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Approaches for measuring the dynamics of RNA-protein interactions.

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Abstract

RNA-protein interactions are pivotal for the regulation of gene expression from bacteria to human. RNA-protein interactions are dynamic; they change over biologically relevant timescales. Understanding the regulation of gene expression at the RNA level requires knowledge of the dynamics of RNA-protein interactions. Here, we discuss the main experimental approaches to measure dynamic aspects of RNA-protein interactions. We cover techniques that assess dynamics of cellular RNA-protein interactions that accompany biological processes over timescales of hours or longer and techniques measuring the kinetic dynamics of RNA-protein interactions *in vitro*.

INTRODUCTION

RNA-protein interactions are pivotal for gene expression from bacteria to human^{1, 2}. In typical eukaryotic cells, more than 1,500 different proteins interact with tens of thousands of distinct RNAs^{3, 4}. Many proteins interact with multiple RNAs and probably all RNAs interact with numerous proteins⁵. The resulting combinations of individual RNA-protein interactions give rise to enormously complex RNA-protein networks, which govern gene expression on the RNA level^{3, 6, 7}. Defects or deregulation of RNA-protein networks often cause disease^{8, 9}.

RNA-protein networks are also dynamic¹⁰. That is, RNA-protein interactions change at biologically relevant timescales. As RNAs traverse the stages of biogenesis, function and decay, a given RNA interacts with different sets of proteins². RNA-protein interaction patterns also change in response to biotic and abiotic stimuli, stresses, and during cell differentiation¹¹. The changes in RNA-protein interaction patterns at individual RNA binding sites are ultimately determined by kinetic parameters, e.g. association and dissociation rate constants of the protein to a given RNA site⁵.

The changes in RNA-protein interactions are carefully orchestrated to maintain orderly regulation of gene expression¹⁰. Accordingly, understanding the regulation of gene expression requires knowledge of the dynamics of RNA-protein interactions⁵. This is a formidable charge, given the sheer number of cellular RNAs and their interacting proteins³. No current method is able to capture the full scale of dynamics of all RNA-protein

interactions in cells, but available methods provide insight into various dynamic facets of RNA-protein interactions. In this review, we discuss the main experimental approaches to measure dynamic aspects of RNA-protein interactions.

It is instructive to classify these approaches into two categories, according to the interrogated timescales (Figure 1). The first category entails techniques capturing and cataloging changes in cellular RNA-protein interactions that accompany biological processes including cell differentiation, and exposure to stresses or to other stimuli. From a chemical perspective, these techniques compare changes in steady state patterns of RNA-protein interactions that occur over hours or days.

The second category encompasses techniques measuring the kinetics of RNA-protein interactions, that is, rates by which proteins bind to, dissociate from, and otherwise interact with RNAs (Figure 1). Kinetic measurements examine reactions over timescales from sub-seconds to hours. Although kinetics ultimately dictates all RNA-protein interaction patterns in cells, monitoring the kinetics by which proteins interact with RNA sites in cells is technically challenging, and, to our knowledge, has not yet been accomplished. However, many RNA-protein interactions have been kinetically characterized *in vitro*⁵. We highlight the most commonly used *in vitro* techniques. We will thus discuss the main experimental approaches to assess dynamics of RNA-protein interactions according to the two overarching categories: (i) approaches to measure the dynamics of RNA-protein interaction in cells, and (ii) approaches to measure kinetics of RNA-protein interactions *in vitro* (Figure 1).

APPROACHES TO MEASURE THE DYNAMICS OF RNA-PROTEIN INTERACTIONS IN CELLS.

Approaches that measure the dynamics of cellular RNA-protein interactions typically interrogate changes in RNA-protein interaction patterns at timescales of hours or longer. In a chemical sense, most of these RNA-protein interaction patterns are likely at steady state, but the steady state changes over the observed time. The cellular approaches are best viewed in two broad categories: (i) approaches that identify RNAs bound to a given protein, and (ii) approaches that identify proteins bound to RNA, either to a specific RNA or to a class or all cellular RNAs (Figure 2). Most methods in both categories utilize RNA-protein crosslinking via ultraviolet (UV) irradiation. Crosslinking is performed at $\lambda = 254 \text{ nm}$ ¹², or at $\lambda > 310 \text{ nm}$, if photoreactive thionucleosides have been incorporated into the cellular RNA¹³. Protein-RNA crosslinking occurs at zero distance¹⁴. Proteins that remain associated with RNA after extensive washes are classified as RNA binding proteins (RBPs)¹⁴. Following crosslinking, biochemical isolation strategies are used to either (i) identify RNAs bound by a specific protein, or (ii) to identify proteins bound to a specific RNA, or to all cellular RNAs or to an RNA class, such as mRNAs¹⁴. Performing these experiments at different conditions or at different stages of cellular development reports dynamic changes of the RNA-protein interaction spectrum at biological timescales^{15, 16}.

RNA-protein crosslinking has also been performed by chemical means^{17–19}. Alternative methods to identify RNA-protein contacts use covalent marks on RNA (e.g. A to I edited

bases, or poly-U addition to the 3' end of the RNA) that are left upon physical protein contacts with the RNA region^{20, 21}. In addition, the dynamics of RNA-protein interactions in cells has been assessed by high-resolution fluorescence microscopy^{22, 23}. The microscopy studies have been reviewed in detail elsewhere²⁴. We focus our discussion on the widely used approaches that utilize RNA-protein crosslinking.

Identification of RNAs bound by protein

The most commonly used method to identify RNAs that are directly bound by a specific protein is CLIP (Cross Linking and Immunoprecipitation)^{14, 25–29}. CLIP methods typically use an antibody to immunoprecipitate (IP) native or epitope-tagged proteins that have been UV-crosslinked to RNA, or affinity precipitation of an affinity-tagged protein^{20–22}. Either before or after precipitation, RNA is fragmented to reduce the size of the region crosslinked to protein. Subsequently, these RNA fragments are isolated, converted to cDNA and then sequenced to identify transcriptome-wide sites of protein crosslinking. Following the first proof of concept study in 2003¹², which depended on plasmid-based cloning and Sanger sequencing, CLIP-based methods now employ next generation sequencing (NGS) platforms, improved reagents, and protocols allowing efficient RNA recovery and cDNA library construction, aimed at reducing the amount of starting material needed^{26, 30, 31}. A panel of bioinformatic tools now exists to maximize information gleaned from CLIP libraries generated by deep sequencing³². The evolution and iterations of CLIP-based methods are discussed in detail in several recent reviews^{25–27, 31}.

CLIP maps sites of protein-RNA contact. However, determining which interactions are functional requires additional information, including data from biochemical assays, integration of RNA profiling data from over-expression or knockdown/knockout models, or a combination of these data^{33, 34}. Combined, these approaches begin to discriminate which protein-RNA contacts may be functional rather than opportunistic, while also revealing general principles by which some proteins bind and control their RNA targets³³. For example, biochemical purification of the LASR complex (Large Assembly of Splicing Regulators), combined with in vitro protein-RNA binding assays shows that the RBP Rbfox1 can be recruited to RNA by other proteins. These observations shed light on the interplay between RNA binding proteins, and provide an explanation for the lack of Rbfox1 consensus sequences in some Rbfox1 binding sites mapped by CLIP³⁵.

Another example of functional interaction between RBPs derived from CLIP maps comes from a comparative analysis of PTBP2-RNA interactions in the brain of wild type mice and mice lacking the RBP NOVA2¹⁶. Incorporating CLIP maps with splicing data from single and double knockouts of NOVA2 and PTBP2 showed that NOVA2 normally represses retention of distinct introns. These introns act as scaffolds for PTBP2 binding and PTBP2-mediated splicing regulation¹⁶. This observation highlights the possibility that loss or over-expression of a single RBP can cause broad remodeling of RNA-protein binding patterns and extensive changes in the regulation of gene expression.

Integrating RNA profiling data with RBP abundance, and RNA-protein binding maps also illuminate the dynamics of RNA regulation by RBPs in developmental programs, including neurogenesis, stem cell pluripotency, spermatogenesis, liver maturation, and development of

muscle, heart, and skin^{36–44}. For example, in postnatal heart development, the RBPs CELF1 and MBNL1 undergo developmentally regulated changes in expression that are critical for establishing alternative mRNA splicing patterns necessary for proper development^{39, 45}. A combination of phenotypic data from gene knockout models and RNA-protein binding maps revealed biological roles of specific protein-RNA interactions at key developmental time points. Examples include NOVA2-dependent regulation of alternative splicing of DAB1 that is required for neuronal migration⁴⁶, and PTBP2-dependent regulation of alternative splicing of SHTN1 that is required for axonogenesis⁴⁷.

Beyond modifications that have made CLIP protocols more efficient^{48, 49}, CLIP has also been combined with powerful genetic tools to provide new perspectives on protein-RNA interactions and alternatively processed mRNAs in specific cell populations in different tissues^{16, 50, 51}. For example, cTag-CLIP (conditionally tagged) utilizes transgenic mice and Cre-lox technology to express GFP-tagged versions of the RBP NOVA2 in specific cell types depending on the promoter used to drive expression of the Cre recombinase^{16, 28, 50, 52}. Combining this approach with CLIP using an antibody against GFP allows isolation of protein-RNA complexes from specific cell types without the need to first obtain enriched populations of cells^{16, 50}. This is particularly advantageous for tissues with high cellular heterogeneity such as the brain, and is similar to the RiboTag strategy for conditional epitope tagging of the ribosome⁵³. Using this approach, comparison of NOVA2 - RNA interactions in motoneurons versus whole spinal cord revealed motoneuron-specific RNA regulatory functions of NOVA2 that promote branching of dendrites⁵¹. Similarly, NOVA2-cTag-CLIP allowed resolution of functional differences in NOVA2-mediated alternative splicing of the same transcripts in either excitatory or inhibitory neurons¹⁶. Cell-type-specific splicing regulation has also been demonstrated by Rbfox1 in different populations of cortical interneurons⁵⁴.

Alternative methods to map protein-RNA contacts utilize formaldehyde or other chemical means to crosslink proteins to RNA¹⁹, or immunoprecipitate without crosslinking⁵⁵. Other approaches involve expression of chimeric RBPs that mark their RNA targets *in vivo*, followed by NGS to identify RNAs bearing these marks. One approach (RBP-PUP) involves fusion of the RBP of interest to polyU polymerase (PUP) which marks RNAs with 3' terminal uridines²⁰. Another approach is TRIBE (targets of RNA-binding proteins identified by editing), which involves construction of a RBP fused to the catalytic domain of the ADAR RNA editing enzyme²¹. An advantage of these methods is that protein-RNA complexes do not need to be biochemically isolated. However, these techniques require the construction and expression of chimeric proteins, and therefore cannot be readily applied to native RBPs.

Identification of proteins that bind a specific RNA

Identifying proteins bound to a specific RNA in cells is key to understanding function and regulation of a given RNA. To delineate the mechanism by which the non-coding RNA Xist mediates X chromosome inactivation, multiple groups developed approaches to identify Xist-interacting proteins using a general strategy that can be applied to other RNAs. These methods, RAP (RNA Antisense Purification)⁵⁶, CHIRP-MS (Comprehensive Identification

of RNA binding Proteins by Mass Spectrometry)⁵⁷, and iDRiP (Identification of Direct RNA Interacting Proteins)⁵⁸ depend on hybridization of biotinylated antisense probes complementary to a target RNA, followed by streptavidin capture of probe: RNA hybrids, and identification of UV-crosslinked proteins by mass spectrometry. These methods are applied to different cell states to identify differences in proteins interacting with a given RNA. For example, CHIRP-MS analysis of four different cell-types revealed a common set of Xist-interacting proteins, as well as proteins that interact with Xist only in embryonic stem cells, epiblast stem cells, or trophoblast stem cells⁵⁷.

An alternative approach to identify proteins bound to a specific RNA species is RaPID (RNA-Protein Interaction Detection)⁵⁹. This strategy uses proximity-dependent protein labeling via BirA*, a modified promiscuous biotin ligase from *E. coli* that biotinylates proximal proteins⁶⁰. In RaPID, BirA* is expressed as a chimeric protein fused to the λ N peptide of bacteriophage lambda, which binds with high affinity to BoxB stem loops engineered into the RNA of interest. Upon addition of biotin to cells containing these two components, proteins in the vicinity of the λ N-BirA fusion bound to BoxB stem loops are biotinylated. These proteins are recovered using streptavidin capture.

RaPID has been used to assess how disease-associated point mutations affect protein-RNA interactions in living cells and to identify host proteins that interact with viral RNA⁵⁹. The *B. subtilis* biotin ligase has significantly higher labeling efficiency after 1 minute, compared to the 18 hour optimal labelling time point for the *E. coli* enzyme⁵⁹. RaPID with the *B. subtilis* enzyme may thus be a powerful approach to measure the dynamics of protein-RNA interactions on specific RNAs during a short time course.

Mapping of protein-RNA interactions across the transcriptome

Strategies to examine protein-RNA interactions across different classes of RNAs, or even all RNAs, have provided unprecedented insights into protein-RNA interfaces and dynamics, and revealed hundreds of proteins not previously known to bind RNA⁴. The first of these methods, RNA Interactome Capture (RIC)^{61–63} uses oligo-dT-coated beads to capture polyA + mRNAs from UV-crosslinked cells. After extensive washes to remove non-crosslinked proteins, the remaining crosslinked proteins are released by RNase digestion and identified by mass spectrometry.

RIC has been combined with pre-treatment of cells with photoreactive thionucleosides (4-thiouridine and 6-thioguanosine)¹³. UV-crosslinking of proteins to RNAs at sites containing these analogs results in base changes in the corresponding cDNA library⁶⁴, which allows the precise localization of protein-RNA contacts⁶¹. RIC has been applied to different cell types for global comparisons of protein-RNA contact sites and identification of dynamic differences that may alter mRNA metabolism⁶⁵. For example, RIC has shown pervasive changes in protein-RNA contacts in HEK293 cells following infection by sindbis virus⁶⁶, hypoxia-associated changes in kidney tubular epithelial cells⁶⁷, and in LPS-induced and untreated macrophages⁶⁸. An enhanced version of RIC (eRIC) uses a locked nucleic acid (LNA)-modified oligodT capture probe, which allows stringent capture and washing conditions, and potentially allows the detection of dynamic changes in the RNA-binding proteome in different biological conditions⁶⁹.

The use of photoreactive thionucleosides and T-to-C mutations to map protein-RNA sites was recently combined with cellular compartment-specific protein biotinylation by APEX2, a highly active, engineered ascorbate peroxidase or APEX^{70, 71}. This method, Proximity-CLIP⁷² (does not contain an IP step) builds upon the APEX-RIP strategy⁷³ where APEX is localized by genetic fusion to different cellular compartments. Upon addition of biotin phenol and hydrogen peroxide, APEX catalyzes the formation of biotin-phenoxyl radicals that covalently biotinylate proximal proteins. Following addition of formaldehyde to crosslink protein to RNA, cells are lysed, and streptavidin-coated beads are used to capture biotinylated proteins and their bound RNAs. Proteins are then examined by mass spectrometry and RNAs by RNA-Seq to reveal the proteome and transcriptome within distinct cellular compartments⁷³. Proximity-CLIP follows a similar workflow, but photo-crosslinks protein-RNA complexes in 4-thiouridine treated cells to examine sites of protein-RNA contact in different cellular compartments⁷².

Incorporation of modified nucleotides into RNA is also used for RICK (RNA Interactome using Click Chemistry)¹⁷ and CARIC (Click Chemistry-Assisted RNA Interactome Capture)¹⁸. In both methods, cells are labeled with 5-ethynyluridine (EU) that is incorporated into newly synthesized RNA and crosslinked. The addition of biotinylated azide to EU occurs via copper (I)-catalyzed cycloaddition reaction (click chemistry), enabling these RNAs to be isolated from cell lysates using streptavidin coated beads, and subsequent analysis of crosslinked proteins by mass spectrometry. Because these methods do not depend on a polyA tail, proteins bound to diverse RNA species can be examined, including circular RNAs, as well as transcriptionally paused and nascent RNA. Combining these techniques with different RNA enrichment methods can potentially reveal dynamics of protein-RNA interactions at different stages of the mRNA lifecycle. However, the need for modified nucleotides restricts these approaches largely to cell culture.

Protein-RNA complexes from UV-treated cells can be efficiently and rapidly isolated by acid-phenol extraction. Three methods, XRNAX (protein-crosslinked RNA extraction)¹¹, OOPS (Orthogonal Organic Phase Separation)⁷⁴, and PTex (Phenol Toluol Extraction)⁷⁵ purify cross-linked ribonucleoproteins based on their physiochemical properties, allowing global purification of protein-crosslinked RNA. XRNAX and OOPS both begin with acidic guanidinium-thiocyanate-phenol-chloroform extraction commonly used to separate RNA and protein into aqueous and organic phases, respectively^{11, 74}. Protein-RNA complexes and DNA localize to the interphase, which is collected. DNase treated and crosslinked protein-RNA complexes are then partially digested and captured on silica. In PTex, a Phenol-Toluol extraction is first performed. RNA, proteins, and protein-RNA complexes accumulate in the upper aqueous phase. DNA and membranes localize to the interphase⁷⁵. The aqueous phase is extracted under chaotropic and acidic conditions to localize crosslinked protein-RNA complexes in the interphase, as in XRNAX and OOPS. These methods do not depend on a polyA tail or modified nucleotides and can therefore be applied to any crosslinked material to identify proteins that interact with all classes of RNAs. Furthermore, mass spectrometry analyses of peptides cross-linked to RNA can reveal unanticipated RNA binding surfaces of proteins. Finally, in proof-of-concept assays, XRNAX and PTex were used to characterize protein-RNA complexes in *E. coli* and *Salmonella Typhimurium*, which lack polyadenylated RNA^{11, 75}. The ability to purify crosslinked protein-RNA complexes by simple and rapid

extractions provides a powerful new approach to study dynamics of protein-RNA interactions in diverse biological contexts.

APPROACHES TO MEASURE THE KINETICS OF RNA-PROTEIN INTERACTIONS

The dynamics of an interaction of a protein with a defined RNA site on the molecular scale is described by kinetic parameters. Given that RNA-protein interaction networks are ultimately comprised of individual RNA-protein interactions, understanding the associated kinetics is required for mechanistic insight into the molecular basis of gene expression^{5, 76}. Every RNA-protein interaction involves the association of a given protein to, and its dissociation from a given RNA site (Figure 3). For enzymes that chemically or physically alter the RNA (e.g., methyl transferases, nucleases, helicases), kinetic descriptions also need to include these enzymatic steps (Figure 3). Kinetic models for proteins that process RNA in sequential steps (helicases, nucleases, polymerases) can thus become highly complex^{77–79}. In addition, many proteins oligomerize⁷⁹, which also needs to be considered. In the past, rigorous quantitative analysis of multi-step reactions often required advanced mathematics to solve complex sets of differential equations. Now, however, software packages allow the numerical determination of kinetic parameters for even complex reactions^{80–82}. Although skill and critical analysis are still needed to validate numerically obtained kinetic parameters for complex reactions, it is possible to quantitatively characterize reactions with dozens of steps and with multiple components⁸³.

The theories underlying kinetic descriptions of molecular interactions have been validated on numerous biological systems over the last century⁸⁴. Rate constants have defined physical meanings, provided the corresponding reaction steps can be experimentally “isolated” and measured. The association rate constant describes the frequency by which a protein and an RNA site interact, as a function of the concentration of the reactants. The dissociation rate constant describes the frequency by which the protein vacates the RNA site. The inverse of the dissociation rate constant ($1/k_d = \tau$) represents the time the protein resides at the RNA site. In addition, rate constants are directly related to free energy terms, a physical property that determines molecular behavior and interactions. For enzymes it is often not trivial to experimentally “isolate” and measure association and dissociation steps. If association and dissociation steps cannot be determined in isolation, composite rate constants are obtained. Since multiple reactions steps contribute to composite rate constants, usually in a non-linear fashion, physical interpretations of composite rate constants are often complicated or even impossible.

In cells, processes or features that alter accessibility of the RNA site, including RNA structure or competing proteins, impact the kinetics of the RNA-protein interaction⁵. Factors that affect the concentration of free protein, such as RNAs or other proteins that compete for protein binding also alter the kinetics of the RNA-protein interaction (Figure 3). In addition, RNA and protein modifications can affect binding or dissociation kinetics, or both (Figure 3). Moreover, most RNA-binding proteins bind to a spectrum of RNAs sites with differing binding and dissociation kinetics^{5, 34}. These layers of complexity all impact RNA-protein

interaction kinetics in biological settings. It is thus challenging to quantitatively link kinetic parameters to the biological role of a given RNA-protein interaction. We are not aware of published work measuring kinetics of protein interactions with individual RNA binding sites in cells. Time-resolved protein-RNA co-localization studies have been reported, which are important for understanding the dynamics of RNA-protein interaction in cells^{22, 23}, but kinetic parameters of RNA-protein interactions in cells could not yet been extracted from these measurements.

Kinetics of RNA-protein interactions have been measured exclusively *in vitro*. Binding and dissociation rate constants for proteins that interact with RNA span a considerable range⁸⁵. Association rate constants have been reported from $k_{\text{on}} = 2.4 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ for the pseudouridine synthetase Pus1, to $k_{\text{on}} = 2.1 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ for bacteriophage MS2 coat protein^{86, 87}. Dissociation rate constants range from $k_{\text{off}} = 7 \text{ s}^{-1}$ for IRP1 binding to iron-responsive elements⁸⁸. This value corresponds to a lifetime of the protein-RNA complex of $\tau = 0.1 \text{ s}$. On the other side of the spectrum lies the dissociation rate constant for CYT-18 binding to bI4 Group I Intron, with $k_{\text{off}} = 3.4 \times 10^{-6} \text{ s}^{-1}$, which corresponds to a lifetime of $\tau = 55 \text{ hours}$ ⁸⁹.

An array of approaches is available to measure the kinetics of RNA-protein interactions *in vitro* (Figure 1). We discuss the main techniques, distinguishing between ensemble and single molecule approaches (Figure 1). For the ensemble approaches, we separately focus on techniques that measure kinetics of individual RNA-protein interactions and then on high-throughput methods that simultaneously interrogate thousands of different RNAs.

Methods to measure the kinetics of individual RNA-protein interactions *in vitro*

Physical separation of RNA-protein complexes from free components.—

Monitoring the kinetics of RNA-protein interactions by physical separation of the RNA-protein complex from the free components is a widely used experimental strategy. In principle, any technique that accomplishes this separation can be employed to monitor the kinetics of an RNA-protein interaction. If the separation procedure is fast, compared to the kinetic parameter in question, the RNA-protein reaction can be directly subjected to the separation technique. If the separation procedure is slower than expected reaction steps, the reaction can be performed in solution, followed by a reaction stop at the desired time. A stopped reaction sample is then subjected to the separation procedure. The main techniques for determining kinetic parameters that utilize physical separation of RNA-protein complexes from free RNA or protein are electrophoretic gel-shifts and filter binding.

Electrophoretic Gel-Shifts

Electrophoretic mobility shifts of RNA, protein, or both, in polyacrylamide or agarose gels are widely used to monitor RNA-protein interactions^{90, 91}. Electrophoretic gel-shift techniques report changes in molecular weight, shape and charge, that occur upon formation of the RNA-protein complex. In most cases it is not possible to resolve smaller conformational changes of the RNA-protein complex. Most experiments monitor shifts of radio- or fluorescently labeled RNA upon protein binding, but it is possible to follow the shift of proteins upon RNA binding with a labeled protein or by Western blotting⁹⁰.

Provided bound and unbound species are sufficiently separated, gel-shifts can be used to monitor RNA-protein interactions. Because molecular weight is directly read out, gel-shift techniques are particularly well suited to monitor binding of multiple protein units to a given RNA. It is also possible to use capillary electrophoresis to separate RNA-protein complexes from RNA, and thereby accomplish a potentially larger sample throughput than traditional slab gel setups⁹².

The kinetic measurements of RNA-protein interactions are usually performed before applying the samples to gels. Dissociation rate constants are measured in a pulse chase regime, where re-binding of protein to the RNA is prevented by scavenger reagents, such as unlabeled RNA or heparin⁹¹ (Figure 4). If the protein shift is followed, other scavenger agents need to be used. To monitor the dissociation reaction, usually aliquots are removed at a given time, and then analyzed by gel-shifts. Measurements of association rate constants requires the association reactions to be stopped a defined times (Figure 4). This is also accomplished by addition of a scavenger⁹¹.

Gel-shift techniques are widely employed because standard molecular biology equipment can be used, and only small amounts of material are needed (few microliters of sub-nanomolar labeled RNA). However, the environment in gels differs substantially from solution conditions. These differences can potentially affect properties of RNA-protein complexes and thus impact obtained data. Problems in this respect can be diagnosed by comparing results from gel-shift experiments with those obtained by solution methods. Despite these caveats, the kinetics of several RNA-protein interactions have been determined by gel-shift techniques^{91, 93}.

Filter binding

Filter binding is based on the differential absorption of RNA-protein complexes and RNA complexes on nitrocellulose or other membranes⁹⁴. RNA-protein complexes are formed in solution. The sample is then filtered quickly through the membrane, usually aided by vacuum pressure. Protein and protein-bound RNA is retained on the membrane, free RNA is not. Usually, labeled RNA is employed, which permits the quantification of RNA in the protein complex on the filter, quantification of not retained free RNA, or both⁹⁴. Like gel-shift techniques, filter binding reports the physical association between RNA and protein.

Since the physical filter binding step is often fast (1–2 s), compared to the kinetic parameters in question, samples can be directly subjected to the filter at the appropriate times. It is also possible to perform the reactions in solution, as discussed above for gel-shift techniques (Figure 4), and apply samples with stopped reactions to the filter⁹⁵. Although samples are rapidly applied to the filter, RNA can potentially dissociate from the protein during the filtering and subsequent washing steps, if those are required, and obtained data can be distorted. To diagnose this problem, it is necessary to validate results by orthogonal solutions methods. Like gel-shift techniques, filter binding requires only minuscule amounts of samples ($\sim 10^{-15}$ mol / sample)⁹⁶.

Solution methods to measure the kinetics of RNA-protein interactions.—

Approaches that monitor RNA-protein interactions in solution bypass the need to physically

separate RNA-protein complexes from the free components. To measure the kinetics of RNA-protein interactions, any technique can be employed that is able to measure a physical property of the RNA-protein complex that differs sufficiently from the free RNA, the free protein, or both, over a timeframe that is short or at least comparable to the kinetic step in question. Several spectroscopic techniques have been employed to measure the kinetics of RNA-protein interactions in solution, including UV-absorption spectroscopy⁹⁷, Circular Dichroism (CD)^{98, 99} and fluorescence spectroscopy¹⁰⁰. Although UV and CD spectrometers are widely available, both methods require RNA and protein concentrations that are often impractical for kinetic measurements. These techniques have thus been only rarely used to determine kinetic parameters for RNA-protein interactions^{101, 102}. Fluorescence spectroscopy has become the method of choice to measure kinetics of RNA-protein interactions in solution^{100, 103}. For this reason, we focus here on fluorescence approaches.

Fluorescence measurements.—Several fluorescence-based techniques are used to measure rate constants for RNA-protein interactions, including fluorescence anisotropy measurements, fluorescence quenching measurements, Fluorescence (Foerster) Resonance Energy Transfer (FRET), and combinations of these techniques. All of these methods allow kinetic measurements in real time and are compatible with stopped-flow equipment to monitor reactions at sub-second timescales¹⁰⁰.

Fluorescence anisotropy

Fluorescence anisotropy is the polarization of polarized excitation light. As the fluorophor changes its position during the excited state, the fluorescence emission is depolarized and fluorescence anisotropy decreases^{104, 105}. The degree of depolarization scales with the rate by which a given fluorophor tumbles in solution¹⁰⁴. The faster the tumbling rate, the higher the degree of depolarization and the lower the anisotropy¹⁰⁴. The tumbling rate of a fluorescently labeled RNA, protein, or RNA-protein complex correlates with molecular weight and shape¹⁰⁴. The smaller the molecule or complex containing the fluorophor, the faster its tumbling rate. Upon formation of the RNA-protein complex in question, size and molecular weight change, compared to the free components. This change is followed over time by recording fluorescence anisotropy (Figure 5A).

In most cases, the kinetics of RNA-protein interactions are measured by placing the fluorophor in the RNA, because it is easier to attach a fluorophor to the RNA, and because proteins are usually larger than RNAs, which results in a bigger change in molecular weight from free RNA to RNA-protein complex, compared with placing the fluorophor on the protein. A potential drawback is the requirement for relatively high concentrations of fluorophor (usually > 50 nM), in order to obtain a sufficient fluorescence signal with most fluorimeters. However, selection of fluorophors with high quantum yield and high extinction coefficient can mitigate this issue to some extent¹⁰⁶. Nevertheless, at RNA concentrations of more than 50 nM, association reactions for most RNA-protein complexes occur in the sub-second range, and usually require stopped-flow equipment to monitor the reaction. While RNA-protein association reactions can be directly measured, dissociation reactions are performed in a pulse chase regime, as outlined above (Figure 4). Fluorescence anisotropy

has been used to measure kinetic parameters for several RNA-protein interactions, including POS1¹⁰⁷, and Gar1¹⁰⁸.

Fluorescence quenching

Fluorescence quenching is seen when the environment of a fluorophor changes, such that either absorption of excitation light by the fluorophor is hindered, its fluorescence emission is quenched, its excited state is physically altered, or a combination of these events occur^{100, 103}. The formation of RNA-protein complexes often changes the environment of fluorophors that are attached to, or incorporated into either RNA or protein (Figure 5C)^{103, 109}. To monitor the kinetics of RNA-protein interactions, the considerations outlined above for fluorescence anisotropy apply. Since only a simple fluorimeter is required, the method is versatile. Fluorescence quenching has been used to measure kinetic parameters for several RNA-protein interactions^{110, 111}.

Fluorescence Resonance Energy Transfer

Fluorescence (or “Foerster”) Resonance Energy Transfer (FRET) is the radiation-less (hence: resonance) transfer of energy from one fluorophor (donor) to another fluorophor (acceptor)^{112, 113}. Upon FRET, the donor fluorescence decreases and the acceptor fluorescence increases, compared to the fluorescence of both fluorophors without FRET (Figure 5B). The FRET efficiency is inversely proportional to the sixth power of the distance between donor and acceptor fluorophor¹¹⁴. FRET is thus highly sensitive to small changes in the distance between the two fluorophors and consequently well suited to monitor formation and dissociation of RNA-protein complexes¹¹⁵. Either donor or acceptor fluorescence, or both, can be measured to monitor FRET (Figure 5B). If donor and acceptor fluorescence are simultaneously monitored, the method is very sensitive¹¹⁶. For this reason, and as discussed below, FRET is the method of choice to measure single molecule fluorescence^{117–119}.

To monitor kinetics of RNA-protein interactions, two fluorophors are required that change their distance in the RNA-protein complex, compared to the free components. In many cases, one fluorophor is placed on the protein, the other on the RNA (Figure 5B). However, both fluorophors can be strategically placed on the RNA, if the RNA structure changes upon protein binding^{118, 119}. Of note, even in single stranded nucleic acids, distances between two strategically placed fluorophors can change significantly upon protein binding, because unstructured RNA and DNA behave like a worm-like chain, but adopt restricted conformations when bound to protein^{120, 121}. Since FRET is highly sensitive to small distance changes, a robust signal can often be measured for even miniscule re-arrangements of RNAs¹²². It is possible to place both fluorophors on the protein, provided they change distance upon RNA binding¹²³. However, it is usually more practical to employ labeled RNA, compared to labeled protein, because fluorescently labeled RNA is commercially available. To monitor kinetics of RNA-protein interactions, the considerations outlined above for fluorescence anisotropy apply. FRET has been used to measure kinetic parameters for RNA-protein interactions including tRNA synthetases¹²³, RNA helicases^{124–126} and RNA binding proteins^{127–130}.

Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) detects molecular events at the surface of a thin metal film^{131, 132}. Polarized light is focused on the metal film, and at a specific “resonant” angle, oscillating free electrons at the interface between the metal film and a medium of a different refractive index (surface plasmons) resonate with the incident light. This resonance causes light absorption, which is detected with high accuracy^{131, 132}. Oscillations of the surface plasmons are extremely sensitive to even small changes in the surface characteristics, and therefore SPR can be used to detect binding of biomolecules to a reactant that is immobilized on the metal surface¹³³ (Figure 6A).

To detect RNA-protein interactions, either the RNA or the protein is immobilized on the metal surface in a flowcell (Figure 6A,B). The reaction partner is added and the binding process can be monitored over time (Figure 6B,C). Apparent binding rate constants can be derived from these “sensograms”¹³¹. Dissociation rate constants are measured by flowing buffer without reactant over the formed RNA-protein complex, and directly monitoring the dissociation of the components over time (Figure 6C).

SPR is conceptually elegant and does not involve labeled components. It requires surface immobilization of one component, which is often accomplished through biotin-streptavidin^{131, 133}. Biotinylated RNAs are commercially available. For these reasons, SPR has become a popular choice for measuring kinetic parameters of RNA-protein interactions¹³⁴. However, both the SPR equipment, and the sensor chips on which the measurements are performed, are costly. In addition, care must be taken to prevent overloading of the SPR chip with sample, which can prevent interpretation of measured sensograms¹³¹. The surface immobilization can, in principle, affect measured kinetic parameters¹³⁵. As for other methods, it is thus useful to validate SPR data with an orthogonal, preferably solution method. Nevertheless, aside from the associated costs, SPR is a versatile method to determine kinetic parameters for RNA-protein interaction, often with only a minimal optimization¹³⁶. Accordingly, SPR has been used to determine kinetic parameters for numerous proteins that interact with RNA. Examples include Rbfox1¹³⁷, HuR¹³⁸, U1A¹³⁹, and pentatricopeptide repeat proteins¹⁴⁰.

Enzymatic approaches to measure the kinetics of RNA-protein interactions

The methods described above allow the determination of kinetic parameters for the physical interaction between RNA and protein. While binding and dissociation rate constants adequately describe kinetics for RNA-binding proteins, several hundred proteins that interact with RNA are enzymes that manipulate the RNA physically, chemically or both^{3, 5}. As mentioned, a kinetic description of an enzyme-RNA interaction needs to include the enzymatic steps (Figure 3). Devising these kinetic descriptions, which can be complex, often requires significant effort, ingenuity and frequently multiple complimentary experimental approaches⁷⁶.

Assays for enzymatic reactions on RNA are frequently more sensitive than methods reporting the physical RNA-protein binding and dissociation. Kinetic parameters of the catalytic steps and of enzyme-RNA binding, dissociation and of product dissociation, can be

obtained from timecourses of the enzymatic reaction at varying enzyme and RNA concentrations, considering enzymological principles¹⁴¹. Michaelis-Menten parameters (k_{cat} and K_{m}) obtained from steady-state reactions describe the dynamics of the enzyme-RNA interaction, and often answer posed questions. In many cases, however, more detailed kinetic descriptions are necessary to understand a given enzyme-RNA interaction, necessitating the use of pre-steady-state approaches¹⁴¹. It is important to keep in mind that enzyme-RNA association rate constants measured with enzymatic assays usually report functional interactions, e.g. those that lead to an enzymatic step. Enzymes can engage in non-productive binding steps, where RNA binds to the enzyme, but no enzymatic step occurs¹⁴². Non-productive events can be biologically significant, and frequently ensure substrate specificity¹⁴³.

Enzymatic approaches have been used to obtain kinetic information for multiple RNA-protein interactions⁵. Prominent examples include tRNA synthetases^{144, 145}, RNA helicases^{77, 79, 83, 146–148}, and non-templated polymerases⁷⁸. Enzymatic reactions are also instructive for the kinetic characterization of systems with multiple components^{78, 83, 149, 150}. An impressive example in this regard is the reconstituted eukaryotic translation initiation system^{151–153}.

Single molecule approaches to measure the kinetics of RNA-protein interactions.—Traditional biochemical approaches measure the “ensemble” behavior of large numbers of molecules. In an ensemble, molecular heterogeneity is often masked, and it is usually difficult to determine kinetic parameters for specific steps of multi-step reactions. Over the last two decades, several techniques have been developed that interrogate individual biomolecules and individual complexes^{113, 154}. For kinetic measurements, single molecule approaches follow time-trajectories of individual molecules or complexes during a reaction. Transitions of individual molecules between different reactions states can be simply read out, and the data directly report molecular heterogeneity of the system under study¹¹⁹. Single molecule methods have provided insight into biological systems beyond the scope of ensemble methods, and have transformed the quantitative understanding of complex reaction kinetics, such as translation and pre-mRNA splicing^{118, 155}. Single molecule techniques have also added critical new ways to characterize RNA helicases^{156, 157} and RNA-binding proteins¹¹⁷.

Although single molecule approaches usually provide more detailed kinetic insight than ensemble techniques, not all biological systems are amenable to single molecule studies. Even systems that can be interrogated at the single molecule level require highly specialized, and frequently custom-built equipment, often laborious preparation and optimization of the biological system for labeling, immobilization, or both. Significant expertise is also needed to perform the experiments and data analysis. Accordingly, most single molecule experiments are conducted in laboratories that specialize in these techniques.

Two types of single molecule techniques have been used to study the kinetics of RNA-protein interactions: (i) single molecule fluorescence approaches, which report time trajectories of fluorescence changes of individual molecules or complexes, and (ii) optical

and magnetic tweezers, which report changes in the mechanical properties of individual molecules or complexes. Both techniques have also been combined¹⁵⁸.

Single Molecule Fluorescence

Most contemporary single molecule fluorescence approaches utilize a fluorescence microscopy setup equipped with a sensitive CCD camera that is able to record fluorescence emission of individual fluorophors^{113, 119}. The fluorescently labeled molecules under study are usually immobilized on a slide in a customized flowcell (Figure 7A). A small area of the slide is imaged through the objective and projected onto the CCD camera (Figure 7A). The fluorophors are excited by total internal reflection (TIR), either directly through the objective, or through a prism on top of the flowcell¹¹⁸. TIR produces an evanescent wave at the glass/quartz water surface, which results in an exponential decrease of excitation light intensity towards the inside of the flowcell¹⁵⁹. Only a small volume of the flowcell is illuminated and the fluorescence background is drastically reduced, compared to wide-field illumination (Figure 7A).

Most single molecule fluorescence studies that measure the kinetics of RNA-protein interactions immobilize one component of the system under study on a functionalized quartz surface in the flowcell. Immobilization allows the straightforward observation of individual molecules over time without losing track of the identity of a given molecule. Most of the current single molecule fluorescence studies employ FRET, although fluorescence changes of a single fluorophor have also been used to monitor reactions^{160, 161}. For single molecule FRET two fluorophors are strategically placed in the system under study, and both, donor and acceptor fluorescence are recorded (Figure 7B.). The anti-correlated fluorescence of the donor acceptor pair provides a robust and specific signal (Figure 7C)¹¹⁹. Since individual fluorophors are recorded, measurements are limited by photobleaching of the fluorophors¹⁶². However, contemporary photostable fluorophors and effective oxygen scavenging systems have significantly increased the time by which individual fluorophors can be observed, compared to two decades ago^{163–165}. Many single molecule FRET experiments place both FRET labels on the RNA, which is immobilized (Figure 7B.), but in some cases one fluorophor needs to be placed on a component that is added in solution. In conventional flowcell setups, a fluorophor in solution cannot exceed low nanomolar concentrations, otherwise the fluorescence background overwhelms the signal. This limitation can be overcome with zero-order waveguides, where the sample is immobilized in wells that are smaller than the wavelength of the excitation light^{166–168}. Single molecule fluorescence has been used to measure kinetic parameters for numerous RNA-protein interactions, including RNA-binding proteins^{169–174}, enzymes such as RNA helicases^{121, 148, 175–178}, and complex systems such as the ribosome^{179–185} and pre-mRNA splicing^{155, 186–188}.

Optical and Magnetic Tweezers

An optical tweezer is a focused laser beam that creates an “optical trap”, which can hold a small, dielectric particle (bead) at its center¹⁸⁹. The system under study is attached between either a fixed plane or another bead and the trapped bead^{189, 190}. Forces in the pN range displace the trapped bead in the laser beam, but do not remove the bead from the trap¹⁹⁰.

The bead displacement corresponds to the exerted force and can be measured with high spatial precision (Figure 8). Optical tweezers allow the measurement of forces exerted by or on single molecules¹⁹¹, a molecular feature that is not accessible by other experimental means.

Although optical tweezer setups are now commercially available, experiments, data analysis and interpretation require highly specialized expertise that is usually beyond the scope of groups that do not focus on these techniques. Most setups allow experiments only on a single molecule at a time, which makes data collection laborious. In addition, careful optimization and constant validation is required to ensure that only a single molecule is present between the beads¹⁹².

Optical tweezers have been mainly used to characterize kinetic aspects RNA-protein interactions that are accompanied by structural changes in RNA (e.g. RNA helicases) or by motor-like systems such as the ribosome or polymerases^{192–195}. Spatial aspects of the reactions, including RNA unfolding at basepair resolution¹⁹², or movement on the RNA at high resolution can be directly measured¹⁹⁶. Optical traps thus provide information that is beyond the scope of other methods. An optical trap has been combined with single molecule fluorescence detection to directly measure coordination of multiple processes in protein-DNA systems^{196, 197}, but to our knowledge, not yet in an RNA-protein system.

Magnetic tweezers function similar to their optical cousins, except that they use magnetic beads^{198, 199}. Currently used setups allow simultaneous measurements on multiple beads²⁰⁰. In addition, magnets allow the application of torsional forces on the system under study²⁰⁰. Magnetic tweezers have been used to measure the kinetics of RNA unwinding by RNA helicases^{201, 202}.

High throughput approaches to measure the kinetics of protein interactions with many RNA substrates in vitro.—In cells, proteins encounter large numbers of potential RNA binding sites⁷. Most proteins inherently discriminate between potential RNA binding sites⁵. This inherent RNA specificity is dictated by the kinetics by which the protein interacts with a RNA variant⁵. Two different approaches have been used to monitor the kinetics of interactions between a given protein and thousands of RNA variants: (i) High Throughput Sequencing Kinetics (HiTS-Kin) converts traditional, biochemical RNA-protein methodology for use with large numbers of RNA variants that are monitored by NGS²⁰³. (ii) RNA on a massively Parallel array, (RNA-MaP) adopts the NGS equipment for kinetic measurements²⁰⁴.

HiTS-Kin

For HiTS-Kin, a pool of RNA substrates with a randomized region is either bound or processed by a protein or a protein complex. Interacting RNA variants are identified by NGS (Figure 9). The approach is conceptually related to the RNAcompete^{34, 205} and the RNA bind-n-seq methods^{33, 206}, although these techniques do not assess the kinetics of RNA protein interactions. For HiTS-Kin, RNAs with 7 to 8 randomized nucleotides can currently be used, limited by the sequencing depth^{203, 207}. Bound or processed RNAs are separated from the unbound, or unprocessed RNA species. The unprocessed (or unbound) RNA is then

analyzed by NGS. It is also possible to analyze the processed (or bound) RNAs. The RNA pool is measured over several time points, which provides the kinetic parameters for all substrate variants. Depending on the reaction setup, obtained kinetic parameters reflect binding kinetics or other reaction steps²⁰⁷.

Because HiTS-Kin interrogates the entire sequence space of an RNA pool, sophisticated kinetic specificity models can be developed^{5, 207, 208}. Most current specificity models for RNA-protein interactions are based on equilibrium binding and Position Weight Matrices (PWMs), which assume independent contributions of each nucleotide position to the free binding energy^{203, 209}. HiTS-Kin provides specificity models that consider energetic coupling between two or even more nucleotide positions^{5, 210}, and allows assessment of specificity at various reaction steps²¹⁰.

HiTS-Kin has been applied to several RNA processing proteins, including C5 from *E.coli* and RNase H^{210, 211}. Detailed methodological aspects of the HiTS-Kin technique have been recently described²⁰⁷. HiTS-Kin and the corresponding equilibrium technique HiTS-Eq (High Throughput Equilibrium Binding) can be combined to characterize kinetic landscapes spanning several reaction steps for all sequence variants of a given RNA pool^{210, 212}. Through free energy relations, which are used in enzymology to delineate enzyme mechanisms, a combination of HiTS-Eq and HiTS-Kin data can determine at which reaction step specificity is accomplished – at substrate association, transition state, substrate dissociation, or through a combination of these steps^{207, 210}. Combined HiTS-Kin and HiTS-Eq analyses have been applied to the C5 protein from *E.coli*²¹⁰.

RNA-MaP

RNA-MaP utilizes a customized NGS machine to directly analyze RNA-protein interactions^{204, 213}. A pool of diverse DNAs, which encode the RNAs that are ultimately analyzed, are immobilized in an NGS flowcell (Figure 10). The DNAs are amplified and identified by NGS, thereby creating a map of the precise localization of each substrate variant in the flowcell. The DNA pool contains sequencing adapters and randomized regions²⁰⁴. The DNAs are converted to RNA by RNA polymerase that remains bound to the DNA (Figure 10). The protein is fluorescently labeled and flowed into the cell. Protein binding to an RNA variant leads to an increase in fluorescence signal at a precise location. Changes in fluorescence for all RNA variants in the flowcell are recorded over time²¹³. Depending on the reaction setup, fluorescence changes correspond to dissociation rate constants, apparent association rate constants, equilibrium dissociation constants, or a combination thereof²⁰⁴.

Once set up, RNA-MaP provides kinetic data in real time for many sequence variants. While this feature is unparalleled by other methods, the customized equipment and specialized technical expertise to maintain the equipment and run the experiments could limit wide applicability of RNA-MaP. In addition, the DNA pool for each reaction setup is a considerable expense. To date, RNA-MaP has been used to characterize the kinetics of the MS2-RNA interaction for several thousand sequence variants²⁰⁴. It has also been used for equilibrium binding experiments for other RNA-binding proteins²¹⁴.

PERSPECTIVE

An impressive array of techniques is now available to interrogate dynamic aspects of RNA-protein interaction in cells and *in vitro* to a degree unimaginable even two decades ago. Today, it is possible to determine how cellular, transcriptome-wide RNA-protein binding patterns change in response to biological cues. Translation can be followed in real time, in cells²¹⁵ and *in vitro*, and kinetics of RNA-protein interactions can be measured at high resolution for systems with multiple interacting components. The existing techniques provide a large selection of tools for any investigator interested in understanding the dynamics of RNA-protein interactions.

Notwithstanding this progress, we still do not understand how RNA-protein dynamics in cells (hours or days timescale) are connected to the dynamics of RNA-protein interactions on a molecular level, their kinetics, which ultimately dictate patterns and dynamics of cellular RNA-protein interactions. Without kinetic information, it will be difficult to devise predictive models of the dynamics of cellular RNA-protein interactions. Efforts to correlate physical parameters of RNA-protein interactions measured *in vitro* with cellular RNA-protein crosslinking data are an important step towards accomplishing this goal³³. In addition, the development of techniques that directly measure kinetic parameters of RNA-protein interactions in cells is important. To our knowledge, no method has yet accomplished this goal, but steps in this direction have been taken. Time-resolved RNA-protein co-localization have been reported^{22, 23}, and fast RNA-protein crosslinking enables the collection of CLIP/CRAC samples over short times²¹⁶. Given the significance of dynamics of RNA-protein interactions for the regulation of gene expression, interest in this field will likely remain high over the next years.

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Figure 1 |.
Classification of approaches to measure dynamics of RNA-protein interactions.

Figure 2 |. Classification of approaches to measure the dynamics of RNA-protein interactions in cells.

For abbreviations of methods, see text. The asterisk denotes that CLIP encompasses multiple specialized techniques. For details, see text.

Figure 3 |. Kinetics of RNA-protein interactions.

The reaction of the protein interaction with a given RNA site is marked RNA-protein interaction. RNA* indicates chemically or physically altered RNA. Differences in the reactions between RNA-binding proteins and enzymes that interact with RNA are marked. Cellular parameters that can impact a given reaction state are marked as biological context. The arrows between the parameters indicate their potential interdependence.

Figure 4 |. Measuring association and dissociation rates with pulse-chase regimes.

Upper panel: Basic reaction scheme of protein binding to a single RNA-site (k_{bind} : association rate constant, k_{diss} : dissociation rate constant). Left panel: Reaction scheme to measure association rates. Separation refers to any technique that separates unbound RNA (or protein) from the RNA-protein complex. Right panel: Reaction scheme to measure dissociation rates.

Figure 5 |. Ensemble fluorescence techniques to measure RNA-protein interactions.
 (A) Fluorescence Anisotropy, (B) Fluorescence Quenching, (C) FRET: Schematic reactions and timecourses of the respective signals for association and dissociation reactions.

Figure 6 | Surface Plasmon Resonance for measuring RNA-protein interactions.
(A) Principle of SPR. (B) Surface immobilization of RNA. (C) Schematic timecourses of the respective signals for association and dissociation reactions.

Figure 7 |. Single molecule FRET to measure RNA-protein interactions.

(A) Principle of smFRET. (B) Protein binding measured with an RNA construct bearing both, donor and acceptor labels. (C) Schematic timetraces of donor and acceptor fluorescence and the corresponding FRET trace for an individual molecule. Rate constants are calculated from histograms of many binding and dissociation events.

Figure 8 |. Measuring RNA-protein interactions with optical tweezers.

(**A**) Measuring an RNA-protein interaction that is associated with RNA unwinding. The schematic setup shows two laser traps, but is possible to perform the measurements with a single trap and another fixed point. A force is applied to one of the laser traps and leads to a small displacement of one bead in the trap. The protein interaction causes partial unwinding of the RNA helix, which causes an extension of the RNA and a movement of the bead in the trap, which can be measured with high sensitivity. (**B**) Schematic timetrace of bead position of an individual molecule. Rate constants are calculated from histograms (at varying force) of many bead displacement events.

Figure 9 |. HiTS-Kin.

Schematic depiction of the method as applied to a reaction involves cleavage of the RNA substrates. The cleavage reaction is followed over time. Separated substrate pools are isolated from the PAGE and a cDNA library is generated and analyzed for each timepoint.

Figure 10 |. RNA-MaP.

Schematic depiction of the method. DNA is shown in black, RNA in blue. The grey circle represents the RNA polymerase. The yellow circle represents the RBP, the star the fluorophor.