LUMICKS application note

Obtain direct evidence of the processes involved in RNA virus replication using a dynamic single-molecule approach

The genetic material of many viruses consists of RNA. To propagate in their host, these viruses have developed a “hijacking” system that takes advantage of the process the host cells use to read genetic information from an mRNA and produce proteins. The process the viruses employ is termed **"programmed ribosomal frameshifting (PRF)", where the viruses alter the ribosomal reading frame to produce their own proteins and multiply.**

Viral ribosomal frameshifting has long been a target for antiviral agents to treat human disease. Understanding the interplay between RNA-protein complexes to enforce ribosomal frameshifting could comprise a big step forward in the design of novel antiviral agents. However, since the **processes involved in PRF are highly dynamic, current approaches in virology, such as electron microscopy and X-ray crystallography that provide only static snapshot images, are not adequate to provide us with a complete picture of the mechanisms at play.**

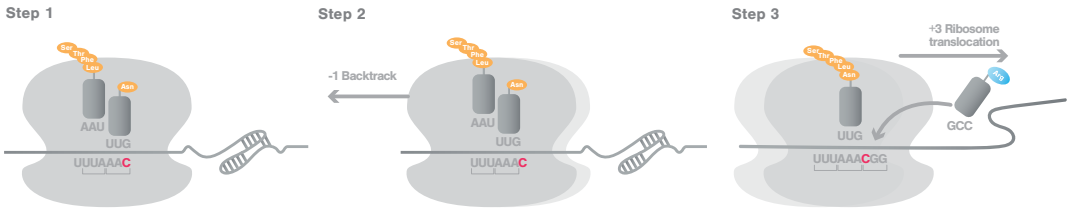

Figure 1. –1 Ribosomal frameshifting. In this alternate mechanism of translation used by RNA viruses to replicate, ribosomes slip by one base in the 5'(-1) direction during translation to produce proteins encoded by two overlapping reading frames.

Here we present two studies that successfully employed **LUMICKS’ C-Trap® technology, which enables you to trap micron-sized objects with optical tweezers (OT) and visualize their interactions in real time,** to shed light on protein–RNA interactions and their effect on ribosomal frameshifting [**1, 2**]. We show how the authors performed OT measurements using the C-Trap with a dual-trap configuration, coupled with a microfluidics system, to **demonstrate two opposite effects of RNA binding proteins on –1PRF – where the ribosome slips back one nucleotide and continues translation in the −1 frame (Figure 1) – resulting in either promotion or inhibition of protein translation.**

More specifically, in the study by Zimmer and Kibe *et al.*, the researchers demonstrated that the protein ZAP-S interacts directly with the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) RNA to interfere with ribosomal frameshifting (**Figure 2A**), which is essential for the viral replicase synthesis, making it a very attractive target for Coronavirus treatment. The study by Hill *et al.,* revealed that cardiovirus protein 2A interacts with and stabilizes the viral RNA to promote ribosomal frameshifting (**Figure 2B**). This event could also potentially be targeted by RNA-based therapies to treat viral infections such as encephlomyocarditis, a disease caused by the cardiovirus EMCV.

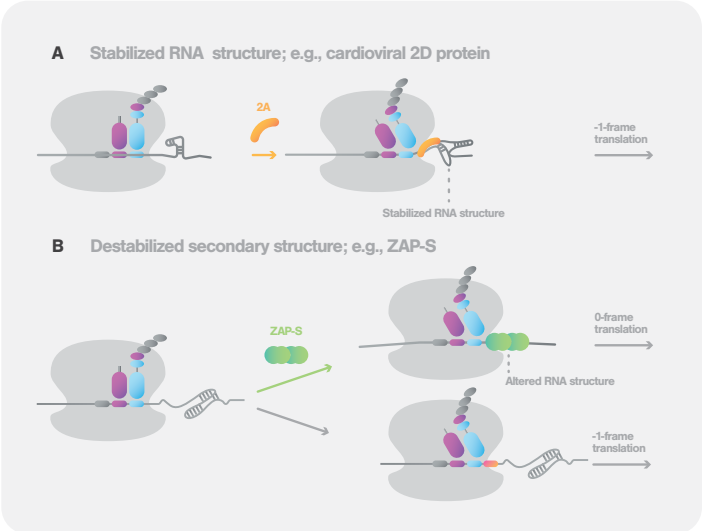

Figure 2. Examples of trans-factor-mediated frameshifting. Two examples, namely (**A**) the cardioviral 2A protein and (**B**) the zinc-finger antiviral protein ZAP-S are shown as representatives of how host- and pathogen-encoded proteins can alter the secondary structure of a frameshift mRNA resulting in enhanced or decreased frameshifting efficiencies. Both proteins specifically bind to the appropriate mRNA and interact with the ribosome. While the 2A protein binds and stabilizes the secondary structure of the EMCV mRNA leading to –1 frameshifting, ZAP-S binding contrastingly destabilizes the secondary structure element on the SARS-CoV-2 mRNA resulting in significantly decreased frameshifting efficiencies. *Created with BioRender.com. Courtesy of Jun. Prof. Neva Caliskan from Helmholtz Institute for RNA-based Infection Research (HIRI).*

The C-Trap is an easy-to-use instrument with a fast workflow that allows you to seamlessly catch and manipulate single molecules. The instrument measures their structural changes or interactions and offers you the opportunity to visualize them in real time with high spatial and temporal resolution. Using the C-Trap to complement other traditional structural biology methods that are unable to provide you with direct evidence of the mechanisms involved in RNA virus replication, you can obtain a complete and detailed picture of protein-viral RNA interactions.

Advantages of using the C-Trap to study RNA virus replication:

- Ability to **study individual RNA molecules** and observe their properties in real time.
- Ability to **directly measure the impact** of protein binding on the **mechanical properties** of the RNA structure.
- Ability to **precisely control the mechanical state** of the RNA molecules as well as the **external conditions,** such as buffer conditions.
- Ability to **observe dynamic transitions** between **different mechanical and structural states,** as compared to classical static structural methods such as electron microscopy and X-ray crystallography.

Methods

Optical tweezer experiments were performed using a LUMICKS dual-trap instrument equipped with laminar flow microfluidics [**3**]. For the experiments, optical tweezers (OT) constructs were mixed with polystyrene beads coated with antibodies against digoxigenin, assay buffer (20 mM HEPES, pH 7.6, 300 mM KCl, 5 mM MgCl2, 5 mM DTT and 0.05% Tween 20) and 1 µl of RNase inhibitor.

The mixture was incubated for 20 min at room temperature and subsequently diluted by the addition of 0.5 ml measurement buffer. Separately, 0.8 µl of streptavidin (SA)-coated polystyrene beads was supplemented with 1 ml of measurement buffer. The flow cell was washed with the measurement buffer and suspensions of both SA beads as well as the complex of OT construct with anti-digoxigenin (AD) beads were introduced into the flow cell.

Per experiment, an AD bead and a SA bead were optically trapped and brought into close proximity to allow the formation of a tether. The beads were moved apart (unfolding) and back together (refolding) at constant speed (0.05 µm/s) to yield the force-distance (FD) curves.

The stiffness was maintained at 0.31 and 0.24 pN/nm for trap 1 (AD bead) and trap 2 (SA bead), respectively. For experiments with 2A or ZAP-S proteins, the protein was diluted to 300nM and 400 nM, respectively, in measurement buffer and added to the buffer channel of the optical tweezer flow cell. FD data were recorded at a rate of ~78,000 Hz. In the case of the cardiovirus 2A protein study, to ensure that the observed effects were indeed a result of interaction with the studied RNA region and not a non-specific binding to handle regions, the researchers also employed constructs containing no single-stranded RNA sequence (No ssRNA control, https://doi.org/10.17632/gkpwngy65h.2). No oxygen scavengers were used during measurements. However, to prevent oxygen damage, all buffers were degassed and contained DTT as a reducing agent.

Results

ZAP-S acts as a direct inhibitor of programmed ribosomal frameshifting in SARS-CoV-2

In SARS-CoV-2, −1PRF allows the translation of different proteins from the same transcript and is essential for viral replication and transcription. PRF relies on the presence of a slippery heptameric nucleotide sequence followed by an RNA-specific secondary structure (a putative pseudoknot). Although several interactors are known for PRF RNA, how these factors alter the mechanical properties of RNA and affect the choice of the reading frame remains unclear.

In this study, HIRI scientists Matthias Zimmer, Anuja Kibe and colleagues demonstrated that the endogenous protein ZAP-S is a direct and specific inhibitor of PRF in SARS-CoV-2 and plays a crucial role in the virulence of this pathogen [**1**]. After employing a multidisciplinary approach to identify the short isoform of ZAP-S as a host-encoded inhibitor of SARS-CoV-2 1a/1b frameshifting in vitro and in vivo, the authors then hypothesized that ZAP-S may interfere with PRF by targeting the putative pseudoknot in the RNA frameshifting element.

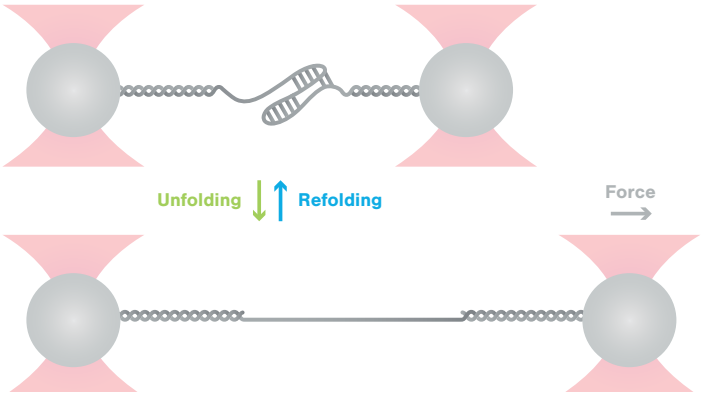

Figure 3. Schematic illustration of optical tweezers experiments. RNA was hybridized to single-stranded DNA handles flanking the SARS-CoV-2 frameshift site and conjugated to functionalized beads. A focused laser beam was used to exert pulling force from one end of the molecule. The force was gradually increased until the RNA was fully unfolded. *Image adapted from Figure 5A, Zimmer and Kibe et al. (2021) Nat. Commun.*

To test this hypothesis and clarify the effect of ZAP-S binding on the structure and mechanical stability of the RNA, the group employed **single-molecule pulling experiments using the C-Trap.** Hereby, they stretched the complex to induce unfolding and relaxed it again to allow refolding of the structure (**Figure 3**).

Strikingly, these experiments showed that **ZAP-S did not affect the unfolding of RNA but did prevent refolding of the RNA structure into its native state (Figure 4).**

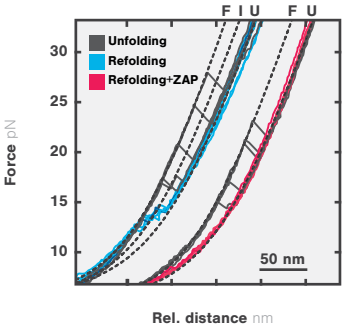

Figure 4. ZAP-S prevents RNA refolding in C-Trap experiments. Example unfolding and refolding traces of the putative form of the SARS-COV-2 pseudoknot RNA in the presence or absence of ZAP-S, as measured using LUMICKS'C-Trap. "F" denotes the folded state, "I" the intermediate, and "U" the fully unfolded state. *Image adapted form Figure 5D, Zimmer and Kibe et al. (2021) Nat. Commun.*

Protein 2A promotes ribosomal frameshifting by binding to and stabilizing an RNA pseudoknot

In cardioviruses, as in SARS-CoV-2, –1PRF is key for protein translation. Activation of –1PRF in these viruses occurs through the virally encoded 2A protein, a multi-functional virulence factor that also inhibits cap-dependent translational initiation. Cardioviruses present a highly unusual variation to conventional viral PRF in which the virally encoded 2A protein is required as an essential trans-activator. Here, the spacing between the slippery sequence and stem-loop is 13 nt, significantly longer than typically seen, and 2A protein has been proposed to bridge this gap through interaction with the stem-loop. This allows for temporal control of gene expression as the efficiency of –1 frameshifting is linked to 2A concentration, which increases with time throughout the infection cycle [**2**].

In their current work, Chris Hill, Lukas Pekarek, Sawsan Napthine and coworkers focused on uncovering how this process is regulated during ECMV viral replication – a virus causing encephalomyocarditis. Protein 2A seems to play a key role in this process. By employing cryo-electron microscopy, the researchers were also able to show that the 2A protein folds in a way completely different to other known RNA-binding proteins in a structure termed the "beta-shell ", and that this folding contributes to the interactions of 2A with the viral RNA. More specifically, binding of the 2A protein to the RNA seems to stabilize its structure and forces a frameshift. **To observe this effect in real time, on the single-molecule level, the authors then made use of the C-Trap optical tweezers (Figure 5).**

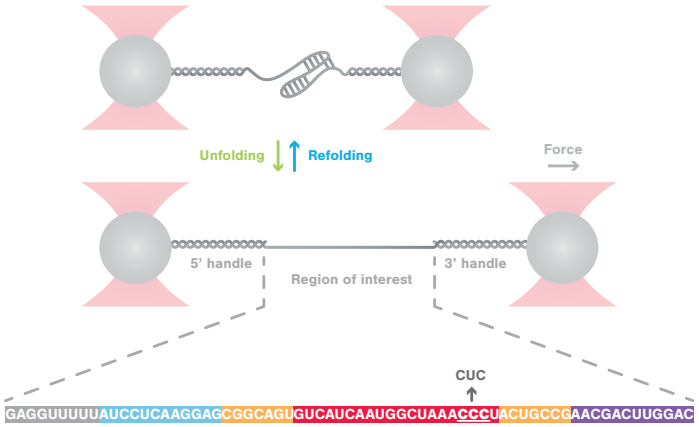

Figure 5. Schematic illustration of optical tweezer experiments. Pulling force is applied to RNA hybridized to ssDNA handles and immobilised on beads. Primary sequence of the construct used in optical tweezer experiments (bottom). *Image adapted from Figure 3A, Hill et al. (2021) Nat. Commun.*

To measure the binding dynamics between the 2A protein and RNA, the scientists employed force-ramp experiments, where a single RNA molecule is gradually stretched and relaxed in several cycles at a constant pulling rate. The applied force allows the RNA molecule to transition between folded and unfolded states, and sudden changes in recorded force-distance (FD) trajectories indicate transitions between RNA conformations. By performing these optical tweezer experiments in the presence and absence of the 2A protein, the authors were able to observe that **2A binding stabilizes an RNA pseudoknot, increasing the force required to unwind it (Figure 6).** These experiments are a great example of how the **C-Trap can be used to complement other classical structural biology methods, such as electron microscopy and X-ray crystallography, to give a complete picture of protein synthesis and role of RNA binding proteins during viral replication.**

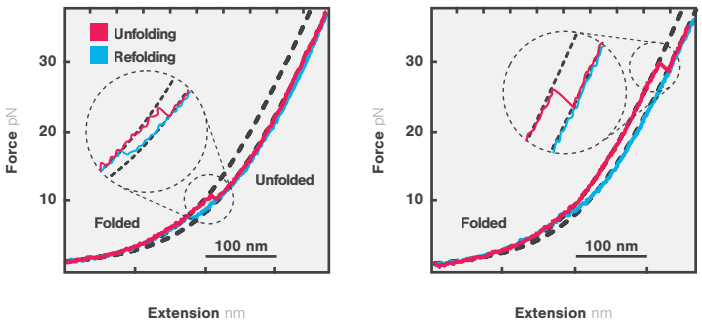

Figure 6. 2A protein stabilizes RNA. Force-distance curves of RNA unfolding and refolding in the absence (A) and presence (B) of 2A. *Image adapted from Figures 3C and 3D, Hill et al. (2021) Nat. Commun.*

Conclusion

Conventional methods used in the study of RNA virus replication, such as electron microscopy and X-ray crystallography, only record static images and cannot provide us with a complete picture of the highly dynamic processes taking place. With LUMICKS' C-Trap technology that allows the real-time study of dynamic processes between micron-sized molecules at high resolution, scientists can now gain more valuable insights into the mechanisms at play during viral replication, on the single-molecule level.

The studies presented here demonstrate the potential of the single-molecule solutions offered by LUMICKS' C-Trap in the study of RNA virus replication. Performing single-molecule experiments, the researchers uncovered two opposing and significant roles of RNA binding proteins. Zimmer *et al.* provided direct evidence that the RNA-binding protein ZAP-S directly impacts the SARS-CoV-2 1a/1b frameshifting and can thus inhibit viral replication [**1**] – a discovery that could lead to the development of novel Coronavirus treatments. Contrarily, Hill *et al.* showed that accumulation of protein 2A in host cells can lead to increased viral protein translation through RNA binding [**2**], allowing for the development of RNA therapies that could help treat encephalomyocarditis.

By using dynamic single-molecule approaches to uncover specific steps in the replication cycle of RNA viruses – from the initial interaction between an RNA virus and its host cell to the shedding of the viral progeny – scientists can gain invaluable insights that will, ultimately, allow for the potential development of new therapies against RNA viruses.

Acknowledgements

Data courtesy of Jun. Prof. Neva Caliskan and colleagues from the Hemholtz Institute of RNA-based Infection Research.

References

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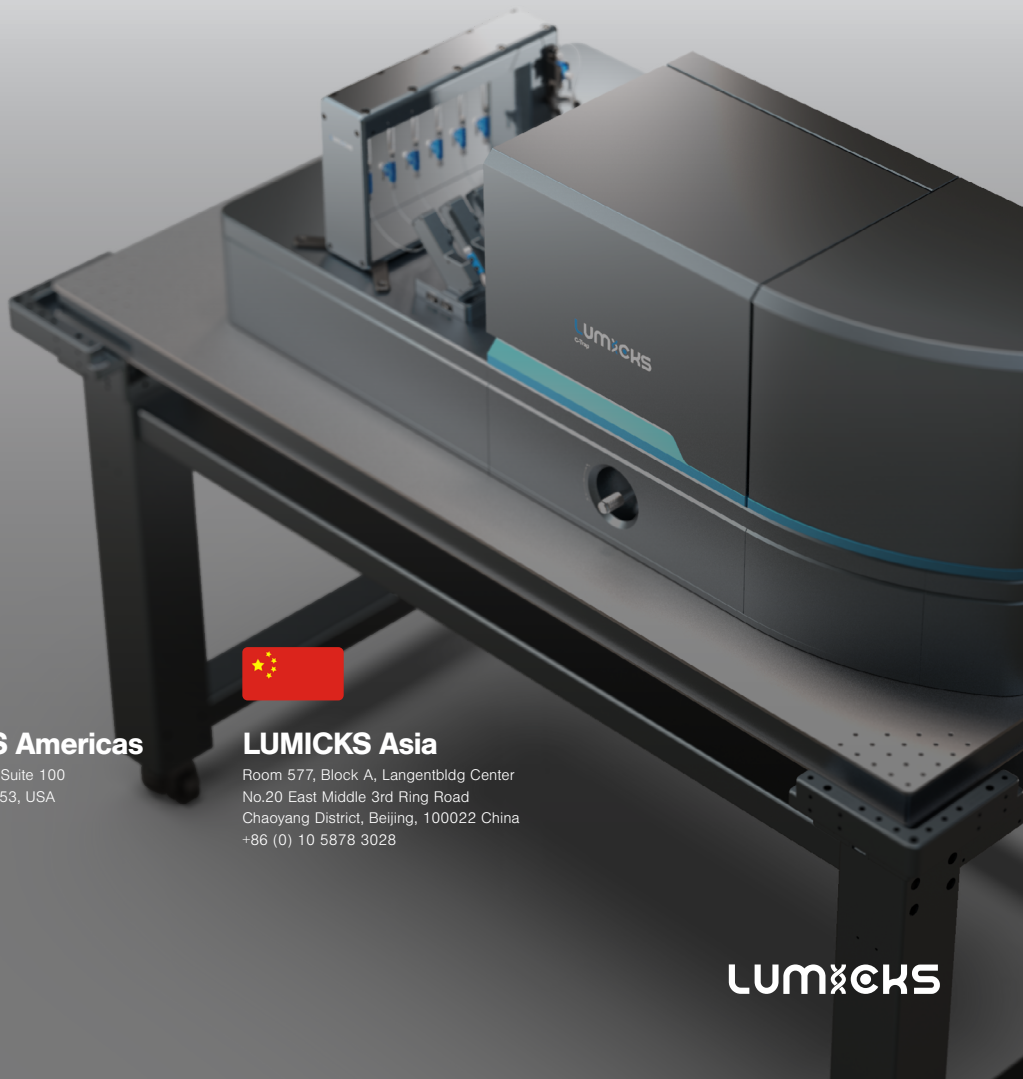

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