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Fluorescent Indicator Displacement Assays to Identify and Characterize Small Molecule Interactions with RNA

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Abstract

Fluorescent indicator displacement (FID) assays are an advantageous approach to convert receptors into optical sensors that can detect binding of various ligands. In particular, the identification of ligands that bind to RNA receptors has become of increasing interest as the roles of RNA in cellular processes and disease pathogenesis continue to be discovered. Small molecules have been validated as tools to elucidate unknown RNA functions, underscoring the critical need to rapidly identify and quantitatively characterize RNA:small molecule interactions for the development of chemical probes. The successful application of FID assays to evaluate interactions between diverse RNA receptors and small molecules has been facilitated by the characterization of distinct fluorescent indicators that reversibly bind RNA and modulate the fluorescence signal. The utility of RNA-based FID assays to both academia and industry has been demonstrated through numerous uses in high-throughput screening efforts, structure-activity relationship studies, and *in vitro* target engagement studies. Furthermore, the development, optimization, and validation of a variety of RNA-based FID assays has led to general guidelines that can be utilized for facile implementation of the method with new or underexplored RNA receptors. Altogether, the use of RNA-based FID assays as a general analysis tool has provided valuable insights into small molecule affinity and selectivity, furthering the fundamental understanding of RNA:small molecule recognition. In this review, we will summarize efforts to employ FID assays using RNA receptors and describe the significant contributions of the method towards the development of chemical probes to reveal unknown RNA functions.

1. Introduction

Ligand-receptor interactions play a crucial role in the regulation of healthy and disease-related processes and can be harnessed to study and/or target these processes. A fundamental understanding of ligand-receptor interactions is thus important for many areas of basic research and drug discovery; however, specific interactions can be difficult to study in a complex cellular environment. These challenges are particularly relevant for RNA receptors, despite increased interest in small molecule:RNA targeting. One method that has facilitated the detection of ligand-receptor interactions *in vitro* is the indicator displacement assay (IDA) [1]. In an IDA, a receptor is first bound by an indicator whose optical properties are

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modulated upon reversible and non-covalent binding (Fig. 1). Then, addition of the ligand, defined as a competitive analyte, can result in displacement of the indicator from the receptor and modulate the optical signal. Pioneered by the Isagawa laboratory [2] and later popularized by the Anslyn laboratory [1, 3], IDAs were first employed as a technique to convert synthetic receptors into optical chemosensors to detect binding and quantify levels of biological ions. The application of IDAs has now been extended to study the recognition of diverse analytes, including small molecules [4–8], anions [9], cations [10], metals [11], metabolite analogues [12], and amino acids [13], using a wide range of synthetic as well as biologically-relevant receptors. Furthermore, IDAs offer a major advantage as a signaling method by avoiding covalent attachment between the indicator and the receptor, which allows for multiple indicators to be potentially used with the same receptor and makes the assay easily adaptable to different receptors and platforms. Fluorescent indicator displacement (FID) assays, which utilize an indicator whose fluorescence properties are modulated upon binding to a receptor, offer additional advantages. FID assays allow for sensitive and quantitative detection, are relatively inexpensive to perform due to convenient availability of a fluorimeter or plate reader, and can be easily formatted to a high-throughput screening (HTS) platform.

In this review, we will focus on FID assays that employ RNA as a receptor to detect the binding of small molecule analytes (Fig. 2). RNA, particularly non-coding (ncRNAs), are a newly discovered class of biomolecules implicated in important biological processes such as gene expression and cellular differentiation [14]. The observed dysregulation of ncRNAs in numerous diseases, including cancers, neurodegenerative disorders, and viral infections, has sparked interest within the scientific community to therapeutically target ncRNAs and elucidate their roles in disease states [15]. Small molecules have proven to be a viable avenue to study disease-driving ncRNAs and are advantageous as chemical probes given their tunability and favorable pharmacological properties [16]. Promisingly, the use of FID assays either as a primary high-throughput screen or as a complementary method to validate target engagement *in vitro* has already greatly aided the development of RNA bioactive chemical probes [17–20].

FID assays offer a promising complementary approach to current methods used to detect RNA:small molecule interactions, which have been reviewed previously [21–23]. In comparison to FID assays, other widespread fluorescence-based methods such as Förster resonance energy transfer (FRET), fluorescence polarization (FP), and site-specific labeling typically require incorporation of a fluorescent handle onto the RNA and/or small molecule. FID assays also avoid immobilization of the RNA and/or small molecule, which is necessary when utilizing techniques such as surface plasmon resonance (SPR), second harmonic generation, and small molecule microarrays. Furthermore, FID assays are high-throughput and require small quantities of material in comparison to other traditional methods, including thermal denaturation, isothermal titration calorimetry (ITC), and nuclear magnetic resonance (NMR) spectroscopy. While FID assays allow for native RNA structures and diverse small molecules to be rapidly evaluated in solution, obtainable data is limited to binding constants whereas other techniques enable measurement of stoichiometry, thermodynamic binding parameters, and/or kinetic parameters. Additionally, attention must

be given when setting up and interpreting results of an FID assay to ensure a high quality and reliable screening method.

Herein we will highlight numerous examples, through the end of 2018, where implementation of RNA-centric FID assays aided the discovery and furthered understanding of small molecule interactions with RNA. In the following section (Section 2), we aim to survey the indicators that have been successfully studied with biologically relevant RNA receptors. We will introduce both synthetic and commercially available indicators, summarizing the known fluorescence and RNA-binding properties of each indicator and the validation of their use through displacement by diverse small molecule analytes. Additionally, we will draw attention to indicators whose fluorescence and/or RNA-binding properties have been tuned by either synthetic derivatization or through labeling of non-fluorescent ligands, thereby expanding the toolbox of fluorescent probes. In Section 3, we exemplify the utility of FID assays to rapidly detect small molecule interactions with RNA and characterize affinity as well as selectivity of RNA-binding small molecules. We will highlight the extensive use of FID assays in academic and industrial laboratories as primary screens for HTS campaigns and as a complementary approach for small molecule structure-activity relationship (SAR) and target validation studies. Lastly, in Section 4, we aim to accelerate the implementation of RNA-based FID assays by providing a resource to guide the selection of indicators, optimization of assay parameters, and interpretation of displacement data. Altogether, we hope to illustrate the versatility of FID assays to evaluate diverse small molecules and RNAs as well as to highlight the valuable contributions of the method towards fundamental understanding of RNA:small molecule recognition and identifying small molecule probes to study the role of RNA in biological processes and disease.

2. Indicators and RNA Receptors

A wide variety of synthetic and commercially available RNA-binding ligands with varying fluorescence properties and affinities have been utilized as indicators in FID assays. In addition, labeling of non-fluorescent small molecules or peptides with a fluorophore has expanded the repertoire of RNA-binding indicators. The fluorescence properties of indicators can be fine-tuned by derivatization, and, in general, emission intensity is measured through standard fluorescence, FP, and/or FRET using a plate reader or fluorimeter. Furthermore, dual functionality has been observed for several indicators that also inhibit RNA-associated processes *in vitro* [24]. In this section, we will review the fluorescence and RNA-binding properties of both small molecule- and peptide-based fluorescent indicators (Table 1) as well as relevant applications. We caution the reader in direct comparisons of reported binding affinities, such as those between indicators and RNA receptors, as measurements are highly dependent on the selected buffer conditions [25].

2.1 Small Molecule Indicators

2.1.1 Cyanine Dyes—Cyanine dyes consist of two nitrogen-containing heterocyclic rings linked by a polymethine bridge (Fig. 3) and have been used widely to stain cellular organelles, nucleic acids, and other biological macromolecules [58]. In aqueous solutions,

free cyanine dye molecules exhibit little to no fluorescence as a result of torsional motion in the polymethine bridge, leading to non-radiative decay of the excited state. Upon binding nucleic acids, torsional motion of the conjugated dye is restricted and decay of the excited state leads to a 100-to 1,000-fold enhancement in fluorescence [59]. The spectroscopic properties of cyanine dyes can be tuned by varying the length of the polymethine chain, altering the identity of the heteroatoms, and adding substituents to either heterocycle. Numerous cyanine dye derivatives have been synthesized and characterized as well as commercialized, providing fluorescent probes that can be monitored from the visible to near-infrared spectral regions [59]. Aside from cellular staining, several cyanine dyes have been evaluated against biologically-relevant RNAs and are commonly utilized as competitive indicators in FID assays [6, 18, 19, 26–32].

Thiazole orange (TO) is a commercially available cyanine dye first used as an RNA stain for reticulocyte analysis [60] that has since been assessed as an indicator with diverse DNA structures [4, 5] and RNA stem-loop structures, particularly aptamers [26–29]. TO consists of an N-methylquinolinium linked by a single methine to a 3-methylbenzothiazoline and has excitation and emission maximums beyond 500 nm, which is advantageous to limit interference with intrinsic fluorescence of small molecules [61]. Studies undertaken by the Stojanovic and Datta laboratories have demonstrated the promising use of TO as an indicator to detect selective ligand interactions with several RNA aptamers, including tobramycin, guanosine triphosphate (GTP), adenosine triphosphate (ATP), 3'–5' cyclic adenosine monophosphate, and theophylline aptamers, binding the structures with moderate micromolar affinity ($K_d = > 10 \mu\text{M}$) [27, 28]. While TO is suspected to bind multiple sites of several RNA aptamers, a significant decrease in TO fluorescence (40–90%) was still observed upon addition of the native ligand [27, 28]. Importantly, RNA aptamers bound by TO were shown to preserve selectivity for the native ligand as little to no TO displacement was observed in the presence of structurally similar negative controls [27–29]. These studies highlight the potential use of TO to characterize the selectivity of RNA aptamers and the chemical space of aptamer-binding ligands.

TO-PRO-1 is a commercially available dye that has been utilized as a general indicator to rapidly screen small molecules against multiple RNAs, allowing for selectivity trends of ligands to be readily assessed. TO-PRO-1 is structurally similar to TO, differing only in charge as a result of a quaternary ammonium substituent on the quinolone ring. Maximum excitation and emission wavelengths of TO-PRO-1 are similar to TO; however, the binding affinity of TO-PRO-1 to double-stranded (ds) DNA has been observed to be 10-fold higher, likely due to increased electrostatic interactions [62]. TO-PRO-1 was first utilized in an FID assay by Asare-Okai and Chow, who characterized and validated the indicator displacement assay using constructs of two well-studied RNAs, namely, bacterial ribosomal A-site and human immunodeficiency virus type 1 (HIV-1) Transactivation Response (TAR) element [6]. Using electrospray ionization mass spectrometry (ESI-MS), displacement of TO-PRO-1 from each RNA by a known ligand was confirmed and correlated with the reduction in the emission intensity of the indicator. TO-PRO-1 was observed to bind A-site RNA with 1:1 stoichiometry and based on the known binding site of each positive control ligand, was thought to bind both RNAs near or adjacent to secondary structures. Furthermore, the authors applied the TO-PRO-1 FID assay to screen aminoglycosides and peptides against

two additional bacterial RNA targets, helix 31 of the 16S ribosomal RNA (rRNA) and helix 69 of the 23S rRNA. Other notable examples of TO-PRO-1 applications are from the Disney laboratory where the indicator has been utilized in FID assays as a primary screen to identify RNA-binding compounds that selectively interact with diverse secondary structures and sequences, including stems [32], r(GGGGCC) expansions [18], and randomized RNA libraries comprised of 6-nucleotide (nt) hairpin loops, 3×2-nt symmetrical internal loops, or 4×4-nt asymmetrical internal loops [31]. Notably, TO-PRO-1 binds RNAs that contain unpaired regions with higher affinity (~0.5 μM) as compared to stems (~5 μM) [31, 32]. Furthermore, TO-PRO-1 has been used by Garcia-Lopez, Scapozza, and coworkers to detect selective small molecule interactions with splicing modifiers such as the cis-regulatory RNA element TSL2, a stem-loop RNA structure that overlaps with the 5' splice site of exon 7 (E7) in the copy of the survival motor neuron 1 gene (SMN2) and regulates E7 inclusion in alternatively spliced transcripts [19]. The TO-PRO-1 FID assay served as a primary screen to evaluate an RNA-focused small molecule library against TSL2 and nucleic acid controls, aiding in the identification of the first bioactive TSL2 structure-stabilizing ligands that promote E7 inclusion and revert spinal muscular atrophy (SMA) molecular phenotypes. Altogether, the examples highlighted demonstrate the versatility of TO-PRO-1 to quickly screen small molecules against diverse and/or underexplored RNAs such as splice sites and identify lead compounds and potential therapeutics.

Both TO and TO-PRO-1 have been used to label non-fluorescent small molecules to generate sequence specific and highly competitive indicators, respectively [29, 30]. For example, the Stojanovic laboratory linked TO to both guanosine monophosphate (GMP) and adenosine monophosphate (AMP) as a strategy to improve the specificity of TO and provide a fluorescent probe that could be used to selectively monitor interactions with corresponding RNA aptamers [29]. Upon binding the respective aptamers, the fluorescence of both TO conjugates increased by more than 100-fold. Both TO conjugates were selective for their corresponding aptamers as the emission intensity was lower or negligible in the presence of other aptamers, mutants, or unrelated nucleic acids. The apparent binding affinity of TO-GMP to GTP aptamers was comparable to that of GMP, while positive cooperativity was observed for TO-AMP binding to ATP aptamers as the affinity increased by several-fold compared to AMP. Titration of the native ligands led to almost complete displacement of each TO conjugate and the selectivity of the aptamer was conserved as other nucleotides were unable to displace TO-AMP or TO-GMP. Similarly, the Disney laboratory linked TO-PRO-1 to an ellipticine derivative (1a) known to bind to the hairpin structure of r(GGGGCC) expansions ($K_d = 9.7 \mu\text{M}$) and inhibit repeat-associated non-ATG (RAN) translation ($\text{IC}_{50} = 11 \mu\text{M}$), preventing the expression of toxic proteins associated with amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) [18, 30]. The indicator (1a-TOQ) exhibited a 3-fold increase in emission intensity upon binding r(GGGGCC)₈ ($K_d = 110 \text{ nM}$) and afforded a dose-dependent decrease in fluorescence upon titration of 1a against the r(GGGGCC)₈:1a-TOQ complex. To aid the identification of potent inhibitors of ALS/FTD-associated RAN translation, 1a-TOQ was utilized in an FID assay to screen a focused library of 40 compounds that were chemically similar to 1a. Several compounds were observed to decrease 1a-TOQ fluorescence by more than 50% and trends in the chemical features that enhanced recognition of r(GGGGCC)₈ were revealed. Ultimately, a single compound (4)

was found to be a more potent inhibitor of ALS/FTD-associated RAN translation ($IC_{50} = 1.6 \mu\text{M}$) and to bind cellular r(GGGGCC) expansions as confirmed by direct imaging through linking of 4 to TO-PRO-1. Overall, the ability to tune the affinity and/or selectivity of TO and TO-PRO-1 by incorporation of non-fluorescent RNA-binding ligands leverages the utility of indicators to monitor interactions with specific RNAs and to identify highly competitive ligands.

There are several cyanine dyes that have yet to be fully characterized and evaluated as RNA-binding indicators for FID assays. For example, YOYO-1 is a dimeric cyanine dye that consists of two molecules of YO-PRO-1, which is structurally similar to TO-PRO-1 except the indicator contains a benzoxazole ring in place of the benzothiazole ring [62]. YOYO-1 and other commercially available dimeric dyes such as TOTO-1 (dimer of TO) bind nucleic acids with higher affinity compared to their monomer counterparts [59], providing fluorescent probes to identify high affinity RNA:small molecule interactions. Additional monomeric cyanine dyes, including SYBR Green I and II, are commercially available and preliminarily have been evaluated against the theophylline RNA aptamer and a randomized 6-nt hairpin library, respectively [28, 31]. The diverse collection of cyanine dyes that can be readily accessed commercially or fine-tuned synthetically highlights the feasibility to characterize and incorporate these dyes as indicators for a given RNA target.

2.1.2 Xanthone Derivatives—Xanthone is a fused tricyclic scaffold comprised of two benzene rings separated by a 4-tetrahydropyranone (Fig. 3) that is found in a variety of biologically active compounds, including antineoplastics, antimalarials, and antimicrobials [63]. Initial photophysical and spectroscopic studies of xanthone with DNA revealed photoreactivity between the excited state of xanthone and DNA that could be diminished by the addition of electron-donating amino groups to the scaffold [64]. The RNA-binding properties of amino-substituted xanthone derivatives have been extensively studied by the Nakatani laboratory, who used these synthetic analogues as indicators to evaluate small molecules against therapeutically relevant RNAs [7, 24, 33–35]. The fundamental studies, several of which are highlighted below, have provided valuable insights towards the utility of amino-substituted xanthenes to develop high quality FID assays and in general, RNA-binding chemical probes.

The Nakatani laboratory first utilized a novel 2,7-Bis(2-aminoethoxy)xanthen-9-one derivative (X2S) as a fluorescent indicator and monitored binding of the indicator to different RNAs, including dsRNA, 1-nt bulges, and the IIB domain of HIV-1 Rev Response element (RRE-IIB) [7]. When bound to RNA, the emission of X2S fluorescence was diminished but recovered upon displacement of the indicator from the RNA in aqueous solutions. Quenching of X2S fluorescence was observed to be more efficient with 1-nt bulges than dsRNA, suggesting that the indicator binds favorably to unpaired regions of RNA. Additionally, binding of X2S was characterized with a truncated sequence of RRE-IIB that retains the critical binding site of Rev peptide necessary for HIV-1 replication [65]. The RRE-IIB construct consisted of a 4-nt hairpin and multiple loops, including a 2×3-nt internal loop and a single adenine bulge. At a 1:2 ratio of RRE-IIB to X2S, the fluorescence of the indicator was ~90% quenched when compared to X2S alone. Measurable fluorescence quenching was also observed with control RNAs that either lacked the hairpin or stem-loops,

respectively, suggesting that X2S binds to RRE-IIB at multiple sites. Notably, the authors highlighted that titration of a truncated Rev peptide known to bind near the purine-rich internal loop of RRE-IIB against a preformed X2S:RRE-IIB complex resulted in a rapid increase in fluorescence, suggesting that Rev binds near an X2S-occupied binding site [66]. In comparison, titration of neomycin B led to a slow but steady increase in fluorescence intensity, consistent with binding at a distal site as characterized in prior work by NMR spectroscopy [67]. To validate the X2S FID assay, the LOPAC1280 library was screened to identify small molecules that bind to RRE-IIB [7]. A portion of the library (59/1280 compounds) was removed prior to screening due to fluorescence overlap between the small molecules and X2S. Similar interference was also noted by Tran and Disney when monitoring X2S emission upon incubation with small molecules alone or in conjunction with a randomized 6-nt hairpin RNA library [31], highlighting a potential limitation of the indicator.

To limit interference with small molecule fluorescence, the Nakatani laboratory synthesized and characterized a series of xanthone and thioxanthone derivatives that emit at longer wavelengths compared to X2S [33]. Modifications to X2S included i) increasing the length of the alkyl chain of the aminoethoxy moieties, ii) adding methyl substituents to either the alkyl chain or nitrogen atoms, and iii) replacing the xanthone core with thioxanthone. While slight red-shifts in maximum absorption (370–377 nm) and emission (450–464 nm) wavelengths were observed for xanthone derivatives with increased alkyl chain length and addition of methyl substituents, considerable red-shifts were observed for thioxanthone (X2SS) and its N,N-dimethyl derivative ($\lambda_{Ex}/\lambda_{Em} = 424/495$ nm). A secondary objective of the SAR study was to improve the selectivity of the indicator for internal loops over stems to prevent non-specific binding of the indicator and to potentially allow for site-specific displacement to be monitored. Dissociation constants of X2S and thioxanthone derivatives were determined using an alternative binding model that assumes independent, stoichiometric, and simultaneous binding of the indicator to both the stem and loop regions of RRE-IIB. The ratio of the two dissociation constants ($K_{d(stem)}/K_{d(loop)}$) yielded a selectivity index that described the binding preference of the indicator for either stem or loop structures. X2S derivatives with a propyl chain, butyl chain, or N,N-dimethyl substitution were observed to be largely selective for loops. Only the N,N-dimethyl derivative (X2SdiMe) had a weaker binding affinity for the stem ($K_d = 4.3$ μ M) in comparison to X2S ($K_d = 1.7$ μ M), and it retained about one third affinity of X2S for the loop ($K_d = 170$ nM). For thioxanthone derivatives, titration data did not fit well with the model and suggested multiple binding events of the indicator to the loop region. Overall, the authors reasoned that the red-shifted emission wavelength of the thioxanthone derivative (X2SS) and the reduced stem affinity of the N,N-dimethyl xanthone derivative (X2SdiMe) make the indicators favorable for use in displacement assays. Indeed, the Nakatani laboratory has demonstrated the utility of these fine-tuned indicators to screen small molecules against pre-micro RNAs (pre-miRNAs) and revealed a dual application of X2SS as an inhibitor of Dicer-mediated processing [24, 34, 35].

2.1.3 Metabolites and Fluorescent Analogs—Metabolites are essential small molecules that are required for cellular processes and can be sensed by regulatory RNA

elements such as riboswitches [68]. Riboswitches are typically located at the 5' untranslated region of bacterial mRNAs and regulate gene expression via metabolite-induced pathways [69]. Upon binding of a cognate metabolite, a riboswitch and its expression platform undergo a conformational change, resulting in modulation of protein synthesis [70]. Inhibition of metabolite binding by competitive small molecules has been validated as a therapeutic strategy to halt gene expression in and prevent growth of bacterial pathogens [17, 71, 72].

Several riboswitch-sensing metabolites are intrinsically fluorescent, providing indicators that allow for direct competition between the native ligand and the small molecule ligand to be monitored. For example, flavin mononucleotide (FMN) is a fluorescent metabolite involved in the riboflavin biosynthesis pathway that binds its native riboswitch with high affinity ($K_d = 1.2$ nM) [17, 73]. The fluorescence of FMN is quenched upon binding the riboswitch and was utilized by Howe, Roemer, and coworkers to validate the RNA target of a lead small molecule identified in a bacterial phenotypic screen. Displacement of FMN by ribocil-B, one of the most selective RNA bioactives to date, demonstrated competitive and direct ligand binding to the riboswitch and highlighted the utility of RNA-centric FID assays for industrial use in target validation studies [17].

Some riboswitches have been found to accommodate and respond to chemically similar metabolites and/or analogs [74], allowing for the design of fluorescent derivatives. For example, the metabolite adenine and its fluorescent structural isomer, 2-aminopurine (2-AP) (Fig. 3), have been shown to bind with similar affinity to both the adenine riboswitch and a mutant guanine riboswitch (GRA) that has a C74U point mutation (0.47 and 0.25 μ M, respectively) [41, 75]. 2-AP strongly emits at 370 nm and can be selectively monitored in the presence of naturally occurring nucleobases [76]. Similar to FMN, the fluorescence of 2-AP is quenched upon binding to RNA via base-stacking interactions. Utilizing 2-AP, Daldrop, Brenk, and coworkers developed an FID assay to experimentally characterize the binding of select small molecules that were virtually screened for binding to the GRA riboswitch [42]. The authors highlighted that the implementation of an FID assay greatly reduced the amount of RNA needed (~99.5%) in comparison to ITC, a commonly used method to assess small molecule binding to riboswitches. Furthermore, 2-AP has been incorporated into other metabolites such as S-Adenosyl-L-methionine (SAM) to provide a fluorescent handle [77]. However, because the 2-AP SAM analog lacks the 6-amino group known to be important for SAM-I riboswitch binding affinity, Hickey and Hammond pursued a SAM analog functionalized at the 3'-hydroxyl of the ribose moiety with a Cy5-labeled linker and developed a competitive FP assay that could be applied in a high-throughput format to screen small molecules against multiple species of SAM-I riboswitches [12]. While riboswitches are particularly well suited for the development of fluorescent analogs, the resulting indicators are likely limited for use with a single riboswitch or class.

2.1.4 Other Intercalating Indicators—Several aromatic-enriched small molecules whose fluorescence is modulated upon binding RNA predominantly through pi-pi stacking interactions have been utilized in FID assays. For example, ethidium bromide (EtBr) is a non-specific ligand known to intercalate into RNA at multiple sites and has been used in FID assays with multiple RNA bulges, a 66-nt construct of RRE (RRE66), and HIV-1 TAR [43,

44, 46, 78]. Furthermore, ICR 191, a commonly used acridine mutagen, has been validated as an indicator with RRE-IIB as emission intensity is recovered upon displacement by Rev peptide or known RRE-binding ligands [51]. Similarly, proflavine is an acridine derivative known to bind RRE with high affinity and 2:1 stoichiometry, competitively inhibiting interactions with Rev peptide [49]. To identify additional competitive small molecules, proflavine was utilized to develop an FID assay that monitors FP at the emission wavelength of the indicator in the presence of an RRE construct that is immobilized to graphene oxide as a means to maximize the change in molecular weight, and thus significantly decrease the FP signal upon displacement of proflavine [50]. Next, triazole orange (TriO) contains a naphthalene core and was first identified to bind a DNA G-quadruplex structure in the promoter region of the alpha subunits of the hypoxia-inducible factor-1 transcription factor and selectively inhibit the growth of cancerous renal and osteosarcoma cell lines [79]. In addition, TriO has been applied as an indicator to characterize the binding of the anticancer agent chelerythrine to biologically relevant RNA G-quadruplexes [47]. Recently, Sato, Nishizawa, and coworkers disclosed an aminoethyl substituted naphthyridine derivative (ATMND-C₂-NH₂) (Fig. 3) that bound bacterial A-site RNA (*E. coli*) with higher affinity (0.44 μM) than any other reported non-aminoglycoside ligand [48]. When bound to A-site RNA, the fluorescence of ATMND-C₂-NH₂ is quenched with higher efficiency (> 95%) compared to other fluorescent indicators that have been used with A-site RNA, particularly fluorophore-labeled aminoglycosides (~65% quenching efficiency) [8, 80]. The authors demonstrated the utility of ATMND-C₂-NH₂ as an indicator and validated its use by screening several known ribosome-targeting antibiotics. Lastly, bisbenzimidazoles such as Hoechst dyes have preliminarily been evaluated as indicators with DNA aptamers [28] and are known RNA-binding ligands [81, 82], providing a potential new class of intercalating-based indicators to be explored in FID assays against RNA.

2.1.5 Dye-labeled Aminoglycosides—Aminoglycosides are polycationic polysaccharides comprised of a 2-deoxystreptamine core substituted with 1,3-diamino functionality and three to four hydroxyl groups that can provide a linkage to aminosugars (Fig. 3) [83]. Aminoglycosides are known to bind RNA, particularly at the A-site of the bacterial ribosome, and function as bactericidal agents towards both Gram-positive and Gram-negative strains [84–86]. Furthermore, aminoglycosides have been shown to bind many other functional RNAs via electrostatic and hydrogen bonding interactions between the amino and hydroxyl functionalities of aminoglycosides and the bases and/or backbone of RNA [36, 87–90]. The capability of aminoglycosides to interact with a number of diverse RNAs highlights the potential of these molecules to serve as general indicators for competition-based studies. While aminoglycosides are non-fluorescent, the synthetic incorporation of a dye moiety has allowed RNA:aminoglycoside complexes to be detected and monitored upon addition of a competitive small molecule [8, 36, 37, 39, 40].

Pyrene-labeled fluorescent derivatives of tobramycin (PYT) have been synthesized and utilized by the Rando laboratory to quantitatively measure RNA:aminoglycoside interactions [36, 37]. Pyrene was selected as the fluorescent moiety because the dye is known to interact with RNA and its emission intensity is quenched upon binding. The dye moiety was preferentially incorporated at the 6'-amino group of tobramycin and observed to enhance the

binding affinity of the aminoglycoside to an RNA aptamer selected from a diverse pool by 200-fold [37]. Additionally, PYT analogs have been evaluated with RRE-IIB, yielding nanomolar dissociation constants that are ~10–20-fold higher compared to unlabeled tobramycin [36]. Using gentamicin C and neomycin B as positive controls, displacement of PYT was observed, while no significant change in fluorescence was observed with the negative controls glucosamine and erythromycin, validating the use of PYT as an indicator [37]. However, because the pyrene moiety of PYT itself can bind RNA, the indicator has been observed to bind non-specifically, which can limit its utility. Incomplete recovery of fluorescence intensity as a result of multiple binding events can hamper affinity and stoichiometry measurements for competing small molecules as the contribution from each binding site to the change in fluorescence quenching cannot be determined.

To mitigate non-specific binding, fluorescent tags whose signal is not dependent on interactions with RNA bases can be incorporated into aminoglycosides. For example, the Arya laboratory has utilized a fluorescein-labeled derivative of neomycin (F-neo) for HTS of small molecules against a truncated, 27-nt construct of A-site RNA (*E. coli*) [8]. Fluorescein was incorporated via a thiourea linkage at the C5 of the ribose ring of neomycin, a position that is not predicted to be involved in binding. The fluorescence intensity of F-neo is dependent on the protonation state of fluorescein where the dicarboxylate anion absorbs strongly at 485 nm and the monocarboxylate anion absorbs weakly, leading to different emission intensities. In solution (pH = 7.0), fluorescein is a dianionic species and emits strongly at 517 nm. Upon F-neo binding to RNA, the dianionic species is destabilized by the negative electrostatic potential of the RNA backbone, raising the pKa of fluorescein and shifting the equilibrium towards the non-fluorescent monoanionic species. Molecular modeling studies with the A-site RNA construct suggested that F-neo binds the major groove with the fluorescein moiety positioned adjacent to the phosphates on the edge of the groove. Scatchard analysis of an A-site titration with F-neo revealed that the indicator binds at a single site with a dissociation constant of 43 nM, consistent with other literature reports for unlabeled neomycin. To validate F-neo as a reliable indicator, aminoglycosides known to bind the A-site construct were screened, and the determined relative binding affinities were observed to be similar to those obtained by other methods. The Arya laboratory has since extended the application of F-neo to rapidly screen peptidic-aminosugars against multiple pre- and mature microRNAs, demonstrating the versatility of the indicator with small RNAs [39]. Furthermore, F-neo has also been used to explore the therapeutic potential of targeting eukaryotic ribosomes such as expansion segment 7 (ES7, 209 nts) of the pathogenic fungi *Candida albicans* [38]. Interestingly, F-neo was found to bind *C. albicans* ES7 with an estimated 13:1 stoichiometry, nanomolar affinity ($K_d = 2.5$ nM), and high selectivity (~2,000-fold) compared to human ES7. Screening of a peptidic-aminosugar library (215 compounds) using F-neo resulted in the identification of ~60 compounds that bound ES7 with higher affinity than neomycin, highlighting the successful application of FID assays to study ligand binding to RNAs of larger size.

Aside from measuring fluorescence quenching, the binding of fluorescently-labeled aminoglycosides to RNA has also been monitored by FP [8, 36, 40]. Labeling of tobramycin with 5-carboxytetramethylrhodamine, a farther red-shifted dye compared to fluorescein, provided an indicator (CRT) that bound an *in vitro* selected RNA aptamer with 1:1

stoichiometry and ~10-fold higher affinity ($K_d = 12.3$ nM) compared to its fluorescein derivative [40]. However, CRT was found to bind more weakly than unlabeled tobramycin ($K_d = 0.77$ nM), highlighting a potential drawback of incorporating fluorophores as the dye molecule may interfere with native interactions. In comparison, a 5-carboxytetramethylrhodamine-labeled derivative of paromomycin (CRP) was observed to bind a construct of the 16S rRNA decoding region with ~10-fold higher affinity ($K_d = 165$ nM) than the unlabeled ligand, demonstrating the importance of evaluating the impact of dye incorporation for each small molecule with every RNA [36].

2.2 Dye-Labeled Peptide Indicators

2.2.1 Tat—Tat is a transcriptional activator protein encoded by HIV-1 [91]. The 86-residue protein activates transcription by binding to the bulge and upper stem region of HIV-1 TAR, leading to increased stabilization and processivity of RNA polymerases [92]. Tat activity is essential for viral replication and therefore, inhibition of the TAR:Tat interaction has been widely viewed as a therapeutic strategy. Tat recognition of TAR has been characterized through the evaluation of peptide fragments, which revealed that a basic arginine-rich subdomain of Tat is critical for binding [93]. Peptide fragments that contain the conserved, positively charged stretch of basic residues have been found to bind multiple RNA bulges *in vitro*, including the 2-nt bulge motif within the TAR element of human immunodeficiency virus type 2 (HIV-2) [94, 95]. Furthermore, labeling of Tat peptide with a fluorophore(s) has provided a general fluorescence-based indicator that has been utilized to detect small molecule interactions with RNA bulges and related structures [53–55, 96, 97].

For example, Masumoto, Hamasaki, and coworkers employed a fluorescent Tat-derived peptide as an indicator for HTS of small molecules against HIV-1 TAR [53]. Utilizing intramolecular FRET, the 16-residue peptide was labeled with 5-carboxyfluorescein (5-FAM) and tetramethylrhodamine (TAMRA) at the N-terminus and C-terminus, respectively (Fig. 4). Binding of Tat peptide to HIV-1 TAR facilitated FRET, allowing for excitation of 5-FAM and emission detection of TAMRA. The truncated peptide was comprised of the basic domain residues 49–57 of Tat with additional alanine residues added to both termini to prevent unfavorable interactions between the fluorophores and RNA. Additionally, a cysteine residue was introduced into the C-terminus to facilitate incorporation of TAMRA. Titration of HIV-1 TAR against the fluorescently-labeled peptide resulted in a 2.7-fold increase in fluorescence with $K_d = 286$ nM, assuming 1:1 stoichiometry. Consequently, a decrease in fluorescence was observed upon titration of unlabeled Tat, affording a dissociation constant of 70 nM. Overall, this study demonstrated for the first time the utility of a fluorescent Tat peptide displacement assay to characterize ligand binding interactions with HIV-1 TAR.

The high affinity of Tat peptide for HIV-1 TAR and literature precedence of Tat peptide to bind other RNAs *in vitro* [94] inspired Patwardhan, Hargrove, and coworkers to extend the application of the fluorescent peptide displacement assay and screen small molecules against four different RNA targets [54]. Using more stringent buffer conditions, the affinity of the 5-FAM/TAMRA-labeled Tat peptide was first re-examined to bulge RNAs HIV-1 TAR and HIV-2 TAR, then assessed for internal loops, including bacterial ribosomal A-site RNA (*E*.

coli) and RRE-IIB. RNA titrations against the fluorescent peptide yielded low-to-mid nanomolar dissociation values for all RNAs, confirming the capability of Tat to strongly interact with different RNA secondary structures and to be used as a general indicator to screen small molecules. Furthermore, the HTS potential of the Tat peptide displacement assay was demonstrated with three sets of small molecules: (1) aminoglycosides, (2) an in-house developed amiloride derivative library, and (3) a representative set of other known RNA-binding compounds from the literature. Small molecules were screened at two concentrations and compounds displaying >25% displacement at either concentration were designated as hits. Statistical analyses, including agglomerative hierarchical clustering and principal component analysis, revealed patterns within the displacement data and relationships between small molecule structure, RNA binding affinity and selectivity. Overall, this study highlighted the utility of the fluorescent Tat peptide to provide a convenient method to simultaneously evaluate small molecule binding affinity and selectivity for RNA.

2.2.2 Rev—Rev is a ~13 kDa viral regulatory protein that binds to HIV-1 RRE and facilitates the export of unspliced genomic viral RNA from the nucleus into the cytoplasm, where the RNA is translated into viral proteins [98]. In the absence of Rev binding, the expression of late phase HIV-1 structural proteins is halted and the replication of new viral particles is inhibited [65]. Similarly, disruption of the RRE:Rev complex by small molecules has been shown to inhibit HIV-1 replication and proven to be a promising therapeutic strategy towards the treatment of HIV-1 [99, 100]. Minimal RNA and protein substrates required to form the RRE:Rev complex have been determined, providing a manageable model system to monitor *in vitro* binding of small molecules [101]. Furthermore, the addition of a fluorescent tag to a 17-amino acid Rev peptide fragment (Rev₃₄₋₅₀) has yielded a useful indicator that can be utilized to monitor peptide displacement upon small molecule binding [36, 56, 100, 102].

Using a fluorescein-labeled Rev peptide (Rev₍₃₄₋₅₀₎-Fl) (Fig. 4), Wang, Hamasaki, and Rando developed a peptide displacement assay that monitors FP at the emission wavelength of fluorescein in the presence of RRE and competing analytes [36]. In solution, the extent of FP or anisotropy value of Rev₍₃₄₋₅₀₎-Fl is inversely proportional to its tumbling rate that is in part, dependent on the size and shape of Rev₍₃₄₋₅₀₎-Fl. Upon titration with RRE, the anisotropy value of Rev₍₃₄₋₅₀₎-Fl increased, indicating complex formation. In this study, a 47-nt RRE construct known to bind both Rev protein and fragments was used, though FP measurements have also been performed with a longer 67-nt RRE construct [57]. Titration of unlabeled Rev₍₃₄₋₅₀₎ against the pre-formed RRE:Rev₍₃₄₋₅₀₎-Fl complex led to displacement of Rev₍₃₄₋₅₀₎-Fl as detected by a decrease in anisotropy, resulting in a dissociation constant of 1.41 nM. Displacement of Rev₍₃₄₋₅₀₎-Fl by small molecules was evaluated for known RRE-binding ligands, neomycin B and tobramycin, and three tobramycin derivatives, revealing that pyrene and arginyl substituted aminoglycosides bind RRE with higher affinity than unmodified ligands. More recently, the peptide displacement assay has been used to screen against commercially-available small molecule libraries, leading to the discovery of new RNA-binding ligands for RRE, including clomiphene,

cyproheptadine, and benfluron, that inhibit RRE:Rev complex formation *in vitro* and interfere with the gene expression processes of HIV-1 in cells [56, 100].

The versatility of the Rev₍₃₄₋₅₀₎-Fl indicator was also shown by Luedtke and Tor, who developed a solid-phase peptide displacement assay and demonstrated its utility to identify selective small molecules that disrupt the RRE:Rev complex [102]. In the assay, a biotinylated 67-nt RRE fragment was first immobilized to streptavidin-modified insoluble agarose beads. Then, Rev₍₃₄₋₅₀₎-Fl was added and the amount of fluorescent peptide bound to RRE was calculated by removing the supernatant and comparing the fluorescence intensity to a calibration curve of Rev₍₃₄₋₅₀₎-Fl concentration as a function of fluorescence. Similar to other literature reports, the dissociation constant for Rev₍₃₄₋₅₀₎-Fl with immobilized RRE was determined to be 2.5 nM, suggesting that the incorporation of a fluorescent tag and immobilization of RNA had no significant effect on the native interaction. The effectiveness of the Rev₍₃₄₋₅₀₎-Fl displacement assay was demonstrated using known RRE-binding aminoglycosides and polycyclic aromatic amidines. Displacement of Rev₍₃₄₋₅₀₎-Fl was monitored as a function of small molecule concentration by collecting the supernatant and comparing its fluorescence intensity to that of the calibration curve. The concentration of small molecule required to displace half of Rev₍₃₄₋₅₀₎-Fl (IC₅₀) was determined and reproducible trends in RRE affinity were observed when compared to values obtained by alternative methods, including FP. However, there were some discrepancies between IC₅₀ values determined by solid-phase displacement in comparison to fluorescence anisotropy, thought to be a result of small molecule fluorescence quenching or interference with fluorescence anisotropy measurements. Indeed, the solid-phase peptide displacement assay can potentially circumvent fluorescence quenching or interference as the supernatant that contains excess small molecule and displaced Rev₍₃₄₋₅₀₎-Fl can be removed, allowing for quantification of Rev₍₃₄₋₅₀₎-Fl that remains on the solid support. Another advantage of the solid-phase Rev peptide displacement assay is the ability to simultaneously assess small molecule binding and selectivity to RRE. Competitor nucleic acids, including transfer RNA (tRNA) and dsDNA, as well as proteins can be added to increase the stringency of the assay and scavenge non-selective small molecules. Under these conditions, only highly selective small molecules will significantly displace Rev₍₃₄₋₅₀₎-Fl and lead to a fluorescence signal in solution. Altogether, these studies highlight the utility of monitoring displacement of a fluorescently-labeled viral peptide, which has aided the identification of high affinity and bioactive compounds that inhibit Rev function, a mechanism of action that is different than currently marketed antiretroviral agents.

3. Example Applications of FID Assays

The ability to use FID assays in a high-throughput format makes the method favorable for screening large small molecule libraries against multiple RNA targets and aiding the rapid identification of RNA:small molecule interactions. For HTS, FID assays have been used as a primary screen, monitoring the percentage of indicator displacement at a single concentration of small molecule in a 384-well plate format. Single-point measurements often provide a “yes or no” response with the percentage of indicator displacement at a specific small molecule concentration denoting whether or not a small molecule binds a

target(s). The Disney laboratory and others have utilized single-point measurements to rapidly evaluate small molecule libraries against multiple RNA targets to identify selective RNA-binding compounds [7, 19, 31, 32, 34, 35, 54, 56, 97, 100]. Several notable small molecule libraries that have been screened by an FID assay include the LOPAC library (1,280 compounds) [7, 31], the MyriaScreen chemical library (10,000 compounds, Sigma-Aldrich) [56], a drug-like small molecule library housed at the University of Michigan (103,498 compounds) [97], and two chemical libraries curated from the Open Innovation Center for Drug Discovery (now Drug Discovery Initiative) at the University of Tokyo consisting of 9,600 and 41,119 compounds, respectively [34, 35]. Importantly, Fukuzumi, Nakatani, and coworkers noted that the entire screening of the 41,119 compound library at 10 μM against three RNA targets by a single person took about 60 days, highlighting the applicability to use FID assays for exploratory study of RNA-binding small molecules in an academic setting [35].

FID assays are also useful to further characterize small molecule affinity and selectivity for an RNA target. Often, binding activity is reported as a competitive displacement value (i.e. CD_{50}) or the concentration of small molecule at which 50% displacement of the indicator is observed. Because FID assays are competition-based, the CD_{50} value of a small molecule can be higher than the value of the dissociation constant measured in the absence of competitor. Dissociation constants can be calculated in FID assays; however, the binding affinity and stoichiometry of the indicator to the receptor must be known [103]. While more time and material consuming than a single-point measurement, determination of CD_{50} or K_d values for small molecules can be useful to rank and compare compounds, providing a more descriptive and quantitative measure than percentage of indicator displacement alone to elucidate chemical properties that enhance RNA binding affinity and selectivity. There are a number of fundamental studies where incorporation of an FID assay helped to identify and characterize structure-activity relationships of RNA-binding small molecules [43, 55, 104]. In one example, the Hergenrother laboratory utilized an EtBr displacement assay to characterize the binding of a synthetic library of spirocyclic compounds to 1-, 2-, and 3-nt RNA bulges as well as to dsRNA, the majority of which had binding constants $> 20 \mu\text{M}$ for all RNAs evaluated. However, there were several compounds with binding constants $< 20 \mu\text{M}$ for bulge RNAs only and notably, a few that displayed differential binding based on bulge size, revealing important chemical modifications that improved small molecule affinity for RNA bulges and dictated selectivity for specific bulge sizes [43]. For indicators that selectively bind an RNA receptor, CD_{50} values can be determined in the presence of other nucleic acid competitors to simultaneously assess small molecule selectivity. For example, fluorescently-labeled Tat peptide binds HIV-1 TAR RNA but does not strongly associate with tRNA, allowing for small molecule displacement to be evaluated in the presence of the tRNA competitor [55, 96, 97].

4. Considerations for Implementing an FID Assay

FID assays are a valuable method for identifying and characterizing selective RNA-binding small molecules; however, when setting up an assay, consideration of several parameters must be given to maximize results. Firstly, an indicator whose fluorescence and RNA-binding properties are favorable for a given system should be selected. If small molecules in

the screening library are suspected to be intrinsically fluorescent, then the use of red-shifted probes would be favorable to limit small molecule fluorescence interference and avoid removal of compounds from evaluation [61]. Obtaining excitation and emission spectra of small molecules at the highest anticipated concentration for screening could help to inform the selection of an indicator whose wavelengths do not overlap. RNA-binding properties to consider for selecting an indicator include the stoichiometry and binding affinity to the receptor. If an indicator binds an RNA at multiple sites, then displacement by a small molecule may not result in a significant change in fluorescence unless the ligand also binds at multiple sites or induces a large RNA conformational change, leading to the displacement of several dye molecules. For the evaluation of monovalent small molecules, an indicator that binds stoichiometrically and near the anticipated binding site of small molecules would be favorable towards the development of a high-quality assay. In addition, the binding affinity of the indicator to the RNA target should be considered as FID assays rely on the competition between a small molecule and indicator for signal modulation. Therefore, the binding affinity between the indicator and RNA should be comparable to that between the small molecule and RNA [1, 103]. High affinity indicators allow for the use of lower amounts of RNA but may restrict the detection of moderate or weak binding small molecules [54].

Secondly, the concentrations of both the indicator and RNA target should be considered as the relative ratio will influence the maximum signal and quality of the assay. Commonly, the quality of an assay is defined by its Z' -factor that describes the signal variation for and separation between the free and bound states of an indicator [105]. Z' -factors can range from 0 to 1, where values equal to or greater than 0.5 describe reliable and high-quality assays. It is important to mention that a selected indicator with a given RNA may not always result in an acceptable Z' -factor. For example, the Z' -factor for an FID assay may suffer from high signal background interference when using a turn-OFF indicator in comparison to a turn-ON indicator [54]. Assay signal prior to the addition of test compound will be highly dependent on the initial fraction of indicator bound to an RNA (Fb^0), calculated using total RNA/indicator concentrations and the experimentally determined indicator binding constant for an RNA [106]. The impact of Fb^0 on FID assays has been demonstrated by del Villar-Guerra, Chaires, and coworkers, where simulations of the expected indicator displacement as a function of test compound binding affinity revealed a minimum affinity required to onset indicator displacement for their system [4]. Furthermore, these simulations can assist assay development to inform the selection of total RNA and indicator concentrations, resulting in an Fb^0 that provides suitable stringency for small molecule screening.

Lastly, upon rational selection of assay parameters, the concentration(s) of small molecules for screening should be considered. Commonly, HTS FID assays have utilized 1, 10, 50, and/or 100 μ M and generally 25% displacement has designated a small molecule as a hit [7, 18, 19, 31, 32, 34, 35, 54, 56]. It is important to note that the observance of little to no displacement does not necessarily confirm that a small molecule does not bind to an RNA as the small molecule may bind to a non-competitive site resulting in the formation of a ternary complex (RNA:indicator:small molecule) (Fig. 5) [4, 6]. Comparably, an indicator may bind an RNA at a second or third site albeit with weaker affinity when displaced from its primary binding site by a small molecule. Using ESI-MS, the displacement of indicator from the

RNA receptor and/or formation of a ternary complex can be monitored as small molecule analyte is added, providing valuable insights on experimental results [6]. Alternatively, the small molecule may interact with the indicator [6], leading to either a false positive or negative result (Fig. 5). Therefore, controls that measure for potential interactions between a small molecule and indicator should be implemented. Additionally, small molecules should be evaluated by a secondary method such as ESI-MS, ITC, or SPR to validate binding interactions and improve interpretation of results. If possible, similar assay and buffer conditions should be used to allow for appropriate and meaningful comparisons. Altogether, careful consideration of each guideline described in this section will help to facilitate the selection of an indicator, optimization of assay conditions, and interpretation of FID assay results.

5. Concluding Remarks

Small molecule targeting of RNA has been largely recognized by the scientific community as a strategic approach to probe the unknown RNA functions in biological processes and disease [16, 107, 108]. Interest towards the rational development of selective RNA-binding ligands has been heightened in both academic and industrial settings as examples of small molecule probes used to attenuate RNA-specific disease-state interactions emerge [17, 109–112]. Additionally, great efforts have been made to fundamentally understand small molecule recognition of RNA and identify key properties that lead to selective interactions that could in turn provide guiding principles for targeting RNA [107, 113]. As a result, methods that can accelerate the identification and characterization of unique RNA:small molecule interactions are critical for the advancement of next generation chemical probes. In this review, we have surveyed the successful application of FID assays to study diverse RNA:small molecule interactions, provided a comprehensive summary of well-characterized RNA-binding indicators, and exemplified the valuable insights that RNA-based FID assays have provided towards small molecule recognition. Future design of novel indicators that are specific for an RNA structure and/or sequence may be advantageous for evaluating small molecule selectivity in the presence of multiple RNAs as well as potentially monitoring displacement of highly specific indicators in cells through standard fluorescence or FRET. Furthermore, we hope that the collection of promising results described here along with guidelines for experimental design will facilitate the use of FID assays to rapidly screen small molecules against underexplored RNAs and aid the identification of first-in-class chemical probes.

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Highlights

- Reviews fluorescent indicator displacement (FID) assays with RNA and small molecules.
- Surveys the fluorescence and RNA-binding properties of known indicators.
- Provides examples where use of FID assays aided high-throughput screening efforts.
- Highlights use of FID assays to quantify small molecule affinity/selectivity for RNA.
- Describes practical considerations for the implementation of RNA-based FID assays.

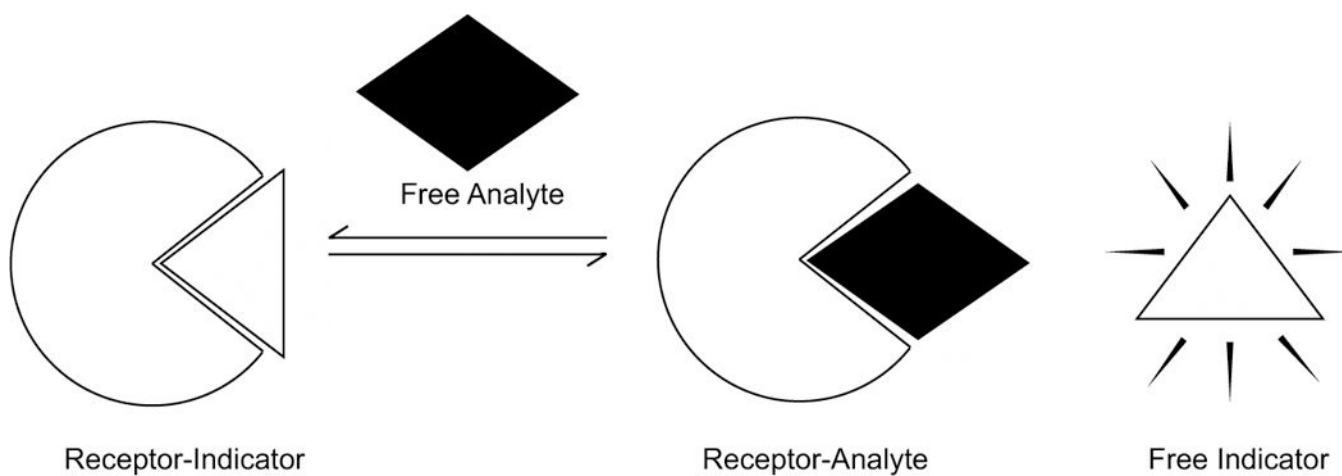


Fig. 1. Schematic of an indicator displacement assay. In this example, optical signal intensity is low when the indicator is bound to the receptor and high when the indicator is free in solution.

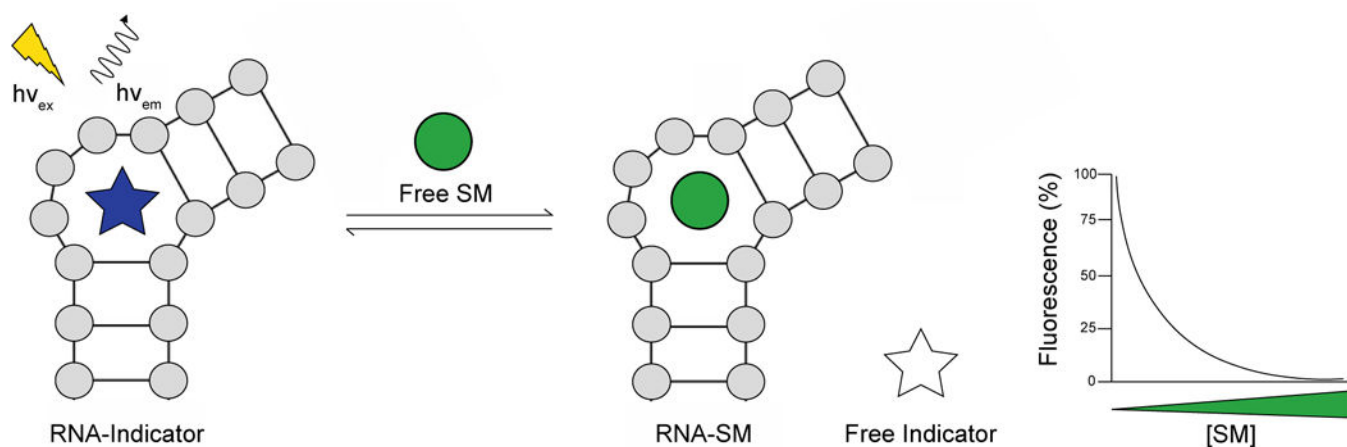


Fig. 2. Fluorescent indicator displacement assay to detect RNA:small molecule interactions. In this example, fluorescence is observed when the indicator is bound to the RNA receptor. As the indicator is increasingly displaced by a small molecule (SM) analyte, the fluorescence decreases. Conversely, displacement of an indicator whose fluorescence is quenched when bound to the RNA receptor will result in an increase in fluorescence.

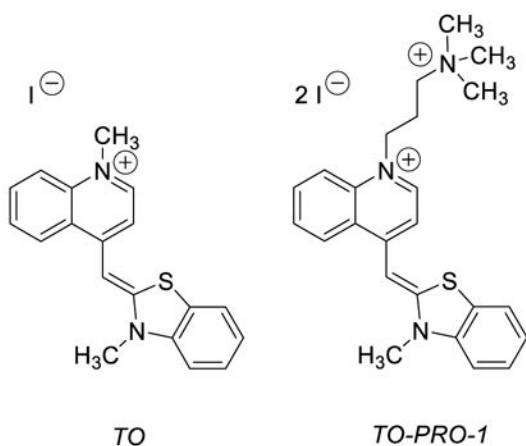
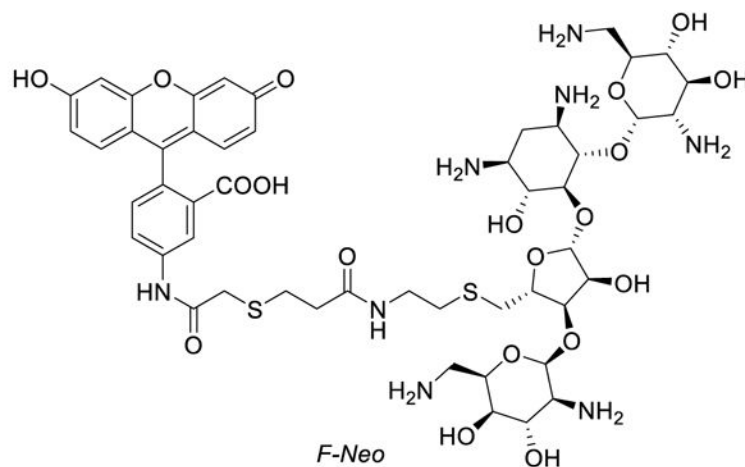
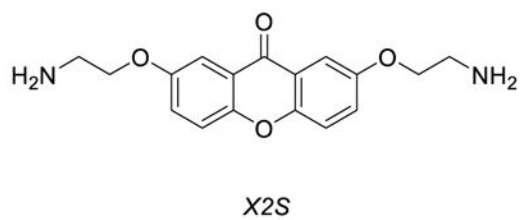
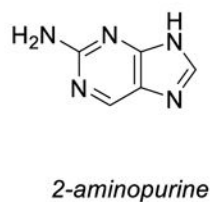
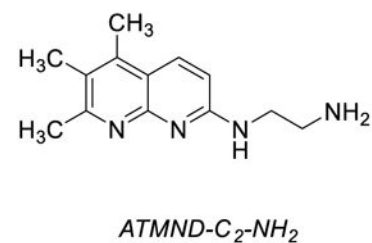
Cyanine Dyes:**Dye-labeled Aminoglycosides:****Xanthone Derivatives:****Metabolites & Fluorescent Analogs:****Other Intercalating Indicators:**

Fig. 3.
Representative structures of RNA-binding small molecule indicators.

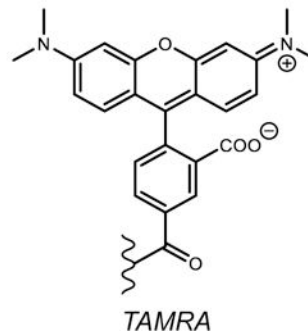
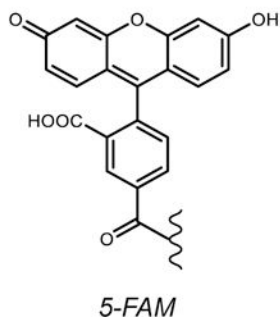
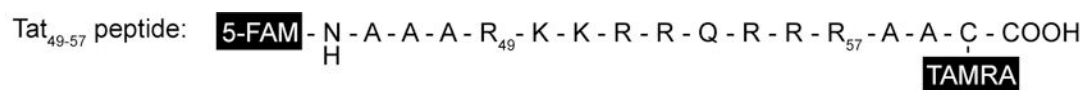
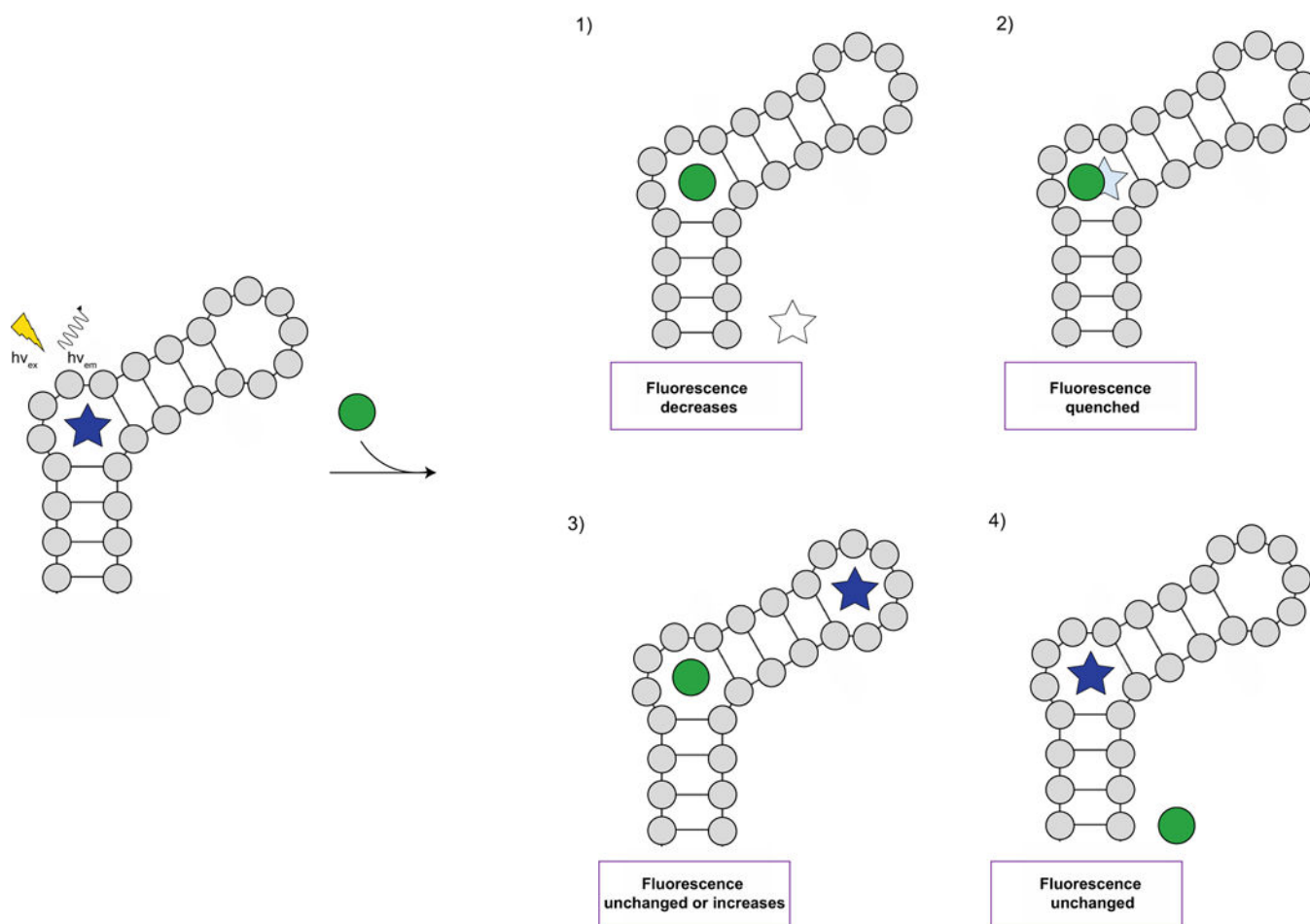


Fig. 4. Sequence of FRET pair labeled Tat peptide and fluorophore labeled Rev peptide. Structures of fluorophores 5-FAM and TAMRA used at the N and C-termini shown. Amino acid abbreviations: A = Ala, R = Arg, Q = Gly, N = Asp, T = Thr, W = Trp, E = Glu, C = Cys.

**Fig. 5.**

Potential outcomes of an FID assay [6]. Initially, fluorescence is observed as the indicator (star) is bound to the RNA receptor. Upon addition of the small molecule analyte (green circle), one of four scenarios can occur. Scenario 1): the small molecule analyte displaces the indicator, leading to a decrease in fluorescence. Scenario 2): the binding site of the small molecule analyte overlaps with the indicator, resulting in quenched fluorescence. Scenario 3): the binding site of the small molecule analyte is different than that of the indicator, leading to unchanged fluorescence or an increase in fluorescence if binding of the small molecule analyte to the RNA receptor enhances the binding of the indicator. Scenario 4): the small molecule analyte does not bind the RNA receptor or cannot outcompete the indicator, leading to unchanged fluorescence.

Table 1

Summary of RNA-Binding Indicators Used in FID Assays.

Indicator	Excitation (nm)	Emission (nm)	Fluorescence Upon Displacement	RNA Receptor(s)	Reference(s)
TO	512	533	turn-OFF	Stem, pre-miR-23b, aptamers (tobramycin, theophylline, GTP, ATP, cAMP, and <i>in vitro</i> selected)	[26–29]
TO-PRO-1	515	531	turn-OFF	A-site (<i>E. coli</i>), HIV-1 TAR, helix 31 of 16S rRNA, helix 69 of 23S rRNA (<i>E. coli</i>), r(GGGGCC) ₈ , TSL2, 6-nt hairpins, 3×2-nt internal loops, 4×4-nt internal loops, stems	[6, 18, 19, 30–32]
SYBR Green I	497	520	turn-OFF	Theophylline aptamer	[28]
SYBR Green II	497	520	turn-OFF	6-nt hairpins	[31]
X2S	370	450	turn-ON	RRE-IIB, dsRNA, 1-nt bulges, 6-nt hairpins	[7, 31, 33]
X2SS	424	496	turn-ON	RRE-IIB, dsRNA, pre-miRNAs (21–1, 29a, 30d-1, 92a-1, 122–1, 132–1, 132–2, 326–2, 433–1, 1297–2)	[33, 34]
X2SdtMe	370	451	turn-ON	RRE-IIB, dsRNA, pre-miRNAs (29a, 122)	[33, 35]
PYT	340	400	turn-ON	RRE-IIB, <i>in vitro</i> selected aptamers	[36, 37]
F-neo	490	517	turn-ON	A-site (<i>E. coli</i>), ES7 (<i>C. albicans</i>), pre-miRNA 504, mature miRNAs (7a-5p, 9–5p, 10b-5p, 15a-5p, 19a-5p, 19b-2–5p, 22–5p, 29a-3p, 31–5p, 34a-5p, 126–3p, 130a-5p, 132–5p, 135–5p, 142–5p, 155–5p, 221–5p, 335–5p, 380–5p, 504)	[8, 38, 39]
CRT	553	577	-	<i>In vitro</i> selected aptamers	[40]
CRP	553	577	-	16S rRNA decoding region	[36]
FMN	455	525	turn-ON	FMN riboswitch (<i>E. coli</i>)	[17]
2-aminopurine	300	370	turn-ON	Adenine riboswitch (<i>B. subtilis</i>), C74U guanine riboswitch (<i>B. subtilis</i>)	[41, 42]
SAM-C ₈ -Cy5	649	666	-	SAM-I riboswitches (<i>B. subtilis</i> , <i>P. fergensis</i> , <i>T. tengcongensis</i>)	[12]
Ethidium Bromide	520	600	turn-OFF	dsRNA, bulge (1-, 2-, and 3-nt), RRE, HIV-1 TAR	[43–46]
Triazole Orange	501	530	turn-OFF	Telomeric RNA G-quadruplex (<i>H. sapiens</i>)	[47]
ATMND-C ₂ -NH ₂	357	406	turn-ON	A-site (<i>E. coli</i>)	[48]
Proflavine	445	505	-	RRE-IIB	[49, 50]
ICR 191	415	490	turn-ON	RRE-IIB, HIV-1 TAR	[51, 52]
5-FAM-Tak ₍₄₉₋₅₇₎ -TAMRA	500	577	turn-OFF	HIV-1 TAR, HIV-2 TAR, A-site (<i>E. coli</i>), RRE-IIB	[53–55]
Rev ₍₃₄₋₅₀₎ -Fl	490	525	-	RRE constructs (28-, 47-, and 67-nt)	[36, 56, 57]

- = indicator monitored through fluorescence polarization