinach. For comparison, we also measured the fluorescence lifetimes of fluorescein ($\tau = 4.1$ ns), as well as RNA Spinach bound to DFHBI ($\tau = 4.3$ ns), both agreeing well with previously published values (Unruh et al. 2005; Han et al. 2013). The TO1-Biotin:RNA Mango complex had a lifetime of 3.5 ns at room temperature and had a 25 nm Stokes shift comparable to the SynTet construct. This lifetime is similar to the lifetime of TO1 intercalated into dsDNA (2.6 ns [Algar et al. 2006]), where quantum yields were also found to be comparable to that of RNA Mango. Interestingly, when TO1-Biotin was bound to RNA Spinach, the fluorescence lifetime nearly doubled to 6.0 ns consistent with the high brightness observed for this complex and with a ~15% decrease in emission peak width relative to the Mango TO1-Biotin emission peak width (Supplemental Fig. S5A). The long lifetime observed with TO1-Biotin:RNA Spinach was also associated with a further 10 nm decrease in the Stokes shift for this complex relative to the 25 nm shift observed for the TO1-Biotin:RNA Mango complex (Supplemental Fig. S5; Supplemental Table S3). Based on these results, it appears highly likely that further selections to increase the quantum yield of RNA Mango will also result in changes in fluorescence lifetime.

**DISCUSSION**

This study demonstrates that the discriminating binding observed for RNA Mango is not generally found in either RNA Spinach or in biologically derived G-quadruplex controls. How exactly the high-affinity complex between RNA Mango and TO1-Biotin is formed from a kinetic perspective is currently unclear, but requires a PEG-biotin side chain or similar derivatives (Dolgosheina et al. 2014) to become fully fluorescent. We infer that a concerted folding process occurs due to the dramatic thermal stabilization of the RNA Mango.
structure observed upon TO1-Biotin binding together with previous data (Dolgosheina et al. 2014) indicates a large change in CD and RNase T1 protection upon addition of the TO1-Biotin ligand. This is significant, as G-quadruplexes are not generally thought to change structure upon ligand binding. While previous work has demonstrated that small molecules can either stabilize or destabilize G-quadruplexes, these studies require significantly higher ligand concentrations to achieve such effects (Bugaut et al. 2012; Yangyuoru et al. 2015). Likewise, the global structure of the promiscuous RNA Spinach aptamer including its G-quadruplex platform is the same in both the bound and unbound states, as shown by crystal structures (Huang et al. 2014) and as corroborated by our UV melting data. In contrast, RNA Mango appears to resemble naturally occurring (but G-quadruplex independent) aptamers such as the purine riboswitches that use a concerted mechanism to achieve ligand specificity (Serganov et al. 2004; Winkler and Breaker 2005; Kim and Breaker 2008; Serganov and Nudler 2013). The adenine riboswitch discriminates between adenine and guanine by 30-fold, but binds 30-fold tighter to 2,6-diaminopurine than to adenine, while the guanine riboswitch does not bind to adenine detectably (Mandal and Breaker 2004; Mandal et al. 2003). RNA Mango compares very favorably in this respect, binding TO1-Biotin more than 100 times tighter than to DFHBI, ThT, or EtBr. While the exact mechanism of this discrimination still remains to be determined, a clear distinction between these natural aptamers and RNA Mango is the complete lack of potential hydrogen bonding interactions found on the TO1 portion of the TO1-Biotin ligand, making the presumably hydrophobic binding mechanism of considerable interest to study further.

Improvements in RNA Mango appear likely, given the data presented in this study. RNA Spinach, which we have demonstrated to ubiquitously bind and enhance the brightness of a broad range of fluorophores, dramatically enhanced the brightness of TO1-Biotin to levels never previously seen. Consistent with our findings, recent work has shown that RNA Spinach binds to novel fluorophores with higher affinity than to DFHBI (Song et al. 2014; Xu et al. 2015; Ilgu et al. 2016). We speculate that the high quantum yield seen for the TO1-Biotin:RNA Spinach complex results from the ability of RNA Spinach to effectively shield bound fluorophores from solvent, as all fluorophores studied showed maximal fluorescence enhancement when bound to RNA Spinach. This solvent shielding is also likely to explain the nearly twofold change in fluorescent lifetime observed when TO1-Biotin was bound to RNA Spinach instead of RNA Mango and is partially consistent with the decreased fluorescence emission peak width observed for this aptamer. It remains to be seen if an RNA Mango aptamer can be evolved to exhibit greater brightness, but experience with RNA Spinach suggests that this should be possible. RNA Spinach has previously been engineered or reselected to improve quantum yield, affinity, and thermal stability, resulting in the Spinach2, Broccoli, and iSpinach aptamers (Strack et al. 2013; Filonov et al. 2014; Autour et al. 2016). In particular, Broccoli and iSpinach were selected using fluorescence-activating sorting methods to directly enhance brightness (Filonov et al. 2014; Autour et al. 2016). This methodology is extremely promising for the identification of new aptamers of superior brightness/fluorescence efficiency. Evolved RNA Mango aptamers could potentially be brighter than eGFP, which, when combined with the already extremely high binding affinity of the Mango system, bodes well for the use of the RNA Mango system in the imaging and purification of RNA complexes in living systems.

MATERIALS AND METHODS

RNA synthesis

RNA Mango, RNA Spinach, and dsRNA were in vitro transcribed by runoff transcription using T7 RNA polymerase with a 0.5 μM single-stranded DNA template strand hybridized to a 20-nucleotide T7 promoter top strand. dsRNA was generated by mixing an equimolar ratio of each complementary RNA strand. PITX1, hTR, and SynTet RNA were purchased from Integrated DNA Technologies Inc. All RNAs were purified using denaturing polyacrylamide gels prior to use.

Fluorophore purification and characterization

TO1-Biotin was synthesized and purified as previously described (Dolgosheina et al. 2014). DFHBI was purchased from Lucernia, Inc. NMM was purchased from Frontier Scientific, Inc. Thioflavin T was purchased from Sigma-Aldrich Co. Ethidium bromide was purchased from Merck KGaA. Fluorophore concentrations were determined using the extinction coefficients listed in Supplemental Table S4. Fluorophores were used with no further purification steps following purchase. Purity were measured by HPLC with a 50 mM TEAA and increasing acetonitrile gradient, which indicated close to >99% purity for all fluorophores with the exception of NMM. NMM has previously been described as a racemic mixture of eight isomers. Historically, NMM has been used as the mixture; hence our study follows the same convention.

Binding affinity and fluorescence enhancement determination

Fluorescence data were gathered using a Varian Cary Eclipse Spectrophotometer unless otherwise stated. Fluorescent titrations in the in vivo mimicking buffer (WB: 140 mM KCl, 1 mM MgCl2, 10 mM NaH2PO4 pH 7.2, 0.05% Tween-20) were performed to determine binding affinities for each fluorophore/RNA pair. Fluorescence was measured at the maximum excitation and emission wavelengths of each complex (see Supplemental Table S3). Curves were fitted using least squares (Kaleidagraph 4.5) using the following equation if $K_D$ was $<100$ nM:

$$F([RNA]) = \frac{F_{\text{max}}([RNA] + [DFHBI])}{2} + \frac{K_D([RNA] + 2[DFHBI])}{2 + F_{\text{bound}}},$$

where $F([RNA])$ is the fluorescence as a function of RNA concentration $[RNA]$, $F_{\text{max}}$ is the maximal fluorescence of the bound...
complex, and \( F_{\text{unbound}} \) is the fluorescence of the unbound fluorophore, or to the following equation if \( K_D < 100 \text{ nM} \):

\[
F([RNA]) = \frac{F_{\text{max}}[RNA]}{(K_D + [RNA]) + F_{\text{unbound}}}.
\]

(2)

When the fluorescence of \( F_{\text{unbound}} \) was undetectable, it was set to zero. Fluorescence enhancement was determined using

\[
F_E = \frac{F_{\text{max}}}{F_{\text{unbound}}}
\]

(3)

\( F_{\text{max}} \) was determined using Equation 1 or Equation 2 as appropriate.

**Temperature dependence of fluorescence**

Temperature dependence measurements were started at 90°C, decreasing at a rate of 1°C/min until 20°C, then returning at 1°C/min until 90°C was reached. Fluorescence measurements were obtained at the maximum excitation/emission of the fluorescent complex used and were measured in WB buffer, 1 µM RNA either with or without 5 µM fluorophore. Temperature dependence of fluorescence and absorbance were measured using a Varian Cary Eclipse Fluorescence Spectrophotometer and a Varian Cary 100 Bio UV-visible spectrophotometer monitoring at 260 nm. Data were fitted to a smooth function (Kaleidagraph 4.5) to guide the eye.

**Temperature dependence of \( K_D \) and \( F_{\text{max}} \)**

Temperature dependence of \( K_D \) for TO1-Biotin:RNA Mango was measured using a SpectraMax M5 Multi-Mode Microplate Reader using Greiner black, clear-bottom, 96-well microtiter plates. The fluorescence measurements were obtained at ex/em of 495/535 nm with a 530 nm cutoff filter using the bottom read function. Temperature measurements were stepped from 25°C to 60°C in increments of 5°C. RNA titrations were done in triplicate with fixed amounts of TO1-Biotin (20 nm) in WB buffer. RNA Mango was titrated from 2048 nM to 1 nM by twofold dilutions. Data were fitted to Equation 1, and \( K_D \) and \( F_{\text{max}} \) (normalized to fluorescence at 25°C) are plotted in Figure 4.

**Time-resolved fluorescence lifetime measurements**

Fluorescence lifetimes were measured using a Horiba-Jobin Yvon Fluorolog spectrophotometer. For TO1-Biotin measurements, the excitation source was a 494 nm NanoLED at a repetition rate of 1 MHz. Emission was passed through a 14.5 nm band-pass filter centered at 525 nm for TO1-Biotin:RNA Mango. For DFHBI:RNA Spinach, the excitation source was a 463 nm NanoLED at a repetition rate of 250 kHz. Emission was passed through a 14.5 nm band-pass filter centered at 501 nm. For the fluorescein standard, the excitation source was a 494 nm NanoLED at a repetition rate of 1 MHz. Emission was passed with a 2 nm filter centered at 521 nm. Each measurement was taken with 100 nM fluorophore and 500 nM RNA.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available for this article.

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