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New Ideas for *in vivo* Detection of RNA

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1. Introduction

The parallel discovery of the catalytic potential of RNA by Tom Cech and Sydney Altman at the beginning of the 1980s completely changed our view of the capabilities of RNA molecules and won them the Nobel prize in 1989 (Kruger et al., 1982; Guerrier-Takada et al., 1983). Since then, many new roles have been discovered and assigned to RNA, including the ability to form complex multitasking, supramolecular machines, such as ribosomes or spliceosomes, in which RNAs play leading roles. While only about 1.5 % of the human genome codes directly for protein sequences, a large fraction of it is nonetheless transcribed to produce many noncoding RNAs (ncRNA) that carry out important cellular functions (International Human Genome Sequencing Consortium, 2004). The structures and functions of some ncRNA have been extensively characterized, for example, ribosomal RNAs (rRNA), transfer RNAs (tRNA), small nuclear and nucleolar RNAs (snRNA and snoRNA). Moreover, applications of recent technological advances are revealing new classes of ncRNA molecules with novel or yet unknown functions, ranging in size from very small (20-30 nts) to thousands of nucleotides long. Small ncRNA (~20-30nts in length) include microRNA (miRNA), Piwi-interacting RNA (piRNA), small interfering RNA (siRNA), trans-acting siRNA (ta-siRNA), natural antisense transcript siRNA (nat-siRNA) and small scan RNA (scnRNA) all of which help regulate various stages of gene expression (Choudhuri, 2009). The functions of "long noncoding RNA" are still not understood, but they seem to be involved in transcriptional and epigenetic gene regulation (Ponting et al., 2009).

Many ncRNAs form distinct yet very diverse three-dimensional structures to carry out their functions (Noller, 2005; Staple & Butcher, 2005; Montange & Batey, 2008). These functions include fundamental roles at all stages of gene expression including chromosome remodeling, transcriptional and translation regulation, mRNA processing, transport, and localization, protein translocation and posttranslational modification. RNA molecules use various mechanisms to participate in these processes. For example, miRNA, siRNA and piRNA inhibit gene expression by hybridizing to complementary sequences of target mRNAs in association with RISC ribonucleoprotein complexes and either temporarily block translation or target the mRNA for destruction (Carthew, 2006; Bushati & Cohen, 2007; Boyd, 2008). Riboswitches, usually located in the 5' untranslated region (5'-UTR) of mRNA, also inhibit translation, but do so by forming specific three-dimensional structures, that respond to small molecule metabolites that are products of the pathway to which the gene belongs. Upon binding cognate metabolites, riboswitches undergo conformational changes in their secondary and tertiary structures which usually result in suppression of

transcription or translation. Some riboswitches actually function as ribozymes, resulting in self-cleavage of the mRNA and complete inactivation of the mRNA (Montange & Batey, 2008; Winkler et al., 2004).

Defects in RNA expression or processing due to mutations and misregulation can lead to serious diseases (Cooper et al., 2009). Recent studies of miRNA suggest their important role in cancer biology (Lee & Dutta, 2009). The expression levels of specific miRNA are highly correlated with tumor, tissue type or disease stage. Aberrant miRNA levels are also involved in other diseases, including Tourette's syndrome, fragile X syndrome, myotonic dystrophy, and schizophrenia (Abelson, 2005; Caudy et al., 2002; Bilen et al., 2006; Perkins et al., 2007).

All RNAs are produced by transcription in the nucleus, where most processing also takes place. Processing may require transport of RNA between specific locations in the nucleus (Vargas et al., 2005). Once mature, most RNAs are exported to the cytoplasm through the nuclear pores (Stewart, 2007; Kelly & Corbett, 2009), where some may be shuttled back to the nucleus (Hwang et al., 2007; Takano et al., 2005). Once in the cytoplasm, RNAs maybe further transported to specific sites to carry out their functions, depending on the cell type, its developmental stage, environmental signals or perturbations.

2. Review of RNA detection/visualization methods

Several general methods for *in situ* or *in vivo* visualization of RNA processing and transport are currently being used. However, none are as powerful and versatile as methods developed to visualize proteins *in vivo* that employ autofluorescent proteins such as green fluorescent protein (GFP) and its relatives. Autofluorescent ptotein (AFP) technology was made possible by a series of advances beginning with the isolation of GFP from jellyfish in 1962 by Osamu Shimomura. It was not until much later, however, that GFP was cloned by Douglas Prasher (1992) and expressed in different microorganisms by Martie Chalfie (Shimomura, 2009; Chalfie, 2009; Shimomura et al., 1962; Prasher et al., 1992; Chalfie et al.; 1994). Later, Roger Tsien contributed to this field by engineering a whole library of GFP color mutants with increased photostabilities and a wide range of absorption and emission frequencies (Tsien, 2009; Shaner et al., 2004; Wang et al., 2004; Heim et al., 1995). In conjunction with advances in fluorescence microscopy and new spectroscopic tools, AFP technology enabled the development of general purpose methods for intra-cellular genetic tagging to monitor the motions and interactions of two or more proteins simultaneously. This methodology involves genetic fusion of an autofluorescent protein to the protein of interest and its localization at the subcellular level by fluorescence microscopy *in vivo* (Bertrand et al., 1998). Unfortunately, no autofluorescent RNA molecules have been discovered to date that can be used to both detect and monitor RNA *in vivo*. Given the important and diverse roles RNA molecules play in living cells, developing new tools for *in vivo* monitoring of RNA dynamics is crucial for better understanding RNA transport, localization, and functional interactions.

2.1 FISH and molecular beacons probes

Traditional methods for determining which RNAs are produced by a given cell population involve cell disruption and total RNA extraction and its analysis *in vitro* using various tools to identify and quantify specific RNAs. Currently available methods include polymerase chain reaction (PCR), to amplify low copy RNA, Northern Blotting, to separate cellular RNA

by size, microarrays and serial analysis of gene expression (SAGE) for high throughput detection of many RNAs at once. Most techniques require the extraction of RNA from cell populations although some have been adapted for single-cell analysis. The ability to study RNA in a single live cell offers new capabilities for investigating gene expression to better understand RNA function in living cells. This chapter focuses exclusively on fluorescence-based methods, which at this time seem to be the best adapted for *in vivo* real-time RNA visualization to yield subcellular spatial information.

Fluorescence-based techniques have gained popularity for several reasons. While radioactive labeling and autoradiography are potentially more sensitive, fluorescent-based methods have the advantages of 1) using labels with long shelf lives, 2) low toxicity and ease of handling, and 3) high resolution imaging at the single cell level. Moreover, a variety of fluorophores and quenchers, which differ in excitation and emission wavelength, fluorescence lifetime, and anisotropy, are commercially available. This makes fluorescent-based methods excellent candidates for high-throughput screening and real-time *in vivo* detection.

Several recent reviews provide a critical evaluation of fluorescent methods that are currently available for live-cell RNA imaging (Bao et al., 2009; Rodriguez et al., 2007; Tyagi, 2009; Schifferer & Griesbeck, 2009, Raj & van Oudenaarden, 2009). Modern fluorescent visualization approaches can be subdivided into the two main groups with respect to how the fluorescent probe is delivered (Figure 1). The traditional and still most widely used method is fluorescent *in situ* hybridization (FISH) (Moter & Gobel, 2000; Amann et al., 2001), which makes use of short exogenous oligonucleotide probes (about 15-30 nts in length) that carry a fluorophore tag and are complementary to a selected sequence in the target cellular RNA molecule. The main limitation of FISH is that the emission signal of bound and unbound probes is about the same, so cells must be fixed prior to hybridization and then extensively washed to remove unbound probes, which otherwise interferes with the desired signal. Newer "quenched probe" methods for *in vivo* RNA visualization employ fluorophore-labeled hybridization probes to which quencher molecules are also attached to quench the emission of the fluorophore when the probe is not bound to the target RNA (Figure 1A). This approach eliminates the need for washing unbound probes and allows direct imaging of RNA in intact cells. A variety of probe designs have been implemented to position the quencher close to the fluorophore in the unbound state while separating them when the probe binds to its target. "Molecular beacons" (MBs) are the best-known examples of this class of probes (Tyagi and Kramer, 1996; Tyagi et al.; 1998). In the simplest design, the quenched probe forms a hairpin stem-loop structure in which the loop sequence is complementary to the target RNA and the fluorophore and quencher molecules are attached to the 5' and 3' ends of the probe. The terminal four to six bases of the 5' and 3' ends are complementary, so that in the absence of the target they form a short double helical stem that brings the quencher close to the fluorophore, thus quenching its fluorescence. Upon recognition of the target sequence, the hairpin stem of the probe unfolds. This separates the fluorophore from the quencher and restores fluorophore emission. For more information about the design and use of molecular beacons as probes for *in situ* hybridization the reader is referred to the recent publications on the subject (Silverman & Kool, 2005; Tsourkas et al., 2003; Wang et al., 2009; Bao et al., 2009). Numerous variations of quenched probes, which aim to improve the signal-to-background ratio, increase *in vivo* stability or target specificity, have been introduced (Bao et al., 2009). A major concern is to balance specificity and binding kinetic on- and off-rates. Increasing the length of the probe sequence complementary
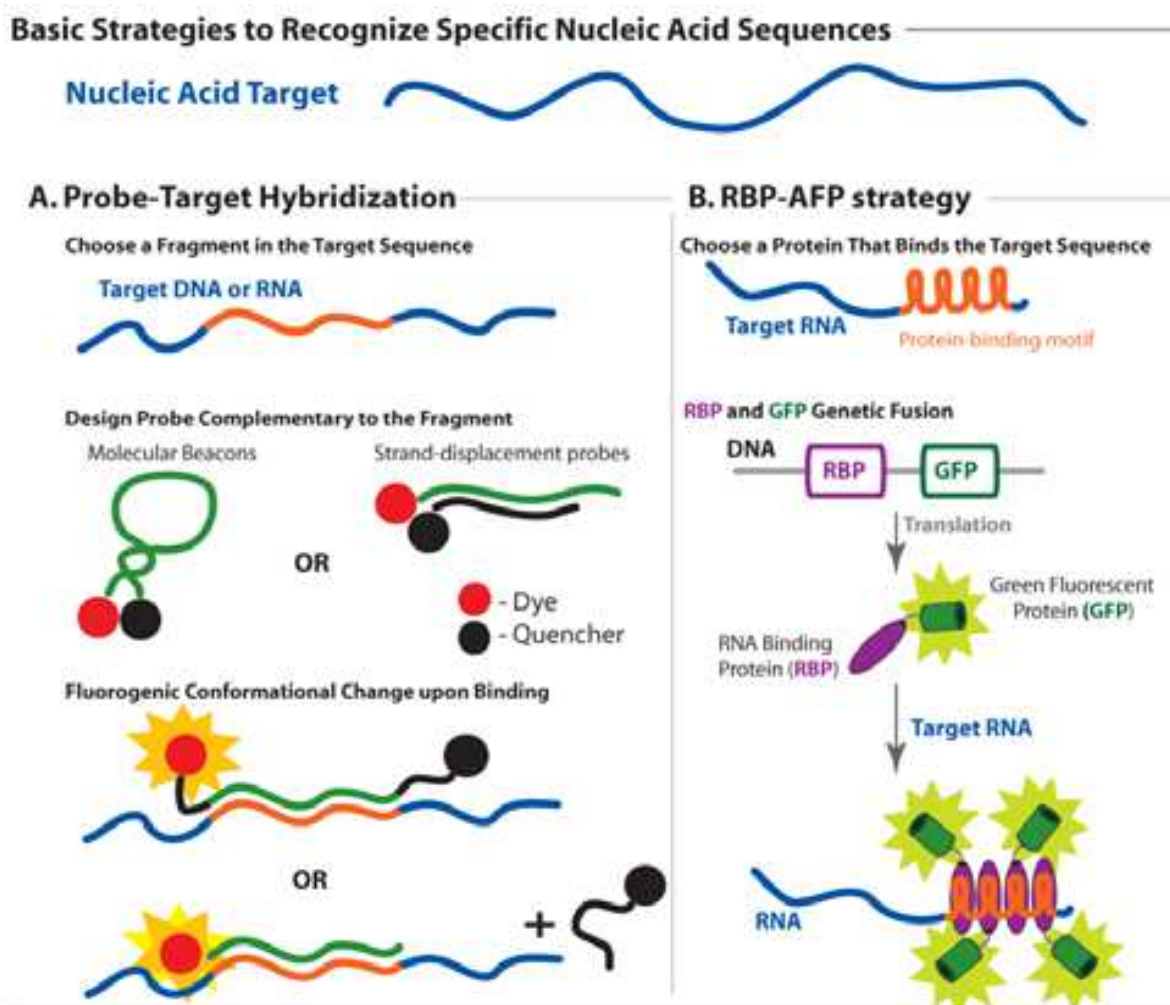
Basic Strategies to Recognize Specific Nucleic Acid Sequences

Nucleic Acid Target

A. Probe-Target Hybridization

Choose a Fragment in the Target Sequence

Target DNA or RNA

Design Probe Complementary to the Fragment

Molecular Beacons   Strand-displacement probes

OR

● - Dye

● - Quencher

Fluorogenic Conformational Change upon Binding

OR

B. RBP-AFP strategy

Choose a Protein That Binds the Target Sequence

Target RNA   Protein-binding motif

RBP and GFP Genetic Fusion

DNA   RBP   GFP

Translation

Green Fluorescent
Protein (GFP)

RNA Binding
Protein (RBP)

Target RNA

RNA

Fig. 1. Schematic representation of several strategies for *in vivo* RNA visualization. The target RNA is represented as a blue strand containing an orange probe hybridization site or protein-binding motifs. A. Probe-target hybridization using "quenched" probes (green strands with attached fluorophore (red) and quencher (black). "Quenched" probes are optically silent in the absence of target. Molecular beacons (on the left) form helical stem that brings quencher close to chromophore. Upon target binding, the terminal stem is disrupted, separating the fluorophore-quencher pair and restoring fluorescence. Strand-displacement probes (on the right) are the pairs of complementary sequences. Upon target recognition, green strand forms more stable probe-target hybridization complex leading to the black strand displacement and yielding emission signal. B. RBP-AFP strategy. Multiple binding motifs for a specific RBP are genetically encoded into the 3'-UTR region of the target RNA (orange region of the sequence). RNA-binding protein (shown in pink) is than fused with GFP (green color) that serves as a fluorescent reporter. When both modified target RNA and RBP-GFP construct are co-expressed in the cell, RBP-GFP constructs interact with target RNA resulting in high localization of fluorescent signal.

to the target increases specificity so long as (i) the melting temperature for exactly matching sequences is greater than physiological temperature and (ii) the melting temperature for single-base mismatches remains below this temperature. If the hybridization sequence is too short, unintended targets may also be bound thus decreasing specificity. If it is too long, one

or more mismatches may be tolerated, decreasing specificity and reducing off-rates from correct targets and thus decreasing the biosensor's responsiveness to decreases in levels of the target RNA.

To increase *in vivo* stability and prevent Rnase H degradation of the probe, oligonucleotides with modified backbone have been introduced, including 2'-O-methyl modified MBs, locked nucleic acid MBs (LNA-MBs) and peptide nucleic acid MBs (PNA-MBs). For further information regarding these modifications, the following publications should be consulted (Tsourkas et al., 2002; Wang et al., 2005, Kuhn et al., 2002).

A number of innovations have been proposed to improve the signal-to-noise ratio of molecular beacons such as dual fluorescence resonance energy transfer (FRET) MBs and quenched autoligation (QUAL) probes (Santangelo et al., 2004; Santangelo et al., 2006; Satterfield et al., 2007). Dual FRET MBs involve a pair of quenched probes that bind to adjacent sites on the target RNA. One probe contains a FRET donor, and the other a FRET acceptor. When both are bound to the target, the donor can transfer its excitation energy to the acceptor, producing FRET emission. A further improvement to dual probes is the use of quenched autoligation (QUAL) probes which bind to adjacent sites and autoligate forming a single long probe that binds to the target with high specificity (Silverman and Kool, 2005). However QUAL probes have the disadvantage of binding essentially irreversibly to targets and hence cannot be used to monitor decreases in target RNAs dynamically.

Quantum dots, gold or silver nanoparticles and photoluminescent polymers were recently introduced as alternatives to organic fluorescent dyes for labeling hybridization probes (Algar et al., 2009; Kim et al., 2008). Quantum dots are brighter and have better resistance to photobleaching than organic fluorophores. Moreover, quantum dots possess broad absorption bands and narrow tunable emission bands that are desirable for optical multiplexing. In comparison with conventionally labeled probes, that have a tendency to accumulate rapidly in nuclei when microinjected, probes conjugated to quantum dots remain in the cytoplasm (Chen et al., 2007). Photoluminescent polymers and gold nanoparticles have better quenching efficiencies as compared to organic dyes (Kim et al., 2008).

Disadvantages of FISH and "quenched probe" techniques include the requirements for time-consuming and expensive chemical modifications of probe sequences to covalently attach fluorophore and quencher molecules. Moreover, these conjugates are not able to diffuse directly into cells and require perturbation or disruption of the membranes.

2.2 RNA labelling with AFP conjugates
2.2.1 General GFP-based strategy

GFP and related autofluorescent proteins, proven immensely useful in visualizing proteins *in vivo*, have also been adapted for detection of RNA molecules *in vivo* (Bertrand et al., 1998; Valencia-Burton et al., 2007). The basic approach involves the genetic fusion of an RNA-binding protein domain (RBP) to an auto-fluorescent protein such as GFP (Figure 1B). The simultaneous genetic modification of the gene for the cellular RNA of interest incorporates an RNA sequence that is recognized by the RBP domain in the target RNA at a location, that does not interfere with its function. This approach was demonstrated by Bertrand and coworkers, who genetically fused the coat protein from the bacterial phage MS2 to GFP to track *in vivo* mRNA encoded from a gene into which multiple MS2 coat protein-binding sites were inserted (Bertrand et al., 1998). When the RBP-GFP fusion and the modified target

RNA are co-expressed in the same cells, multiple copies of RBP-GFP bind to the target RNA, producing a strong fluorescent signal. However, RBP-GFP constructs also emit in their unbound forms and contribute to the background signal. Thus, the need to bind multiple copies of RBP-GFP to the target RNA is to achieve adequate signal-to-background ratio. The disadvantage is the relatively large size of the fluorescent probe complex, which can potentially interfere with RNA transport and localization.

2.2.2 "Split-GFP"

To reduce the background signal from unbound RBP-GFP, the "protein fragment complementation" (PFC) or "split-GFP" methodology was introduced (Valencia-Burton et al., 2007). This method involves the application of rational protein design to separate the autofluorescent protein sequence into two parts that do not fluoresce individually and can be expressed separately. Each part is fused to a different RBP domain. In the presence of a target RNA that is engineered to contain adjacent binding motifs specific to the two RBPs, the two protein fragments are brought together to reconstitute an intact fluorescent protein. In addition to reduced background signal, this method has the virtue that it does not require introducing exogenous RNA fluorescent probes, and can therefore be considered a label-free method. The main disadvantage of this method is the fact that signal generation is essentially irreversible, limiting its use for real-time dynamics of RNA expression levels. An additional limitation of AFP-based methods is the relatively large size of the protein tag (~270 kDa for multiple copies), which raises concerns that transport and localization of the target RNA may be perturbed (Bao et al, 2009; Tyagi, 2009).

We compare the features and advantages/ disadvantages of the latter-mentioned methods for *in vivo* imaging of RNA in Table 1. Rows specify probe and target parameters or additional limitations/ advantages. Hybridization probes are subdivided into FISH probes (column 2) and "quenched probes", including MBs (Column 3); autofluorescent probes (AFP), including GFP, are subdivided into fused (Column 4) and split formats (Column 5). An alternative imaging strategy involves fluorogen-binding aptamers (column 6,7) and will be discussed in the next section, starting with a brief overview of aptamer selection technology and extending to a detailed discussion of how aptamers have been adapted for use as biosensors. These methods are not as developed as RBP-AFP and hybization methods but show potential for further development for *in vivo* use.

3. Aptamer-based RNA detection

3.1 Overview of aptamer technology

Aptamers are functional, single-stranded RNA or DNA oligonucleotides, usually 30 to 100 nts in length, artificially selected from combinatorial libraries for high binding affinities to specific molecular targets. Combinatorial libraries of oligonucleotides, randomized at desired sequence positions and comprising up to 10^{16} different sequences, can be easily generated by modern nucleic acid synthesizers. The process of screening a randomized oligonucleotide pool to identify and amplify sequences that perform a specific function is called "Systematic Evolution of Ligands by Exponential Enrichment" (SELEX) and was introduced almost 20 years ago (Tuerk & Gold, 1990; Ellington & Szostak, 1990). SELEX has been used to obtain high affinity aptamers to almost any conceivable target and has even been adapted to obtain catalytic nucleic acids (ribozymes and DNAzymes) by selecting for

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
Method	**Hybridization probes**		**AFP-based**		**Fluorogen-binding aptamers**	
	Fluorescent in situ hybridization (FISH)	Quenched probes	General	split-AFP	Genetic fusion of aptamer into target RNA	Allosteric aptamer-based approach
Suitable for in vivo study	No (cells must fixed)	YES	YES	YES	YES	YES
Average probe size	15-30 nts (5-10 kDa)*	< 50 nts (16.5 kDa)	Multiple probes (about 270kDa)	Single probe (< 100 kDa)	< 60 nts (20 kDa)	
Unbound probe	Fluorescent	Nonfluorescent or low fluorescent	Fluorescent	Nonfluorescent	Nonfluorescent	Nonfluorescent
Delivery of the probe	Exogenous		Transfection or genetic fusion		Genetic fusion	Exogenous
Target RNA	Unmodified or modified		Genetically modified		Genetically modified	Unmodified
Additional advantages	Well-adapted	1) High potential for optical multiplexing; 2) better specificity compare to FISH; 3) low background; 4) easy adapted for FRET		1) Low background; 2) the tag size is reduced. 1) No perturbation of the cell; 2) not as sensitive to target secondary structure.	no need for additional co-expressed constructs and to balance the AFP probe levels with the target RNA	no chemical attachments of fluorophores and quenchers - label free 1) free dyes penetrate cells better than hybridization probes; 2) signal generation is reversible
Additional drawbacks and limitations	Real-time in vivo RNA imaging is not possible.	1) Unmodified probes are subject to degradation- leading to false positives; 2) Hybridization site selection is difficult due to target secondary structure and multiple protein binding sites; 3) probes tend to accumulate in the nucleus.	1) Genetic manipulation of RNA may not always achievable; 2) Large probe size may interfere with RNA transport; 3) AFPs are phototoxic to the cells.	GFP reconstitution is irreversible (not possible to monitor the gene downregulation).	1) the number of suitable fluorogenic dyes is rather limited; 2) low number of fluorophore-binding aptamers with light-up properties are available; 3) not adequately tested in living cells.	
Challenges and opportunities	New bioinformatic methods to achieve better hybridization site selection.		1) Infrared emission proteins for tissue and organism studies; 2) new bioinformatic methods for positioning of protein binding motifs.		1)Expanding the number of available fluorogenic dyes; 2) Expanding light-up pairs to fully cover the visible-near-IR spectrum; 3) adapt for *in vivo* use; 4) improving bioinformatic tools to identify aptamer insertion or hybridization sites.	

* calculate 330Da per nucleotide

Table 1. Comparative analysis of in vivo RNA visualization methods. *-calculations based on 330 Da per nucleotide.

transition-state analogues (Weigand et al., 2006; Schlosser et al., 2006). This technology has therefore revolutionized the field of sensing chemistry by enabling researchers to systematically generate numerous aptamers targeting a diverse range of analytes. Large numbers of RNA and DNA aptamers have been obtained by this method in the past 20 years, targeting a broad range of small molecules including organic dyes, amino acids, cofactors, carbohydrates and nucleotides, as well as proteins and nucleic acids (Klussmann, 2006). Aptamers against different classes of antibiotics, proteins and even whole cells have already found wide applications in biosensing, diagnostics, drug development and nanomedicine, as recently reviewed by other authors (Liu et al., 2009; Cho et al., 2009).

Aptamers provide a nucleic acid-based alternative technology to monoclonal antibodies for specific recognition of diverse molecular targets. Aptamer selection exhibits a number of advantages over monoclonal antibody technology including: 1) versatility in designing selection strategies; 2) lower cost; and 3) easy scale-up and production as no cell or animal culture is needed. Other advantages of using nucleic acid aptamers in biosensors include their relatively small sizes, high analyte specificities and high affinities (K_ds down to pM range), low toxicities and immunogenicities, and amenability to chemical modification to suit the desired application. Finally, they are small enough that their 2D structures can be reliably predicted and their 3D structures solved by NMR solution methods, which facilitates "tuning up" their chemical and biological properties.

 SELEX begins with chemical synthesis of a randomized DNA pool. If selection of an RNA aptamer is the goal, the DNA pool is transcribed prior to the selection procedure. Next, the pool is incubated with the target molecule to achieve efficient binding. This is followed by a carefully designed selection step to sequester those DNA or RNA oligonucleotides in the pool exhibiting the desired functional properties - this selection process being the most crucial step. Finally, the amplification step enriches the pool, and the entire cycle is repeated for a sufficient number of times to obtain a pool comprising only molecules with a desired functional property. The original SELEX procedure has been modified to achieve higher affinity and specificity of aptamer products (e.g. Negative SELEX, Counter SELEX), to improve selection towards more complex targets (e.g. Genomic SELEX, Deconvolution-SELEX, Tissue-SELEX) or to select aptamers with novel functional properties (e.g.Covalent SELEX, Photo-SELEX) (Stoltenburg et al., 2007).

The Ellington group established a database of aptamers that is current as of 2006 and provides aptamer targets, sequences, details regarding selection procedures and references to original publications. It can be accessed at http://aptamer.icmb.utexas.edu/ site (Lee et al., 2004).

3.2 Fluorogen-binding aptamer-based systems with light-up properties

Organic dyes were the first targets used to develop the SELEX methodology for RNA and DNA (Ellington & Szostak, 1990; Ellington & Szostak., 1992). Recent work has been directed to obtain aptamers that target fluorogenic dyes, with the aim of developing new *in vivo* tagging systems for cellular RNA molecules as alternatives to RBP-GFP fusion constructs. However, initial selections of fluorophore-binding aptamers were actually intended for other purposes. For example, Malachite Green (MG) dye has long been known to generate hydroxyl free radicals when irradiated with visible light. Liao *et al* exploited this property in 1994 by conjugating MG to antibodies to target proteins *in vivo,* a technique called "Chromophore-assisted laser inactivation" (Liao et al., 1994). This inspired Grate and

Wilson to select an RNA aptamer to bind MG and selectively target specific RNA molecules for inactivation *in vivo* (Grate & Wilson, 1999). An RNA aptamer to Hoechst dye 33258 was also selected to study gene regulation while RNA aptamers for Sulforhodamine B and Fluorescein were selected for potential *in vivo* RNA labeling (Werstuck & Green, 1998; Holeman et al, 1998). Werstuck and Green showed that they could regulate a gene *in vivo* which had been modified by the insertion of the Hoechst dye by adding the dye to the medium (Werstuck & Green, 1998).

This earlier work was subsequently adapted to obtain high-affinity aptamers that specifically bind to fluorogenic dyes, defined as molecules which exhibit minimal emission when free in solution, but which fluoresce intensely when tightly bound. Any strategy where an increase in emission signal occurs upon specific binding to a target is called a "light-up" methodology, and fluorogen-aptamer complexes are often referred to as "fluoromodules" or "light-up pairs". The performance of sensing systems is usually quantified in terms of "fluorescence enhancement" and so it is important to define what is meant by this term. For classic molecular beacons or FRET probes, fluorescence enhancement is defined as the ratio of the emission of the probe bound to its cognate target divided by the emission of free probe in the absence of the target. For light-up dye/ aptamer pairs, fluorescent enhancement is defined as the ratio of the emission of the light-up dye bound to its aptamer divided by the emission of the free dye in solution. For biosensors employing light-up dye/ aptamer pairs allosterically linked to target recognition domains, fluorescence enhancement is defined as the ratio of the emission of the dye in the presence of both biosensor and target divided by the emission of the dye in the presence of the biosensor alone. In genetic fusions of dye-binding aptamers with target RNAs, fluorescence enhancement is the ratio of the emission of the dye bound to the aptamer fused with the target RNA divided by the emission of the dye in the presence of the target lacking aptamer modules.

We focus the rest of this section on efforts to design light-up pairs and to adapt them for use as biosensors to detect and image specific molecules *in vitro* and *in vivo*. We begin with the MG aptamer light-up pair, which exhibits the most dramatic increase in fluorescence (Figure 2). Triphenylmethane dyes such as MG exhibit low fluorescence when free in solution due to deactivation channels associated with torsional motion of phenyl groups. When placed in environments that restrict these motions, these dyes become strongly fluorescent. Babendure et al were the first to observe that MG fluorescence increased dramatically when the dye is bound to the aptamer selected by Grate and Wilson, and thus subsequent works focused on adapting this light-up pair for biosensing (Babendure *et al.*, 2003; Grate & Wilson, 1999). Crystal structure analysis of the MG aptamer bound to tetramethylrosamine (TMR), a MG analog, revealed that it comprises an RNA helix containing a large and asymmetrical internal loop (Baugh et al., 2000). The nucleotides of the internal loop form a series of stacking and non-Watson-Crick basepairing interactions to create a unique binding pocket that restricts the flexibility of the dye and keeps it in a highly fluorescent state (Figure 2A, 2B). The high affinity of this aptamer for MG (K_d=117 nM) and the high fluorescence enhancement that results from binding (2360-fold) have encouraged several groups to apply it to create programmable nucleic acid biosensing platforms (Babendure et al., 2003; Stojanovic & Kolpashchikov, 2004; Hirabayashi et al., 2006; Afonin et al., 2008). These will be described in more detail in the next section after we cover other efforts to develop new light-up pairs.

3.3 Novel approaches for "light-up" dye/aptamer pairs selection

Sando and coworkers suggested a new route for generating specific light-up pairs starting with a conventional fluorescent DNA-staining dye that exhibits a number of favorable characteristics, including cell permeability, solubility, and stability in cellular environments (Sando et al., 2007; Sando et al., 2008). The key idea to this approach was demonstrated using Hoechst dye 33258, a widely used fluorescent stain for dsDNA molecules that fluoresces upon binding to the minor groove of dsDNA. To adapt this dye for more specific light-up applications, a variety of chemical modifications of the dye were employed to reduce or completely suppress its nonspecific affinity for dsDNA. The most effective modification of the dye for this purpose was the addition of two t-Butyl groups to its terminal phenol ring (The resulting "Hoechst 33258 derivative" is shown in Figure 2C). Next, SELEX was used to obtain a DNA aptamer that bound Hoechst 33258 derivative with K_d = 878 nM and enhanced fluorescence emission by 191-fold (Sando et al., 2007). Further work obtained an improved RNA aptamer showing higher affinity for the dye (K_d=35 nM) but somewhat reduced emission enhancement (56-fold).

A similar strategy was adapted by Constantin and coworkers to design a novel fluorogenic cyanine dye, Dimethyl Indole Red (DIR) (Constantin et al., 2008). Cyanine dyes such as thiazole orange (TO) and its derivatives are fluorogenic intercalation dyes that comprise two heterocycles separated by a methine group. Substitution in TO of a bulky dimethylindole heterocycle in place of the thiazole ring sterically hindered intercalation. Addition of an anionic propylsulfonate group further minimized nonspecific interactions with nucleic acids by increasing electrostatic repulsion (DIR is shown in Figure 2C). An RNA aptamer was obtained for DIR by SELEX that binds with K_d=87 nM and 60-fold emission enhancement. An advantage of the cyanine dyes is that they have relatively narrow emission bands, their emission wavelength maxima easily varied by chemical modification. As a group, their emission maxima cover much of the visible spectrum and extend into the near infrared.

Another recently developed approach for creating light-up dyes is to start with a dye that emits when free in solution and to covalently attach electron donor groups that quench its emission by a photo-induced electron transfer (PET) process (Sparano & Koide, 2005; Sparano & Koide, 2007). Aptamers that bind exclusively to the quencher moieties are then selected. The idea is to obtain aptamers that can block electron transfer and thereby restore emission of the fluorophore moiety. The PET approach was demonstrated in principle using 2',7'-dichlorofluorescein (DCF) as a fluorophore and N-(p-methoxyphenyl)-piperazine (MPP) as a quencher (Figure 2D). An RNA aptamer for the MPP quencher moiety was obtained by SELEX and was indeed found to enhance DCF fluorescence in the DCF/ MPP conjugate in a concentration-dependent manner. As the authors pointed out, however, this particular DCF-MPP/ aptamer system is unsuitable for *in vivo* applications because the fluorescence enhancement is only about 13-fold at 100 μM aptamer concentration (Sparano & Koide, 2007).

A novel class of light-up probes for RNA detection was developed by Stojanovic's group by conjugating nonspecific fluorogenic intercalating dyes such as thiazole orange (TO) to small molecules such as GMP and AMP, for which RNA aptamers have previously been obtained (Pei et al., 2009). The GMP aptamer binds the GMP-TO conjugate with K_d=60 nM and a 500-fold fluorescence enhancement (Figure 3). Similar results were obtained with an AMP aptamer and AMP-TO conjugate. To adapt this approach for *in vivo* use, multiple copies of the appropriate aptamer would have to be inserted into the gene encoding the cellular RNA to
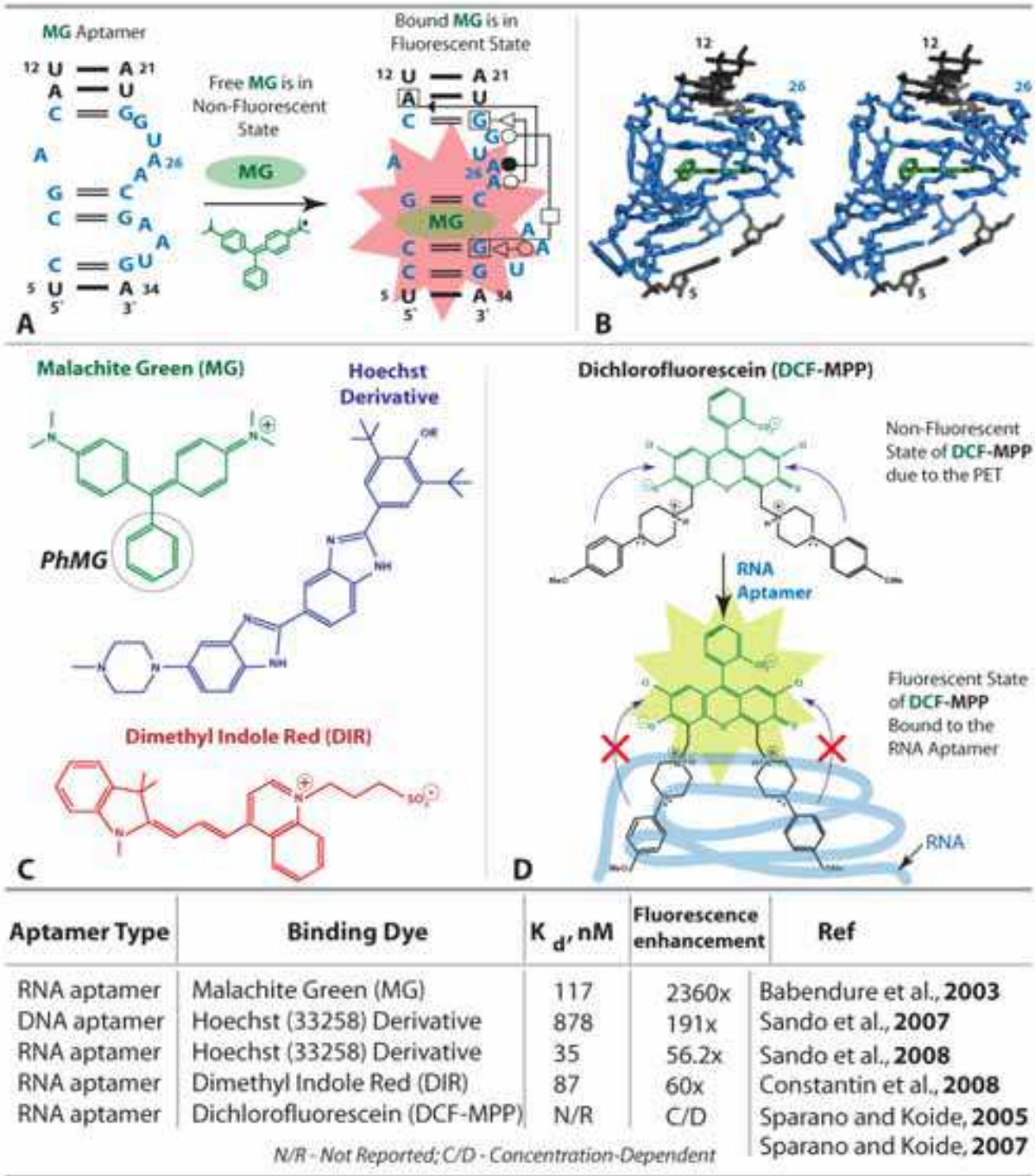
Fig. 2. Light-up dye/ aptamer pairs and their properties. A. Malachite Green (MG) aptamer 3D structure with basepairs annotated with Leontis-Westhof nomenclature. Watson-Crick Edges are shown in circles, Hoogsteen Edges in squares, and the Sugar Edges in triangles (Leontis & Westhof, 2001). Hollow symbols indicate trans basepairs and filled symbols, cis basepairs. Boxes indicate nucleotides pairing with more then one base. B. 3D stereo view of the X-ray structure of the MG aptamer binding to tetramethylrosamine (PDB file: 1f1t). Nucleotides are colored to match the annotated 3D structure. C. Several examples of fluorogenic dyes for which nucleic acid aptamers have been obtained to create "light-up" pairs. In case of MG, phenyl ring (PhMG) mentioned in text is circled. D. Schematic

representation of a strategy use to obtain a new "light-up" pair (Sparano & Koide, 2005). Dichlorofluorescein (DCF, shown in green) is attached to two quencher molecules (shown in black). Photoinduced electron transfer (PET, blue arrows) quenches fluorescence. Binding of an aptamer selected to bind exclusively to the quencher moieties blocks PET, leading to the recovery of emission. Lower panel. Binding affinities and fluorescence enhancements of the light-up pairs described in this chapter.

be visualized, and the small-molecule dye conjugate would have to be delivered into the cell by an appropriate mechanism (for a review of cellular delivery systems, see Bao et al., 2009). The main drawback of this approach is competition for aptamer binding by endogenous molecules (for example, cellular GMP or AMP). Nevertheless, this approach has great potential, as the number of small molecules that can be used here is almost limitless.
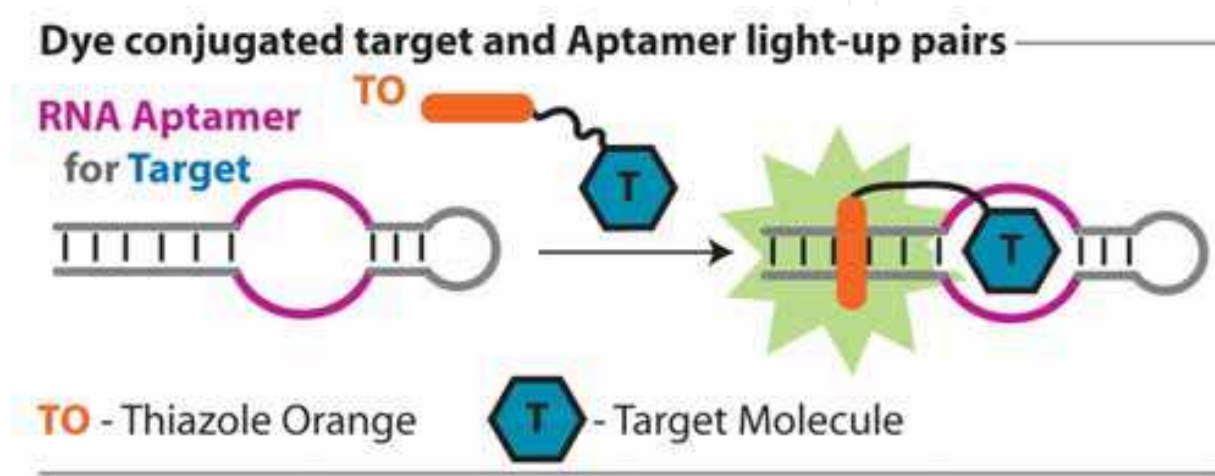
Fig. 3. Stojanovic's strategy for RNA detection. Thiazole Orange (TO), a fluorescent intercalator, is conjugated to a small molecule (blue hexagon). RNA aptamer (pink region) specific for its target binds the small molecule and forces TO binding to double-stranded region at the specific location.

3.4 Fluorogen-binding aptamers as signalling elements for label-free, specific nucleic acid detection

Some of the disadvantages of other RNA imaging methods (described above in section 2.1 and 2.2) may potentially be overcome using label-free approaches based on fluorogen-binding aptamers. First, aptamers can be entirely composed of unmodified RNA, allowing genetic fusion to genes coding for cellular RNAs of interest (Figure 4B). This has the advantage of endogenous synthesis of the targeting sequence. Moreover, these aptamers, even when used in multiple copies, do not significantly increase the size of the cellular RNA, and only the imaging dye needs to be introduced exogenously, as most organic dyes are able to diffuse into cells. Finally, dyes with suitable light-up characteristics and low affinity for cellular targets can be expected to give low background signals.

However, selecting appropriate sites for aptamer insertion remains a critical issue. To obtain valid imaging results one must ensure that the insertion of the aptamer molecules does not disrupt functionally important target RNA secondary or tertiary structure or protein-binding sites. In cases where the organism of interest is difficult to genetically modify, these aptamers can be coupled to a specific target recognition domain and introduced exogenously using a variety of delivery methods (Figure 4A).

3.4.1 Allosteric aptamer-based sensors

Aptamers have been conjugated to other aptamers forming "allosteric aptamers" or to ribozymes forming "aptazymes" (Soukup, 2004; Silverman, 2003). Stojanovic and Kolpashchikov first reported the design of novel allosteric sensing systems produced by coupling the MG-binding RNA aptamer through a "communication module" to each of a series of specific aptamers for ATP, FMN (flavin monomucleotide), and theophylline. These "allosteric aptamers" are capable of transducting binding information between the two aptamers (Stojanovic & Kolpaschikov, 2004). They showed that binding of the target molecule to its respective aptamer domain caused a significant conformational change in the RNA sensor that was relayed through the communication module to the MG aptamer module so as to enable it to bind MG and produce a fluorescent signal.

Within a year, Kolpashchikov adapted this concept to create an allosteric RNA aptamer probe system to sense specific nucleic acid targets (Kolpashchikov, 2005). The MG aptamer domain of the probe was divided into two fragments, each of which also contained half of the sequence complementary to the target DNA (see Figure 5). In the presence of the target, the complementary "binding arms" of the binary probe hybridize cooperatively to the target, forming a three-way junction structure and reconstituting the MG aptamer by stabilizing the helix containing it. The probe-target complex then binds MG and renders it
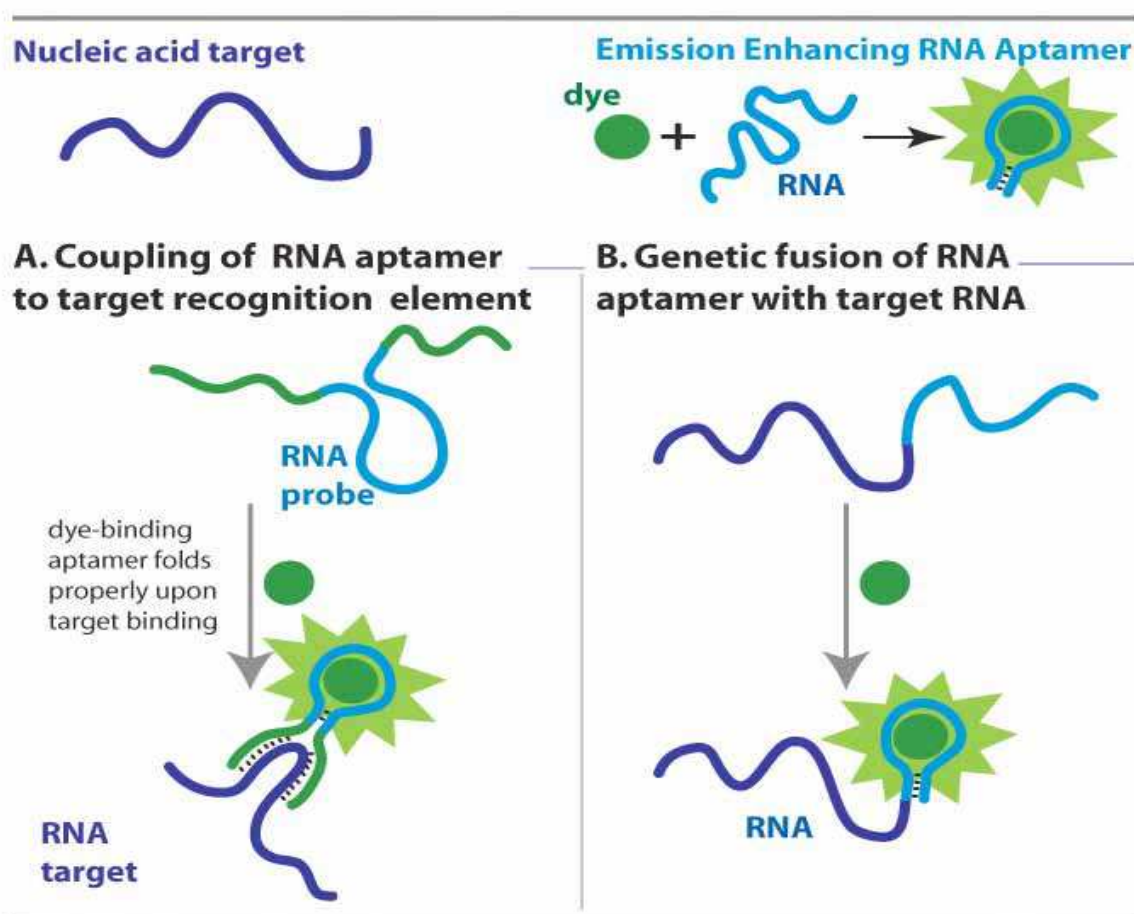

Fig. 4. Label-free approaches for in vivo RNA imaging. A. Allosteric coupling of fluorogen-binding aptamer to another target recognition element. B. Genetic fusion of the fluorogen-binding aptamer to target RNA sequence.
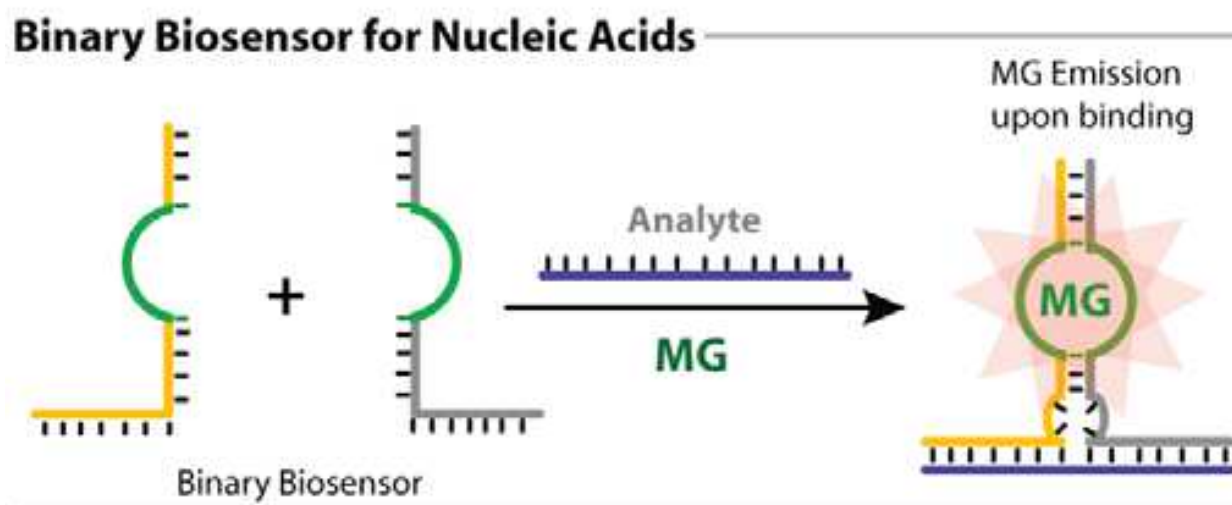
Fig. 5. Binary MG-aptamer based sensor (Kolpashchikov, 2005). Analyte-promoted assembly and stabilization of binary biosensor to form MG aptamer (shown in green color).

fluorescent, signaling the presence of the analyte. A 20-fold fluorescence enhancement was reported for this biosensor in the presence of 2 μM concentration of the target, with discrimination factors greater than 20-fold for half of the sequences differing from the intended analyte by single nucleotide substitutions. Sando and coworkers tested the applicability of Kolpashchikov's strategy to design a binary probe using their novel light-up Hoechst derivative/ DNA aptamer light-up pair (see section 2.2 above) (Sando et al., 2007). The same design principle was used: the DNA aptamer for the Hoechst dye was split into two molecules, each of which contains half of the sequence complementary to the target. When the target DNA binds to the complementary regions of the binary probe, a three-way junction forms which restores the DNA aptamer structure and allows it to bind to the Hoechst dye. These binary DNA probes showed 70-fold fluorescence enhancement; correct analyte was discriminated from single-mismatches by a factor of 15.

A major hurdle in designing probes for specifically detecting or imaging biological RNAs in complex environmental or whole cell samples is the presence of secondary structure in the target RNA, which may compete with probe binding, thus reducing the binding affinity of the biosensor. Paranemic binding between two pre-formed nucleic acid structures was originally developed for DNA nanotechnology by Seeman and coworkers (Shen et al., 2004) and subsequently demonstrated in RNA (Afonin et al., 2008). Paranemic binding provides the means to design probes targeting internal loop structures in the RNA analyte in a programmable, sequence-specific manner that obviates the need to unfold the target to expose extended single-stranded regions. Paranemic binding can be applied for recognition of symmetrical internal loops ranging in size from 4 to 8 nucleotides (Afonin et al., 2008), as well as asymmetrical loops which have different numbers of bases in each strand (Novikova et al, unpublished observations). Afonin *et al* coupled the MG aptamer to an RNA paranemic binding motif to establish proof-of-principle for programmable sensing of pre-folded RNA secondary structures using paranemic binding (Afonin et al., 2008). To make MG binding contingent upon binding of the target RNA, the position and orientation of the aptamer motif was optimized relative to the paranemic binding motif. In the resulting constructs, crucial non-WC basepairs that "zip-up" the MG binding site and fix MG in its

fluorescent state only form in the presence of the target RNA, as shown in Figure 6. Using several different probe and target sequences, the generality of this approach was demonstrated. Target sequences were bound with low nM affinity and fluorescence enhancements ranged from 6 to 15-fold. Furthermore, it was shown that single-base mismatches could be discriminated by about a factor of 13.

The key principle of the sensing strategies described above involves the incorporation of a fluorogen-binding aptamer within the probe structure in such a way that fluorogen binding depends upon the specific recognition and binding to the target.
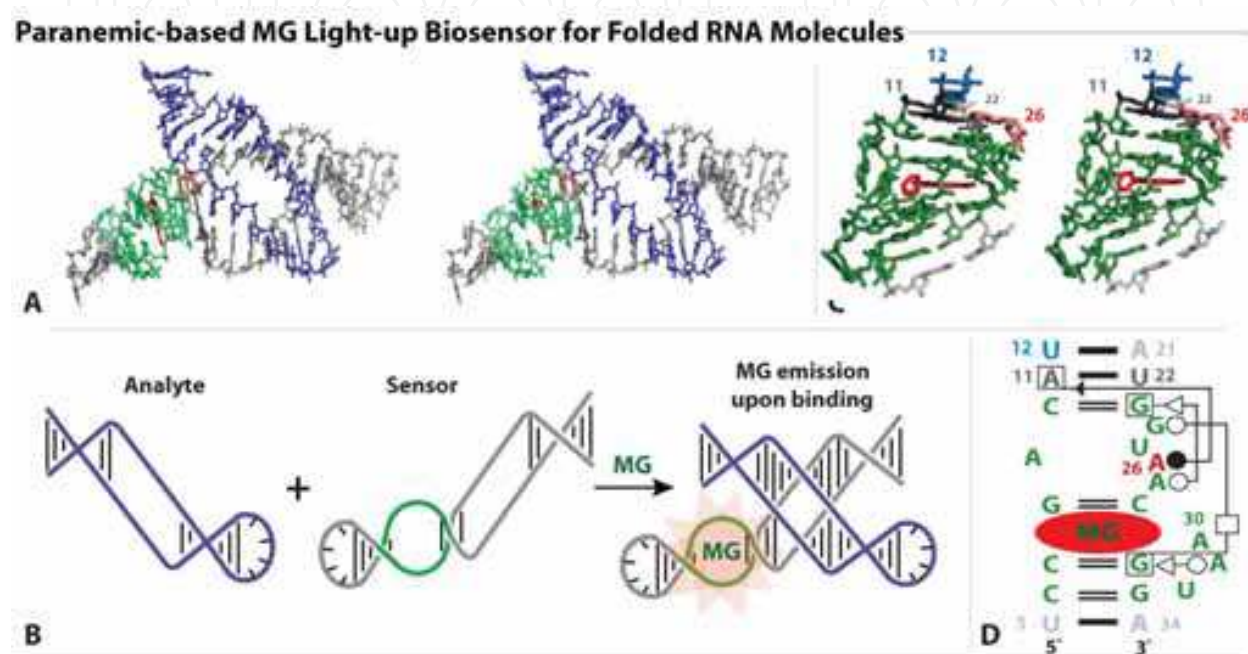
Fig. 6. Paranemic RNA biosensor for folded RNA molecules (Afonin et al., 2008) **A.** 3D model of the biosensor-target complex. **B.** Schematic representation of the paranemic assembly of the MG-aptamer containing biosensor (grey)with the pre-folded analyte RNA (in blue). Formation of the recognition complex stabilizes the Malachite Green (MG) aptamer structure (in green). **C.** 3D stereo view of the MG aptamer (Baugh et al., 2000; PDB file 1f1t). **D.** Corresponding annotation of the 3D structure using Leontis-Westhof basepair annotations (Leontis and Westhof, 2001). Bases are numbered as in X-ray (1f1t). U12 (blue) of target RNA interacts with A21 of biosensor probe RNA (in grey), stabilizing the flanking A11/ U22 (grey) basepair of the biosensor, which interacts with A26 (red) in the aptamer to "zip-up" the MG binding site.

3.4.2 Genetic fusion of fluorogen- or fluorophore-binding aptamers and target RNA sequences

In the previous section, we described strategies for RNA detection and visualization using hybridization probes allosterically coupled to fluorogen-binding aptamers. A related approach is to genetically encode the fluorogen- or fluorophore-binding aptamer directly into the gene sequence of a cellular RNA of interest. Sando *et al.* recently reported the selection of an effective RNA aptamer for Hoechst 33258 derivative and the insertion of five tandem repeats of this aptamer sequence on the 3' side of the stop codon of the luciferase gene (Sando et al., 2008). They showed that transcription of the mRNA containing the

tandem aptamers could be monitored *in vitro* by fluorescence in the presence of the dye, and observed a 5-fold enhancement compared to parallel transcription of the same mRNA lacking the aptamer sequences. The authors call this method "blue fluorescent RNA" and reported K_d=35 nM for binding of the dye to the isolated aptamer, but did not report apparent K_d's in the context of the mRNA construct.

Eydeler *et al.* reported detailed measurements of the Sulforhodamine B-binding aptamer (SRB2m), selected by Holeman et al (1998), fused to a variety of genetic constructs, including intact EGFP-mRNA produced *in vivo* and assayed *in vitro* in the presence of other cellular RNAs (Eydeler et al., 2009). They used fluorescence correlation spectroscopy (FCS), a method that allows one to obtain diffusion constants and other kinetic parameters from fluorescence fluctuations generated by molecules diffusing in and out of the focal volume. They reported K_d's ranging between 120 nM and 1130 nM for different constructs containing single aptamer modules compared to K_d~238 nM for the isolated aptamer. Tandem repeats of the aptamer sequence in these constructs showed a significant increase in binding affinities, with K_d values of 60-70 nM. The lowest apparent binding affinity of Sulforhodamine B was observed for aptamer embedded into EGFP-coding mRNA (k_d~1.13 μM), which was attributed to improper RNA folding. Even though the binding of Sulforhodamine B to its aptamer SRB2m does not cause a significant emission change (this is not a "light-up" pair), this work shows the value of using multiple tandem copies of aptamers and points out the need for careful consideration of target secondary structure to obtain optimal *in vivo* results.

Zhang and co-workers inserted the MG aptamer sequence into the cloned gene of *V. proteolyticus* 5S rRNA so that the aptamer motif replaced Helix 5 and Loop C of the 5S molecule (Zhang et al., 2009). They transcribed the 5S/ MG aptamer chimera *in vitro* and showed that it binds to MG and enhances its fluorescence to a comparable extent as free aptamer (Kd ~ 900 nM for 5S/ MG chimera vs. Kd ~ 800 nM for isolated aptamer, Baugh et al., 2000; cf. Kd ~ 117 nM, Babendure et al., 2003). They also expressed the 5S/ MG chimera from a plasmid in *E. coli* cells and observed fluorescence enhancement of MG dye added to the medium, although they did not report detailed *in vivo* measurements.

Applications of light-up dye/ aptamer pairs are not solely limited to sensing nucleic acids. Other applications for these technologies have been proposed and are under development. For example, MG dye was conjugated to kinesin-driven microtubules to develop mobile bioprobes that can capture MG aptamer-tagged mRNAs from a transcription mixture and sequester them in a microfluidic device for high-throughput screening (Hirabayashi et al., 2006).

4. Conclusions

To date, remarkable progress has been made to visualize specific RNA molecules with high spatial and temporal resolution in living cells. These studies have begun to reveal detailed mechanisms of RNA trafficking, localization and turnover (Bratu et al., 2003, Lange et al., 2008; Zimyanin et al., 2008). However, significant challenges remain to render these methods more generally applicable and to overcome shortcomings of currently used methods. The ideal RNA biosensing system should be easy to deliver to living cells, non-toxic and non-perturbing to cellular functions. It should penetrate all parts of the cell homogeneously and be chemically stable in different cellular environments. The fluorescent

reporter of the biosensor should have high absorptivity and quantum yield (brightness) as well as good photostability and low phototoxicity. The sensor system should be easy to implement, so that different RNA targets can be studied without extensive optimization. It should allow optical multiplexing so that interactions between two or more cellular RNAs (or RNA and protein) can be monitored. It should only fluoresce when bound to the intended target and otherwise produce low background fluorescence. It should bind specifically to the intended target with minimal binding to targets having similar sequence and should exhibit fast binding kinetics to monitor rapid changes in RNA concentration. Finally, it should allow for studying specific RNAs in whole tissues or organs. Of course, no current method possesses all these desired characteristics.

Currently, hybridization probes offer the greatest variety and versatility in choice of fluorescent reporters as they can be conjugated to semiconductor quantum dots, in addition to a wide variety of organic dyes. However, they still require exogenous delivery which can perturb cell functions. Hybridization methods suffer from signal sensitivity issues for two reasons. Unmodified targets generally bind only one hybridization probe and degraded probes release fluorescent reporters. To minimize degradation, hybridization probes are usually synthesized with resistant, modified backbones (see above). To increase sensitivity, multiple copies of the complementary binding sequence can be introduced in the target gene (van den Bogaard & Tyagi, 2009). Whether or not the target is genetically engineered, the use of hybridization probes raises several concerns related to choosing appropriate binding sites on the target RNAs. First the target sites should have relatively weak secondary structures to allow the probe to bind with high affinity with minimal competition. Second the selected binding sites should not participate in crucial functions, especially related to the processing, localization, or regulation of the target RNA. Hybridization probes targeting RNA sequences that bind other cellular factors can potentially interfere with these processes and produce spurious imaging results.

Autofluorescent protein (AFP) probes are synthesized intra-cellularly from gene fusions directly encoded in cell lines or from plasmids that can be introduced by transfection. These methods are less perturbing than those used to deliver hybridization probes. Moreover, AFP probes can be directed to desired cellular compartments with appropriate peptide targeting sequences. However, the use of AFP probes requires insertion of RNA binding protein (RBP) recognition sites into target RNA sequences, which also requires attention to target RNA sequence to avoid interfering with RNA function, but is potentially less sensitive to RNA secondary structure. One disadvantage of the general AFP strategy is that unbound probes are also fluorescent. This has been largely solved by the "split-AFP" approach, but this method has the disadvantage that signal generation is irreversible upon reconstitution of the fluorescent protein. Contrary to common perceptions, autofluorescent proteins also have phototoxicity issues, as under irradiation, they produce reactive oxygen species that can damage cells (Remington et al., 2006).

New methods, employing fluorogen-binding aptamers, hold promise of addressing many of the shortcomings of the two currenty used methods. First, aptamer-based methods are generally simpler and potentially more flexible and can be readily adapted to the particular application. Like AFP probes and unlike hybridization probes, the aptamers themselves can be produced endogenously. They can be either genetically encoded in the target RNA or produced from plasmids. Of course, they can also be introduced exogenously like

hybridization probes if that is required. Like hybridization probes and unlike AFP probes, there is no need for co-expressing additional fusion constructs that require additional time to mature and that need to be balanced in their expression to target RNA expression levels– a significant simplification of the procedure. Additionally, because fluorogen-binding aptamers can be genetically integrated in their targets, signal generation is rapidly and completely eliminated upon RNA degradation, thus reducing background and false-positive rates providing further improvement over both AFP and hybridization methods. Finally, the aptamer size is quite insignificant compared to protein-based probes. However, it is not yet well established how many tandem aptamers are required to achieve adequate sensitivity for single-molecule detection *in vivo*. Based on recent studies, it appears that tandem repeats of aptamers domains in target RNA are preferred to achieve proper aptamer folding and significant increase in binding affinities. Unlike hybridization probes, only free fluorogenic dyes need to penetrate cells when genetically encoded aptamers are used and there is no need to covalently attach the fluorescent reporters and quenchers to the probe. However, a number of challenges must be met for fluorogen-binding aptamers to become practical alternatives to the currently used methods. First of all, the number of available fluorogenic dyes is still quite limited and they have not been adequately tested for suitability *in vivo*. Further work is needed to identify which fluorogenic dyes have low *in vivo* toxicity and phototoxicity, low background fluorescence, and produce sufficient signal when bound specifically to their target aptamers to make single-molecules detection possible *in vivo* with modern imaging methods. For example, MG, while exhibiting low background fluorescence, has substantial phototoxicity issues. Cyanine dyes appear promising. In addition to DIR, a number of cyanine dyes with reduced affinity to nonspecific nucleic acids and substantial light-up signal have been synthesized by Armitage's group and tuned to emit across the visible spectrum and even into the near-infrared (Ozhalici-Unal et al., 2008; Shank et al., 2009). Second, a challenge for future work is to expand the available choices of light-up pairs by selection of new aptamers that specifically bind new improved fluorogenic dyes, including near-IR emitters. Just as new AFPs emitting in the NIR have recently been reported (Shu et al., 2009), new aptamer/ NIR dye light-up pairs will make it possible to detect signals from specific molecules located deep inside tissues (Amiot et al., 2008). While, a number of innovative approaches for aptamer/ dye pair selection have been developed *in vitro*, most of them have been tested only *in vitro*. Thus, more *in vivo* testing is a high priority to determine which fluorogenic dyes and aptamer-based biosensing platforms are suitable for *in vivo* use. Finally, improved bioinformatic methods are needed to identify optimal sites for aptamer insertion or hybridization into target RNAs.

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Biosensors

Edited by Pier Andrea Serra

A biosensor is defined as a detecting device that combines a transducer with a biologically sensitive and selective component. When a specific target molecule interacts with the biological component, a signal is produced, at transducer level, proportional to the concentration of the substance. Therefore biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. Bringing together researchers from 11 different countries, this book covers a wide range of aspects and issues related to biosensor technology, such as biosensor applications in the fields of drug discovery, diagnostics and bacteria detection, optical biosensors, biotelemetry and algorithms applied to biosensing.

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