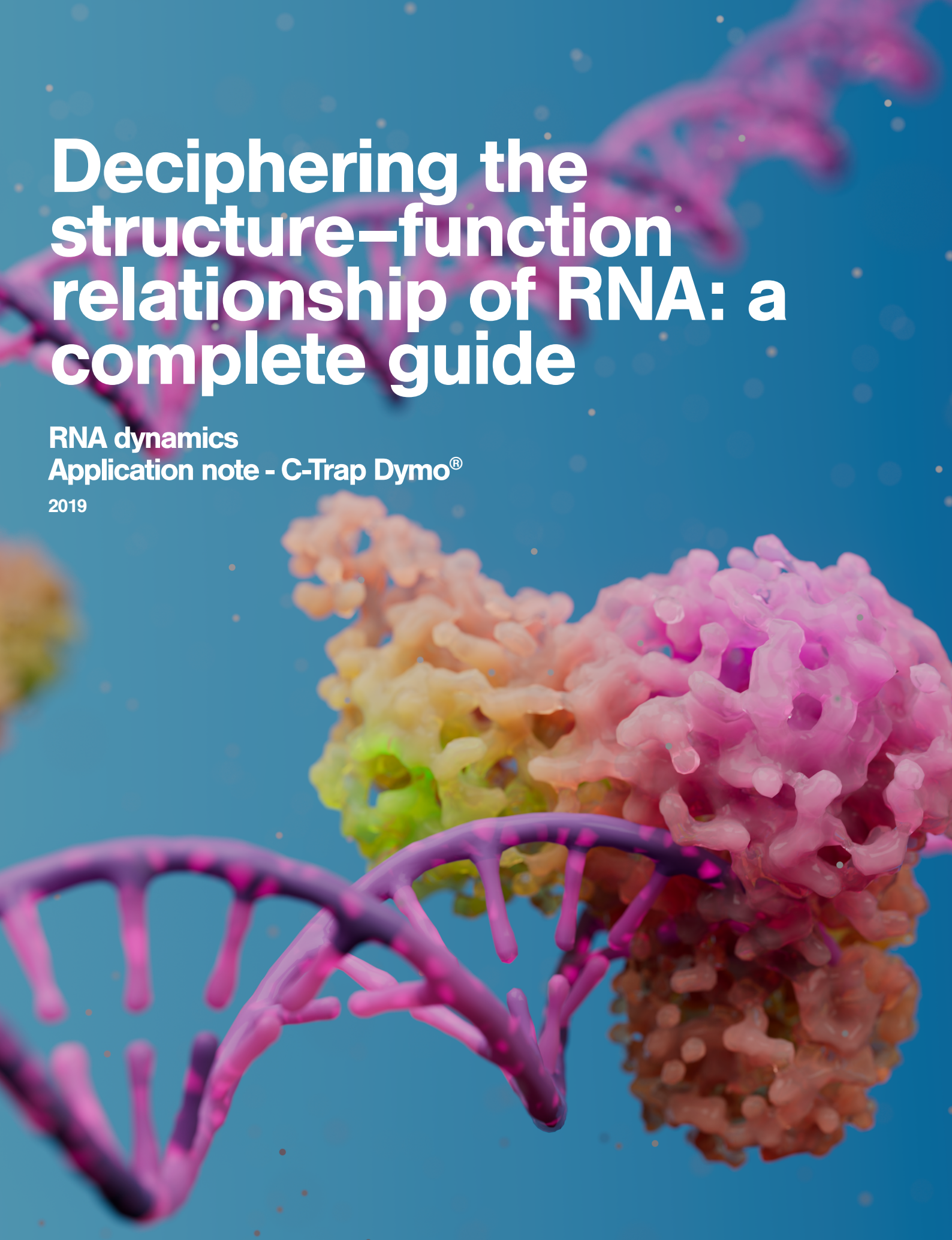
Deciphering the structure–function relationship of RNA: a complete guide

RNA dynamics
Application note - C-Trap Dymo®

2019



LUMICKS

Table of Contents

One RNA Molecule: Many functions and architectures

Study the dynamics, structure, and function interplay

The structure-function relationship of RNA molecules is ubiquitously tied to many biological processes. These include gene regulation, coding and transport of genomic information, and protein synthesis among others. However, when trying to establish how a particular structure or conformation enables a specific function using modern imaging and biochemical methods, the scientific community has to overcome specific challenges:

- RNA molecules can adopt many alternative structures. The consequence of this is that multiple structures and their relation to different modes of activity can be overlooked when using ensemble assays or when recording static data.
- Some structural conformations occur stochastically or more frequently than others and are only accessible under specific conditions.
- Biological functions are deployed over time. So those assays addressing static features will not capture specific activity features (e.g., speed, processivity).

RNA molecules can adopt complex structures which are tightly coupled to their biological function and often exhibit rich dynamics. To truly understand these systems and overcome the aforementioned challenges, dynamics, structure, and function need to be studied simultaneously.

This interplay is very relevant for many different types of RNA molecules. Riboswitches, for instance, are regulatory folded RNA segments that can adopt different structural conformations after binding a metabolite or ligand, turning the gene on or off. Similarly, RNA molecules are known to fold into functional structures as they are being synthesized by transcription. RNA folding dynamics can be studied in detail by observing the co-translational folding at the single-molecule level as it happens.

Since three-dimensional RNA structures cannot be predicted based only on sequence, it is crucial to experimentally study these structures to understand how protein synthesis is regulated. Furthermore, there is a number of RNA-binding proteins (RNPs) that are essential in fundamental biological processes, such as splicing or ribosome assembly, but whose function, structure, and dynamics are still unknown.

Scientists can investigate the interplay between these elements and probe the dynamics of protein-RNA interactions using high-resolution optical tweezers. Further combining optical tweezers with simultaneous multicolor fluorescence measurements (e.g., with FRET) allows correlating the mechanical properties of the structure with specific conformational states.

In this guide

With this guide we aim to equip scientists with the necessary knowledge to perform optical tweezers experiments to study the dynamics, structure, and function relationship of RNA molecules.

In the following sections, you will learn how to:

- Design assays to study RNA molecules and their interactions with proteins
- Prepare RNA samples for optical tweezers experiments
- Perform experiments using the C-Trap, and analyze and interpret the obtained data

Features of the C-Trap®

LUMICKS' C-Trap™ optical tweezers–fluorescence microscopy enables the study of RNA structures and the investigation of their interactions with proteins.

This can be done by exerting mechanical forces on single molecules to induce structural transitions which mimic those that occur physiologically upon local temperature fluctuations or binding of small molecules.

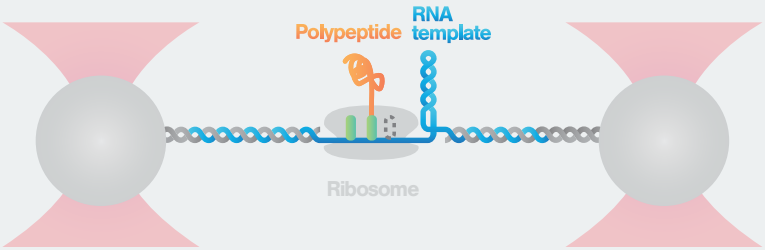
At the same time, measuring force over time allows the quantification of the stability and the dynamics associated with the different conformers. Ultimately, the information obtained can be used to establish the complete energy landscape of a specific RNA molecule.

Designing the assay: what are the possibilities?

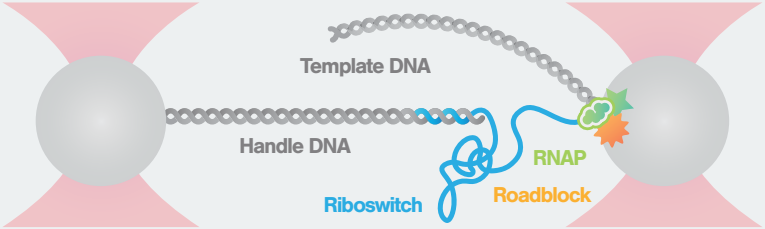
RNA folding has been known for decades. Modern techniques have revealed that the tertiary structures of RNAs are variable and directly dependent on chemical modifications and their physiological state for nearly every class of cellular RNAs. For mRNA, these structures could modulate splicing and translation. For tRNA or rRNA, the tertiary structure could play a role in the biogenesis and the structure-dependent function of these molecules. Regulation of RNA plays an important role in various diseases and much remains unknown about how the dynamics of RNA structure affects the physiology of a cell and an organism at large.

With the C-Trap scientists can use optical tweezers to trap beads and tether an RNA molecule in between. The RNA template can then be mechanically manipulated by moving the beads, while the force and extension are measured and recorded. Fluorescent-labeled proteins can also be visualized with confocal or STED fluorescence microscopy simultaneously with the force measurements. One could use the C-Trap to study RNA folding upon synthesis by RNA polymerase. Similarly, one could measure the effect of ligands which induce alternative or meta-stable structures of RNA. Figure 1 shows examples of different experimental designs that can be used to assess the properties of RNA molecules and their interactions with proteins. Elucidating the thermodynamics and the kinetics of these processes would take us further into the understanding of the precise mechanisms of these systems.

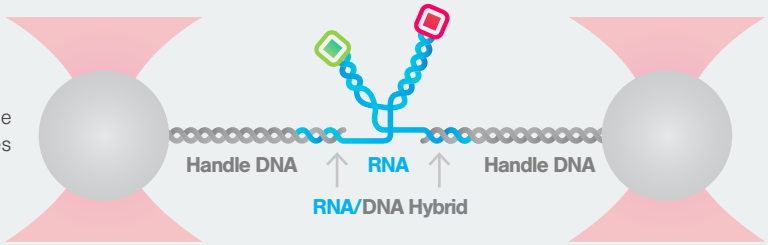
Assay 1: Protein folding starts co-translationally when the emerging peptide is still attached to the ribosome. Using single-molecule methods it is possible to follow in real time the moment when co-translational folding begins. In this way, it is possible to: dissect the role of the ribosome in guiding the nascent peptide towards its folded 3-dimensional structure, and characterize the translation elongation kinetics modulating protein folding.



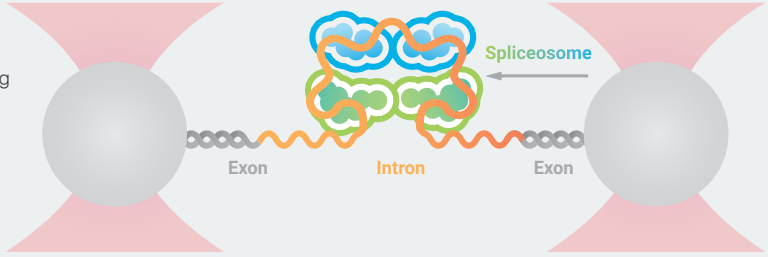
Assay 2: RNA molecules begin to fold immediately as they emerge from the RNA polymerase. Interactions between nascent RNAs and ligands can direct the formation of alternative RNA structures. This is a feature exploited by riboswitches, which modulate downstream gene expression by a tight coupling between ligand-dependent RNA folding kinetics and the transcription rate.



Assay 3: RNA folding pathways leading to functional ON and OFF regulation involve the formation of metastable states. Using single-molecule force spectroscopy and fluorescence it is possible to determine the landscape of the thermodynamically stable states adopted by riboswitches in either the presence or absence of metabolite for mRNA transcripts.



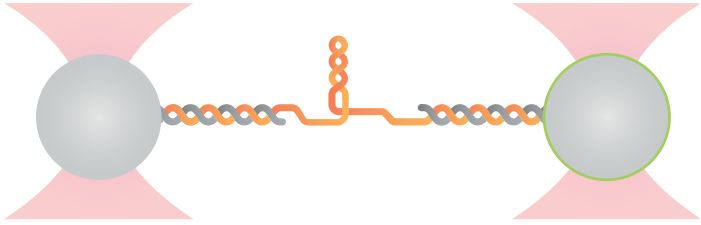
Assay 4: Pre-mRNA molecules are processed by the spliceosome, a large RNA-protein complex composed of several small nuclear ribonucleoproteins which produces protein-encoding mature mRNAs. Because of the triplet nature of the genetic code, a mistake in cutting the RNA of only one nucleotide will produce an mRNA that has an altered reading frame. This leads to the production of a message that cannot encode the correct protein. The splicing process can also be studied in detail using single-molecule methods.



Biochemical preparation in 7 steps: how to make a suitable sample?

Configuration of the experiment

In the most common unfolding experiments, an RNA sample — in this example a hairpin — consists of the RNA of interest flanked by two double-stranded DNA/RNA hybrid handles (Figure 2). The DNA/RNA handles are labeled with digoxigenin (Dig) or biotin (Bio) on each side. We can choose the length of one handle in a range between 900-2000 nucleotides to cover a wide variety of assays, while obtaining optimum resolution.



2 RNA hairpin structure tethered between optically-trapped beads via DNA/RNA hybrid handles. In order to ensure specific interactions with the coated beads, the handles are modified at their ends with the complementary interacting moiety to the beads.

Having functionalized the DNA/RNA hybrid handles with Bio and Dig labels, it is then possible to tether the entire molecule between two differently functionalized micron-sized silica or polystyrene beads. These beads are coated with streptavidin and anti-digoxigenin (AntiDig) which bind to Bio and Dig, respectively. The laser traps will then optically hold the beads and the user will be able to change the relative distance between them by steering one of the traps. Using this approach, we can stretch and relax a single RNA molecule and subsequently measure its mechanical and structural properties.

Preparation of the sample

For the preparation of RNA samples for tweezing experiments we recommend the protocols presented by Stephenson et al. [1] and Wen et al. [2]. First, clone the DNA sequence corresponding to the RNA of interest into a plasmid vector. Next, perform three PCR reactions to the same plasmid to generate both the two handles and a template for transcription. The transcription template encompasses the handle regions and the inserted sequences, while the full-length RNA is synthesized by in vitro transcription. Last, anneal the RNA and chemically-modified handles together to generate the molecules for optical tweezers manipulation.

The 7-step recipe for RNA sample preparation for tweezing experiments

- **Cloning the sequence of interest:** clone the DNA sequence corresponding to the RNA structure into a vector.
- **Synthesis and purification of the transcription template:** synthesize a transcription template by PCR. Next, purify the PCR products using a PCR purification kit, and concentrate the sample to >200 ng/µl. Because the purified DNA is used for transcription, RNase free water should be used in the elution and concentration steps.
- **In vitro transcription:** synthesize RNA by in vitro transcription at 37 °C overnight to maximize RNA yield. The reaction happens in 1x T7 RNA polymerase reaction buffer. After transcription, add 1 µl DNase I to digest the DNA template for 15 min at 37 °C. Purify the RNA using a silica spin column. Verify the length and integrity of the products by denaturing agarose gel electrophoresis.
- **Synthesis of the biotinylated handle A:** First, produce the unmodified handle A by PCR using A_forward and A_reverse primers and concentrate the amplified DNA to >200 ng/µl. Next, incorporate biotin modifications into the PCR product using a primer extension reaction. You can directly use the biotinylated handle A (biotin-HA) in the annealing without purification. As the biotin-HA is to be annealed with RNA, it is critical to use RNase free water in both steps.
- **Synthesis of the digoxigenin-modified handle B:** Synthesize digoxigenin modified handle B (dig-HB) by PCR using primer Bf and digoxigenin-modified oligo Dig-Br. Next, purify the PCR product and concentrate the sample to >200 ng/µl. The same approach as described above could also be used for the synthesis of biotinylated handle A, using a biotin-modified oligo instead.
- **Annealing RNA to the handles:** Use approximately equal molar concentrations of RNA and each of the DNA handles in the annealing buffer to carry out the reaction. Use 800 µl of formamide, 2 µl of EDTA (0.5 M, pH 8.0), 40 µl of PIPES (1 M, pH 6.3) and 80 µl of NaCl (5 M). Incubate the mixture at 95 °C for 10 minutes, 62 °C for1 hour, 52 °C for 1 hour, and finally ramp it to 4 °C.
- **Purification of the annealed sample:** Recover the hybrid constructs by ethanol precipitation. Afterward, dissolve them in RNase free water. The sample is ready to be used and can be stored at -20 °C.

Useful Tips

Incorporate multiple Dig and Bio at the ends of your DNA/RNA handles to apply and measure high forces and have the tether stable for a longer time.

Minimize steric hindrances during functionalization of your handles by leaving 2 or 3 unmodified base pairs between every Dig or Bio modification.

Refer to the protocol developed by Wei Cheng [3] if you want to make an RNA construct with homopolymeric AT or GC sequences.

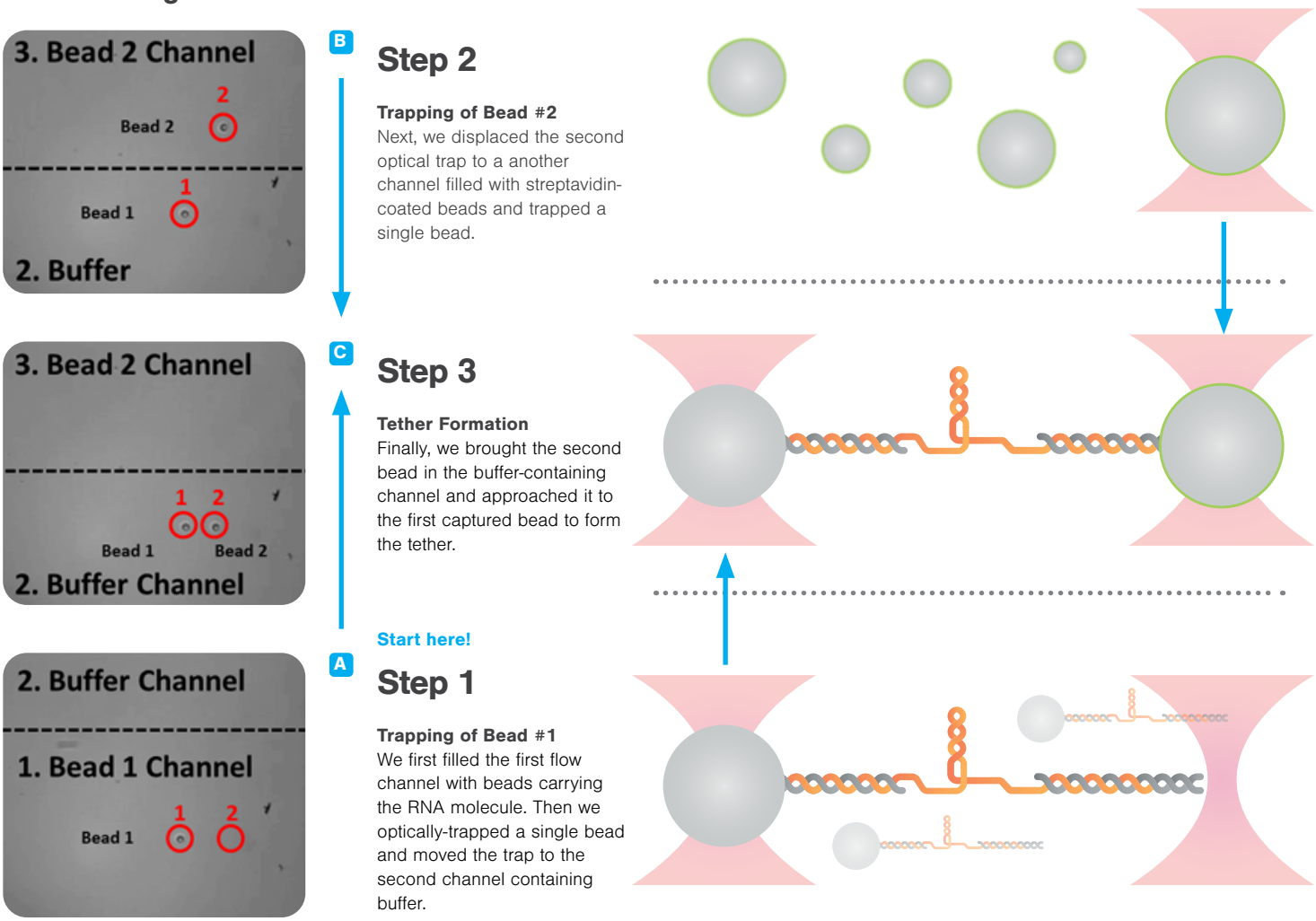
The C-Trap® experiment: manipulation of a single RNA molecule

Setting up the system

In the following examples, we show the results obtained from measuring an RNA sample that has been prepared following similar protocols as described in the previous section.

First, we assembled our single-molecule assay using the C-Trap's integrated laminar flow microfluidic channels (**Figure 3**). In brief, we incubated the sample with AntiDig-coated beads. We then flushed the beads carrying the RNA molecule through the first laminar flow channel and subsequently, we optically trapped a single bead. Next, we brought the trapped bead to the second channel containing buffer. We then displaced the second optical trap to a third channel filled with streptavidin-coated beads and trapped a single bead. Finally, we brought the second bead in the buffer-containing channel and approached it to the first captured bead to form the tether as depicted in **Figure 3.** On average, the RNA assay was efficiently assembled in less than two minutes.

In situ tethering of an RNA molecule



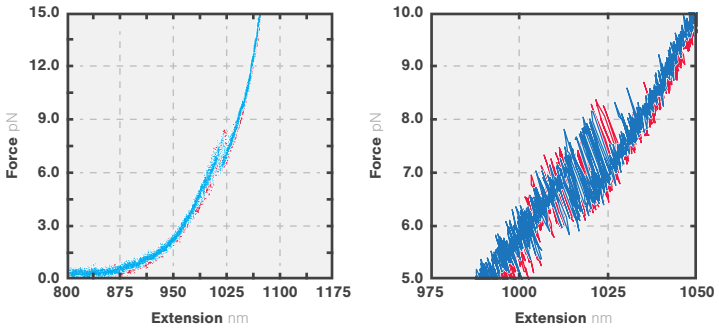
3 In situ tethering RNA molecules. (A) A single RNA-carrying AntiDig bead is trapped in the optical trap #1, in a channel containing those beads (channel 1). (B) The large field of view and manipulation capabilities of the C-Trap allow to position the trap #2 in the laminar flow containing Streptavidin-coated beads and trap a single-bead in the optical trap (channel 3). (C) The beads are positioned in close proximity again in a channel containing the desired buffer and the RNA tether can be formed, in channel 2. At this stage, the force and the distance can be continuously recorded. Sample courtesy of the lab of Prof. Shi at Tsinghua University.

Studying RNA structural mechanics

Having assembled our RNA tether, we proceeded to manipulate and measure our sample. In the first experiment, we stretched and relaxed the RNA molecule with a constant load of 10 nm/s, while recording force-extension curves (**Figure 4, left**).

At approximately 8 pN, we observed an unfolding rip of around 15 nm. When we relaxed the molecule, the refolding curve followed the same path as the unfolding one, which corresponded to a refolding step of similar size. Looking at the magnified section of the transition we observed that the molecule appeared to fluctuate between folded and unfolded states over time (**Figure 4, right**).

These findings show that the unfolding of the RNA molecule is a reversible process, which under these conditions (out of equilibrium), requires 8 pN to overcome the energy barrier between the folded and unfolded states. Cycles of unfolding and refolding could be repeated multiple times with the same result



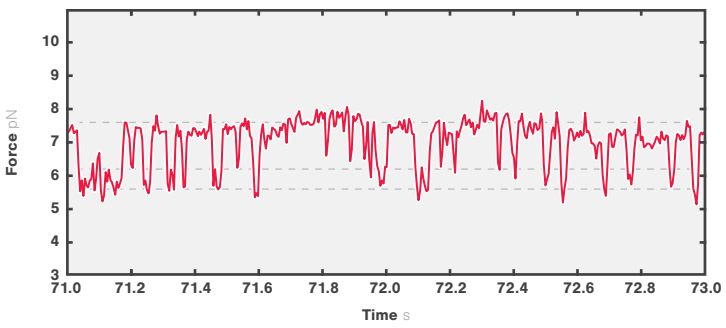
4 Force-distance curves corresponding to the extension (red, 10 nm/s) and retraction (blue, 10 nm/s) of a single RNA tether (left). Zoomed-in section of left image showing unfolding and refolding transitions (right). Data points were decimated to 200 Hz from the high-resolution dataset collected at 50 kHz.

Studying RNA equilibrium dynamics

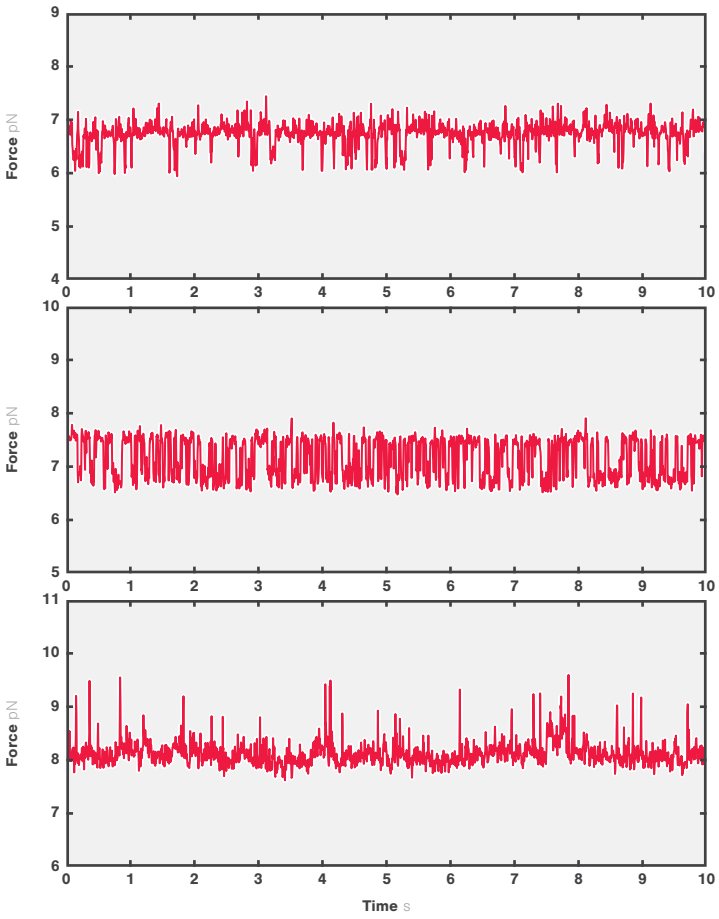
In the second experiment, we evaluated the structural transitions of our RNA molecule over time (in equilibrium). To this end, we brought a single RNA molecule to a specific initial tension and studied its transitions. Interestingly, while two major states could be observed at tensions around 6.2 pN and 7.2 pN, other transient conformational states were also occasionally accessed (**Figure 5**).

When increasing the pretension at the beginning of the measurement, we observed that the occupation of the different states gradually changes, favoring the open states at higher forces and the closed states at lower ones (**Figure 6**). Using high-resolution optical tweezers, small and transient RNA conformational changes can be monitored in real-time with unprecedented stability, providing scientists with valuable information on the number and frequency of various conformational stages.

The next section shows how to confirm the presence of multiple conformational transitions with more advanced analysis.



5 Two seconds time trace of a single RNA molecule at an initial tension of ~7 pN. The dashed grey lines show the two major visited states and a third potential transient state.



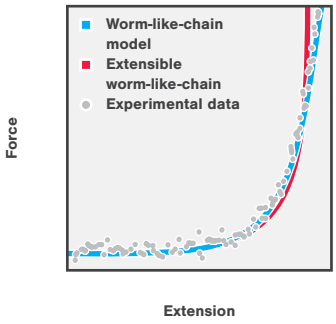
6 Ten seconds time traces at different initial values of tension 6.5, 7.5 and 8.2 pN. Points were decimated to 200 Hz from the high-resolution dataset collected at 50 kHz.

Data analysis and interpretation: when to apply which model?

Force–extension curves

Force–extension or force–distance curves (FEC) are used to characterize the elasticity and mechanical properties of polymers, such as DNA or RNA (**Figure 7**). In a typical experiment, the RNA molecule is tethered between two attachment points. To manipulate the RNA molecule, typically one optically-trapped bead can be stirred, thereby applying tension to the molecule. Thus, both the exerted force and the distance between the two attachment points are parameters that we can control and measure during the assay. As the distance between the two attachment points is actively modified in time, so does the state of the molecule, which can become relaxed at smaller extensions, or tensed at larger ones. It can even display conformational unfolding transitions at different tensions, which directly reflect the energy required to destabilize a specific structure.

The FEC quantifies the molecule's response regarding the force that it experiences as we change the distance between the attachment points. It is intuitive that, for any given distance between attachment points, a more flexible molecule will experience less force than a stiffer polymer of the same length. These characteristics are utterly crucial for the molecules' biological function. For instance, the mechanical properties of the RNA determine how the RNA is processed during translation.



7 Force extension curve and worm-like-chain fit.

How to analyze these data?

The FEC data can be fitted to the worm-like chain (WLC) model. The classic model has two free parameters: the contour length (L) and the persistence length (LP) (**Figure 7, red line**). The contour length corresponds to the extension of the fully extended polymer, whereas the persistence length characterizes the bending properties of the object of interest (higher Lp values correspond to stiffer molecules). The model is described by the following equation, where F is the force measured at any given extension x:

$$F = \frac{k_B T}{L_p}\left(\frac{1}{4\left(1-\frac{x}{L}\right)^2} - \frac{1}{4} + \frac{x}{L}\right)$$

The extensible WLC extends the classic model by taking into consideration that polymers elongate when subjected to external forces, and hence, it characterizes the elastic properties of that object (Figure 7, blue line). The model has the same free parameters as the classical model plus an additional one: the stretch modulus (S) which measures the deformation from the molecule's contour length. The following equation gives the model:

$$F = \frac{k_B T}{L_p}\left[\frac{1}{4}\left(1-\frac{x}{L}+\frac{F}{S}\right)^{-2} - \frac{1}{4} + \frac{x}{L} - \frac{F}{S}\right]$$

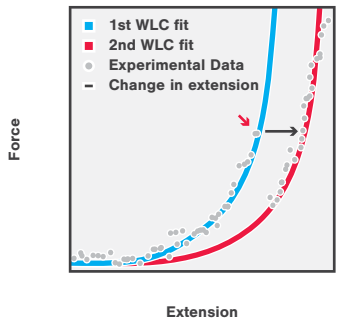
How to interpret sudden changes in the force-extension curve?

In many applications, the DNA is used as a molecular handle to manipulate other molecules, such as RNA structures. As the distance between the attachment points increases, the force experienced by the DNA handle and the object of interest also increases. The deviation of the measured FEC from the typical FEC of a simple DNA molecule corresponds to the response of the object of interest to the applied tension.

At the appropriate tension, we typically observe sudden drops in force indicating that the molecule has instantly relaxed (**Figure 8**). Such sudden relaxation can be due to cooperative unfolding, such as a hairpin opening, leading to a sudden increase in extension of the tethered molecule and, therefore, tension relaxation. This sudden change in extension will be observed at higher tensions for objects that are very stable mechanically compared to those that are less stable.

Thus the FEC curve allows to both:

- characterize the mechanical stability of the RNA molecules by measuring the tension applied at which we observe those sudden drops in force, and
- quantify the extent of the sudden increase in extension, which allows the identification of the specific segments of the molecule that were affected during the experimental procedure.



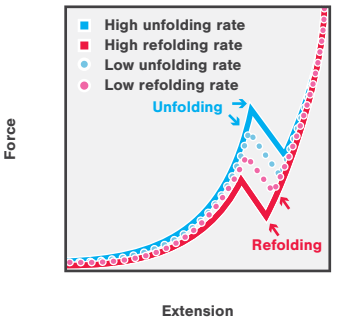
8 Sudden changes in the force-extension curve provide valuable information regarding the object of interest.

The FEC data is split into sections as separated by the sudden drops in force observed. Each section of the data is fitted to the WLC model (blue and red lines in **Figure 8**). The highest data point at which the sudden force in drop first appears determines the force or tension required to induce the change in extension in the object of interest (reflecting an unfolding event, red arrow in Figure 8). A horizontal line at this force is drawn to connect the current WLC fit with the consecutive one (black line in **Figure 8**). The length of this horizontal line corresponds to the increase in extension in the object of interest.

How to analyze these data?

Because a FEC is a plot of the molecule's response as it is being manipulated, the way in which the molecule is being manipulated (also known as the manipulation protocol) further affects such response.

It is typical to observe that the tension at which a given object experiences a sudden increase in extension correlates with the rate at which the molecule's extension is changed. In other words, at lower rates the sudden drop in force occurs at lower values (dotted lines in **Figure 9**), whereas at higher rates the sudden drop in force occurs at higher values (**solid lines in Figure 9**). The reason behind this phenomenon is that slow changes in extension give more time for the molecule to relax between applied tensions and to remain close to equilibrium. Faster changes, on the other hand, keep driving the molecule away from equilibrium. It is known that equilibrium (slow changing) processes require less work than non-equilibrium (fast changing) processes and, hence the difference in behavior depends on the manipulation protocol [4]. This can be used as an advantage: if we perform multiple measurements at different rates we can then extract the unfolding and folding rates together with the ΔG unfolding energy, as well as the number and distance to intermediate states. The way to calculate this is by using the Bell-Evans theory [5].



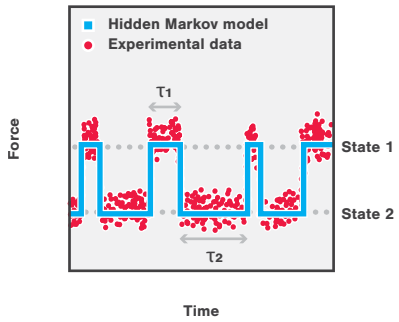
9 The force at which sudden relaxation of force takes place is dependent on the rate of pulling or relaxing.

Equilibrium Measurements

Equilibrium measurements (EM) are measurements in which we do not change the distance between attachment points, and which are typically used in combination with FEC. In EM, a predetermined fixed tension is applied to the molecule, while its response is being followed as a function of time (**Figure 10**).

Tethered objects that show sudden change in extensions in the appropriate tension range display rich dynamics of the molecule transitioning between the not-extended and extended states. These dynamics are solely driven by the thermal fluctuations and best represent the intrinsic properties of the molecule in solution at that specific tension.

Moreover, unlike FEC, where we can observe only one sudden change in extension per realization, EM allow measuring multiple transition events, often within a fraction of a second. Furthermore, with EM it is possible to visualize short-lived events and to resolve very small changes in extension that might represent intermediate states that cannot be easily resolved with FEC. Because of all these features, EM allow to determine thermodynamic properties, such as the free energy associated with a folded state or a given transition and to measure the frequency and lifetime of those states. From a biological standpoint, these properties are fundamental to understand complex macro-molecular reactions within the cell, such as co-translational protein folding and protein aggregation, among others.



10 Equilibrium measurements allow the identification of states and the dynamics of the system as it transitions between those states.

How to analyze these data?

Equilibrium measurement time series are analyzed with hidden Markov models (HMM's). HMM's are statistical algorithms that assume the existence of well-defined states — for instance the unfolded and folded states in the RNA hairpin dynamics — that are obscured by a source of noise, such as Brownian motion. By determining the most likely distributions of states and their interconversion rates while simultaneously calculating the most likely time sequence of underlying states for each trajectory, HMM's provide answers to questions such as:

- How many states are visited by the molecule (**Figure 10**)
- How long does the molecule stay in each state (**Figure 10, τ1 and τ2**)
- What state will be visited next for a given state and time?

HMM's algorithm requires deep statistical knowledge to be implemented from scratch. Fortunately, there are several open source HMM's algorithms available that can be used to analyze equilibrium measurement traces.

In this guide

HaMMy: originally designed to analyze smFRET trajectories. It has its own user interface. This software is available at:

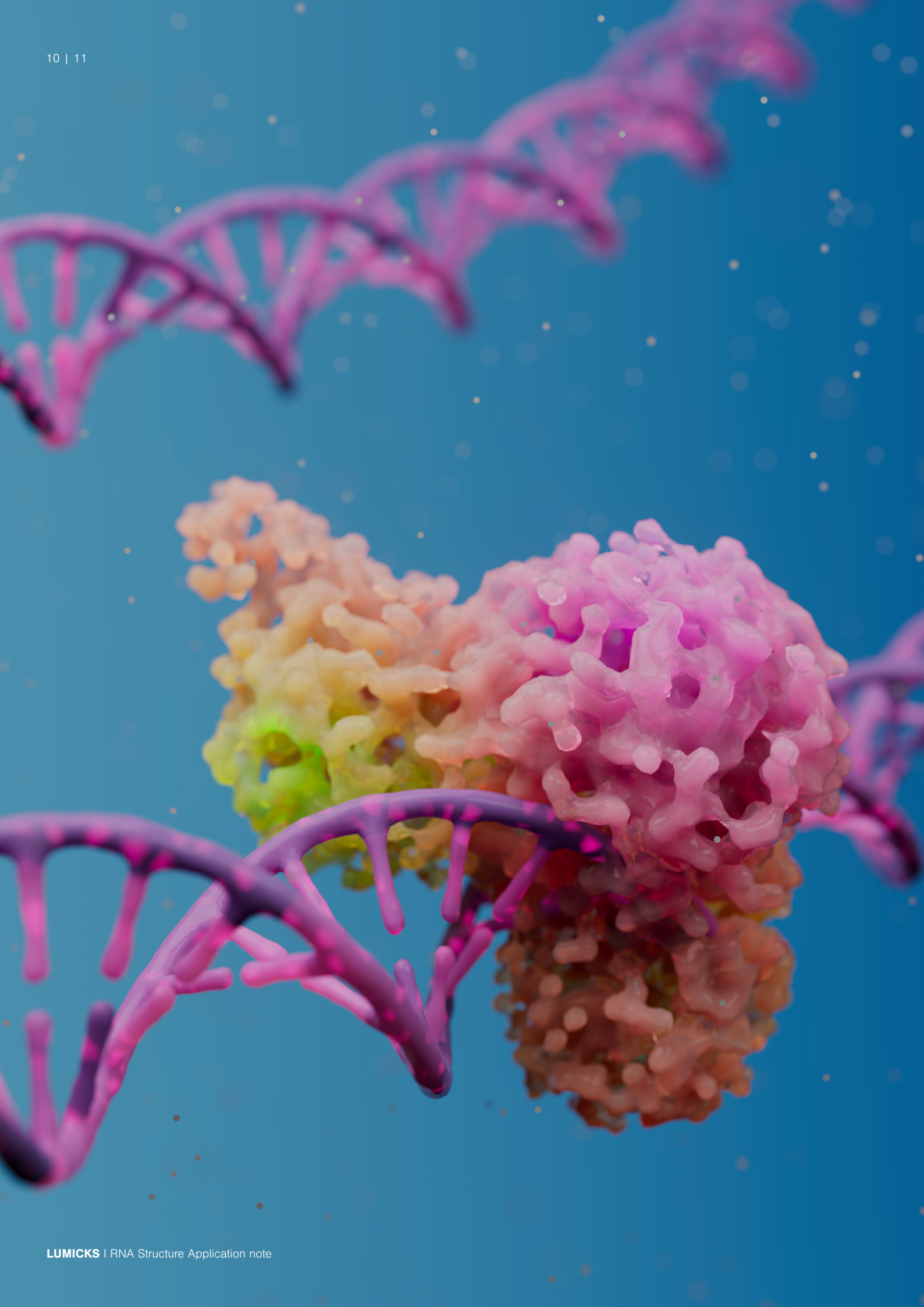
www.ha.med.jhmi.edu/resources/#1464200861600-0fad9996-bfd4

QuB: originally designed to analyze ion channels trajectories. It also has its own user interface. This software is available at:

www.qub.mandelics.com/

vb-FRET: originally designed to analyze smFRET trajectories. vbFRET is a Matlab executable. Available at:

www.vbFRET.sourceforge.net



Conclusions and perspectives

"Biologists and life-scientists can easily and quickly design and perform experiments using this technology, interpret the data obtained and access information that was previously unattainable."

Determining the structure-function relationship of RNA molecules is relevant to understanding how these interact with the molecular machinery and other proteins inside the cell. The interplay between dynamics, structure, and function of many RNAs is tied to the regulation of gene expression and protein synthesis, while protein stability determines remodeling processes that are essential for signaling and protein homeostasis.

This guide has demonstrated how the C-Trap can be used to study single-RNA molecules and has presented the whole experimental process, spanning from sample preparation to data interpretation. It used to be the case that these experiments could only be performed by specialized researchers with advanced knowledge in the matter. However, with the new technological advances, freely open software, and new generation easy-to-use instruments, this is not necessary anymore. Biologists and life-scientists can easily and quickly design and perform optical trapping **experiments using this technology, interpret the data obtained and access information that was previously unattainable.**

Features of the C-Trap

Optical traps:

An optical trap is a highly-focused beam of coherent light capable of moving dielectric objects (cells, microspheres etc.) in the 3D space, while actively measuring its displacement with a high temporal and spatial resolution. The C-Trap has an advantage that it comes with dual-optical traps that can move relative to each other controllably and with very small steps in the 3D space. RNA unfolding is performed best with dual-traps since they allow us to perform experiments deep into the buffer, away from surface effects like non-specific binding and complex surface forces.

Recording of the fastest and the smallest change:

The C-Trap's high spatiotemporal resolution allows you to detect a change in RNA structure about the size equivalent to a single water molecule. At the same time you can easily detect events occurring in a millionth of a second.

Long, repeatable experiments:

The C-Trap is robust and built to minimize drift and maximize stability, making it possible to perform sensitive measurements of a slow RNA-unfolding process.

Full automation and scripting:

The C-Trap is integrated with state-of-the-art scripting interface, allowing you to control even the smallest nuance of your experiment in an automated way. This would free up your time for other experiments, writing papers or that much-needed coffee-break.

Laminar-flow microfluidics:

RNA experiments are often performed with limited sample volumes, at multiple buffer conditions, and with multiple protein interactors. The integrated automated microfluidics optimizes each of these parameters. In this way, you can take measurements in the presence of diverse conditions and protein interactors with the same single RNA molecule.

Temperature control:

RNA processes usually occur at physiological temperatures. Using the C-Trap you can measure them at temperatures ranging from room temperature to 37 degrees or more. This would allow you to get meaningful insights into their cellular behavior.

Precise force feedback:

The C-Trap can respond to stimuli of the folding/unfolding RNA molecule and the motion of RNA binding proteins. It is possible to hold RNA or a protein with a constant force which would be adjusted automatically to the effector force or controllably ramp up or down the force.

References

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