Real-time detection of kinase conformational changes in the presence of a small-molecule inhibitor

Protein Folding
Application note - C-Trap® Dymo

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LUMICKS

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While current methods applied in structure biology have laid the foundation for our understanding of protein conformations, they have been unable to record dynamic processes. Such knowledge may be crucial to identify protein features, and dynamic single-molecule applications are becoming pivotal for discovering relationships between conformational changes and functions. Optical tweezers are ideal to study protein dynamics through their high-resolution readouts of transient and discrete properties of single enzymes.

In the field of structural biology, traditional techniques have uncovered relationships between protein transition states and functions through structural snapshots. X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and circular dichroism spectroscopy all have offered invaluable insights for assessing structural alterations of proteins and connecting them to functions.

However, while being central to our understanding of conformational states and function, these approaches alone can be insufficient for a thorough understanding of the dynamics of proteins. On the one hand, most functional assays rely on bulk measurements that average out transitions at comparable free energies. On the other hand, structural assays merely provide snapshots of the different protein states and might thus exclude conformational states that are hard to stabilize. The two approaches are typically decoupled, and researchers often rely on post-factum correlations between the two to understand structure–function relationships.

Single-molecule tools like optical tweezers address these limitations by allowing researchers to measure transient macromolecular states at a nanometer resolution. The C-Trap® Optical Tweezers – Fluorescence and Label-free Microscopy is a prime example of an instrument that can capture discrete conformational changes that reveal functional properties. Dynamic single-molecule assays enable you to directly correlate functional and structural properties of a protein, for example, in the presence or absence of enzyme-specific substrates or small-molecule inhibitors. As an extension, the correlated imaging can provide you with additional real-time information about intramolecular or intermolecular interactions upon conformational changes of the structural target.

With an ever-growing demand for accelerated drug development, scientific research requires simple, fast, and accurate tools that measure transient conformational processes at high resolution. In this experiment, we show how the C-Trap fulfills these needs, allowing you to interpret enzymatic processes and structural properties through an easy-to-use workflow with standardized kits and automation scripts.

In this application note

This **application note introduces you to a new approach** for measuring dynamic and highly transient conformational states of proteins and collecting the data in real time. It showcases how to perform these measurements with the C-Trap® Optical Tweezers – Fluorescence and Label-free Microscopy system from start to end. We also introduce you to the adopted features that support and simplify your dynamic single-molecule experiments, regardless of your experience level.

Features of the C-Trap

The C-Trap combines optical tweezers, the latest imaging techiques, and an advanced microfluidics system to capture, manipulate, and visualize conformational and functional properties of proteins.

The optical tweezers can capture a protein between two optically trapped micron-sized beads with a highly focused laser beam. You can then manipulate the conformation of the captured protein by moving the beads apart, hence stretching the protein, or releasing the applied tension.

The real-time imaging lets you visualize the trapped protein as you manipulate the tension on the structure. For example, you can label specific protein sites with different fluorophores to assess conformational distances (for example, smFRET microscopy).

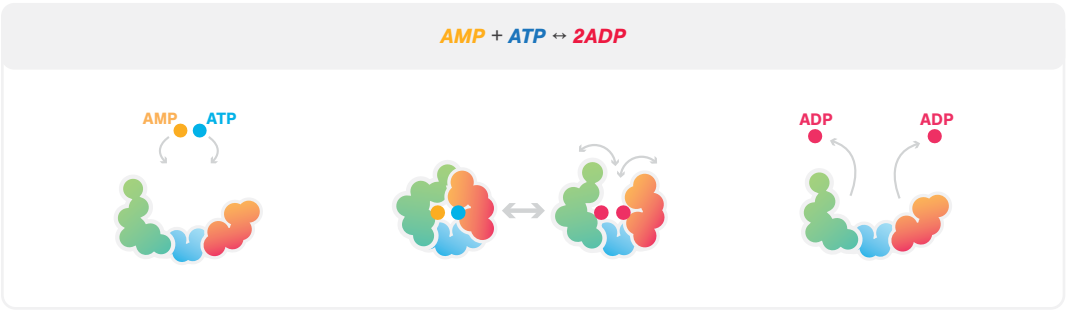
The microfluidics system improves the experimental workflow and helps you to add reagents to the system in separate channels. No physical barriers separate the highly stable flows, making it easy for you to control and navigate the different reagents.

Experimental workflow for protein conformation assessments with optical tweezers

Choice of protein

Kinases are attractive drug targets due to their involvement in several chronic diseases, for example, when exposed to genetic mutations. Thus, understanding the structural dynamics of kinases and assessing their effects on catalytic activity can aid both our general understanding of enzymatic transitions and the development of enzyme-specific inhibitors.

Here, we examined adenylate kinase (AdK), a phosphotransferase enzyme that regulates cellular energy homeostasis by transferring phosphate groups between adenine nucleotides: **(Figure 1)**



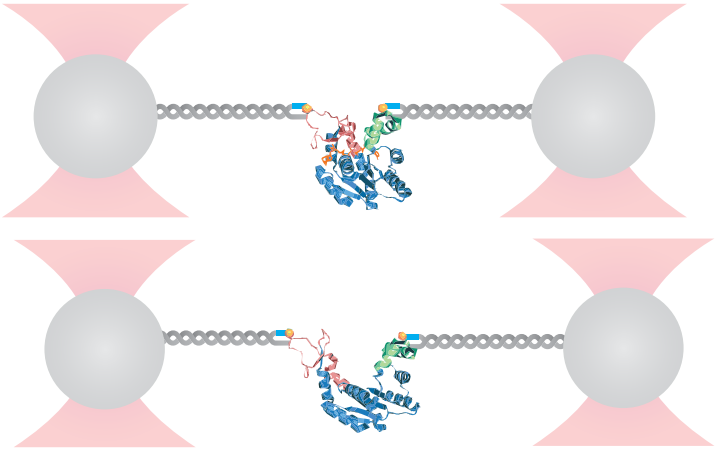
AdK contains a lid domain that closes upon substrate binding, bringing the two substrates into proximity to each other, subsequently activating the enzyme. To understand the conformational properties of AdK, we studied the resulting transitions upon opening and closing of the lid in the presence of a bisubstrate inhibitor.

1 Schematic representation showing the reversible conversion of the nucleotides AMP, ADP, and ATP, catalyzed by an AdK protein. Binding of AMP and ATP induces closing of the catalytic lids (red and green), which initiates the phosphoryl transfer. The opening of the lids releases the final product (two ADP molecules).

Tethering the protein

We studied kinase conformational states upon exposure to small-molecule inhibitor di(adenosine-5') pentaphosphate (AP5A) that pushes the equilibrium of the kinase conformation towards the active (closed) state as opposed to the inactive (open) state. To study these conformations with optical tweezers, we first tether the protein between two polystyrene beads to form a so-called dumbbell configuration **(Figure 2)**. This configuration allows us to stretch (unfold) the protein and measure conformational changes with nanometer precision.

To tether AdK between the beads, we first introduced point mutations to the protein, inserting cysteines at positions 42 and 144. The integration points were chosen to maximize the measured distance change during lid opening and closing. The inserted cysteines anneal to maleimide-functionalized DNA handles, forming a protein–DNA construct through which the complex can tether to the two trapped beads in the C-Trap (see text box below).



2 Schematic representation of an AdK protein that is tethered between two polystyrene beads in a so-called dumbbell configuration. The protein is tethered to the two beads through the attached oligonucleotides (blue) that subsequently hybridize with complementary overhangs on the DNA handles (red). Displacement of the right-hand side bead results in a conformational change (bottom figure).

The **protein labeling is a three-step procedure** that establishes the final protein construct attached to two 529 bp DNA handles (5' digoxigenin modification and 5' biotin modification) in preparation for subsequent C-Trap experiments.

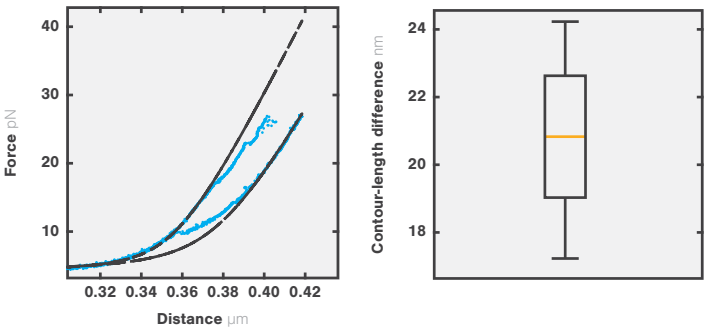
- **Step 1:** DNA oligonucleotides with maleimide- or CoA-modifications are attached to their respective incorporated feature (cysteines or ybbR tags) in the protein of interest.
- **Step 2:** The protein, coupled to two oligonucleotides (forming a protein–oligo complex) is purified from the excess of unreacted oligonucleotides from Step 1.
- **Step 3:** The respective oligonucleotides hybridize to the complementary overhangs on the DNA handles.

The final product from Step 3 is next tethered between an anti-digoxigenin coated bead and a streptavidin-coated bead in the microfluidics system of the C-Trap.

Confirmation of protein tethering

Once we have created the dumbbell configuration (see above), we can confirm proper tethering as an extra line of validation. Tethering validation is achieved by moving the two optically trapped beads apart from each other to reach the expected contour length of the tether, that is, its maximal extension length. Subsequent higher pulling forces will unfold the protein, which is observed by a rapid decay in pulling forces and increased extension of the tethered complex. **Figure 3** shows a total contour length of approximately 360 nm, which corresponds to the expected combined length of the protein and DNA handles. The measured length confirms a stable and proper tethering between the protein–DNA construct and the two beads.

The same setting can also reveal the contour length of the tethered protein alone by measuring the distance changes upon complete extension. Unfolding the protein to its maximal extension length established a contour-length difference of 21.6 nm (+/- 1.14 nm; **Figure 3**), which is in agreement with the literature [1].

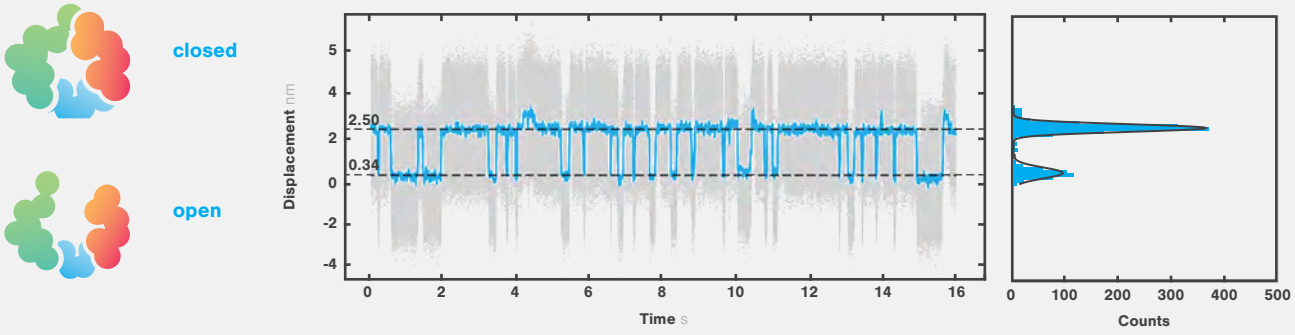


3 Left: A representative graph showing the changes in contour length of the captured protein (including the associated oligonucleotides and DNA handles) upon increased pulling of the beads. Right: A representative box plot depicting the changes in contour length of the protein alone (excluding the contribution from the associated oligonucleotides and DNA handles).

Small inhibitor AP5A induces the closed active state of AdK

To study the equilibrium dynamics of AdK and evaluate properties associated with open and closed conformation states, we pulled the protein at constant low forces (between 6 and 10 pN). At these forces, the molecule is in a state of continuous transition, which enables us to acquire the lengths corresponding to structural changes during lid opening and closing. We investigated the equilibrium between these two states upon treatment with AdK-specific inhibitor AP5A to understand its mechanism of action and effect on the conformational properties of the kinase.

In accordance with the literature [1], the AdK protein adopts an exclusive open-state conformation in the absence of AP5A, possibly due to inherent protein properties. By contrast, in the presence of 50 nM of AP5A, we could measure the two-state conformation of AdK over time. Specifically, we measured a distance range of approximately 2 nm between the open and closed states **(Figure 4)**. The observations demonstrate how the C-Trap can extract data from dynamic processes to reveal conformational properties of a protein, such as distance between states, with nanometer precision.



4 Graph depicting the two-state equilibrium dynamics of AdK in the presence of 50 nM of AP5A at low tension (10 pN). Data was recorded at 78 kHz (grey dots) and decimated to 100 Hz (red line). The lower state of the bead displacement was normalized to 0 nm to highlight the displacement difference between the two states. The two most populated states can be clearly distinguished also from the histogram (right panel), showing two peaks separated by 2.16 nm.

Making single-molecule analysis fast and simple

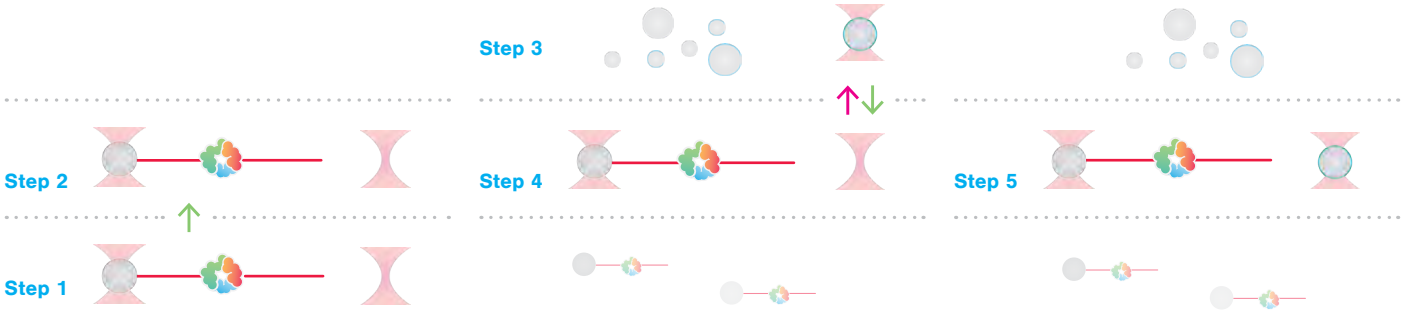
The full workflow of the experiments mentioned in this application note was supported by our kits, reagents, software, and automation scripts, which speed up your experiments and results. We have developed the C-Trap and its workflow to allow any operator to set up and perform experiments in a matter of minutes. The features described below have been optimized to facilitate your dynamic single-molecule experiments so that you can perform these from start to end without prior experience.

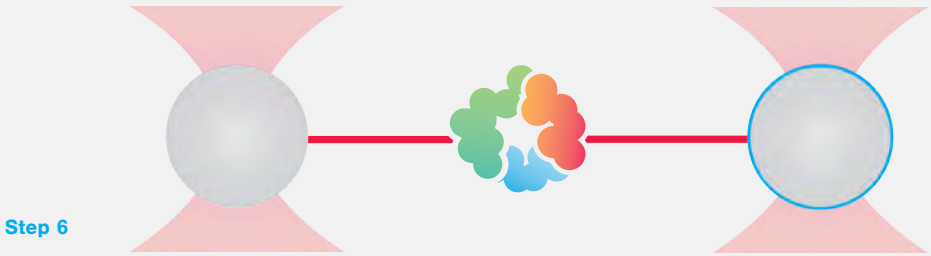
Reagents and kits – Start your experiments

An extensive selection of ready-made reagents and kits ensures that you can easily prepare and perform your experiments. The reagents are optimized for C-Trap specific assays, which means that you don't need to fine-tune the concentrations. In this study we used the **Protein tethering kit (cysteine)** to attach DNA handles to AdK and subsequently tether the complex to the two trapped beads. Visit **www.store.lumicks.com** to learn more.

Workflow – Set up your single-molecule assay

The microfluidic flow cell provides multiple adjacent laminar flow channels – without physical barriers – where you can introduce your reagents. You can easily move your optical traps between channels, gather any reagent from one channel to the other, and assemble your experimental constructs.

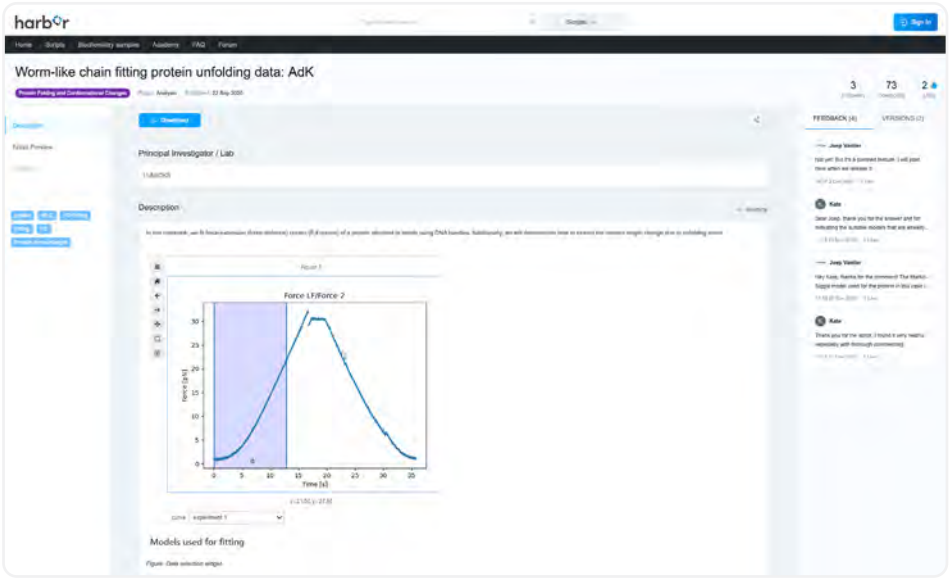




5 Schematic representation of the protein-tethering workflow inside the microfluidics system of the C-Trap – supported by an automation script. **Step 1**: Trapping and positioning of a protein-carrying bead under an applied flow. **Step 2**: Repositioning of the trapped bead into a new (buffer) channel in the flow chamber. **Steps 3 and 4:** Capturing a functionalized bead from the upper channel and transferring the trapped bead into the buffer channel. **Step 5:** Stopping the flow to position both beads for tethering. **Step 6:** The final dumbbell configuration.

Harbor – Automate your experiments through a scripting community

Our new scripting platform is a portal where users can publish, download, review, and cite scripts to automate protein-measuring experiments and analyze the resulting data. Beginners and experts can join the scripting community of C-Trap users to download and upload scripts that have been reviewed by LUMICKS' scientists and community users. The scripts can be used directly to automate your experiment through the Bluelake software or to easily analyze your data. Visit **www.harbor.lumicks.com** to learn more about the platform.



6 Representation of the Harbor page, including the introduction of an uploaded script for automation of a dynamic single-molecule C-Trap experiment. The scripts can be found at **www.harbor.lumicks.com.**

Conclusions

Whereas single-molecule techniques used to be restricted to the realm of physicists, they are now powerful tools that can improve our knowledge in molecular biology. We have designed the workflow of the C-Trap to enable all types of users to perform experiments and extract results from day one.

This study aimed to functionalize a kinase for conformational assessments and showcase how the approach can help us better understand the relationships between conformation and function. We show that high-resolution measurements with the C-Trap can record transient and minuscule conformational dynamics that can explain energy landscapes associated with enzymatic activities.

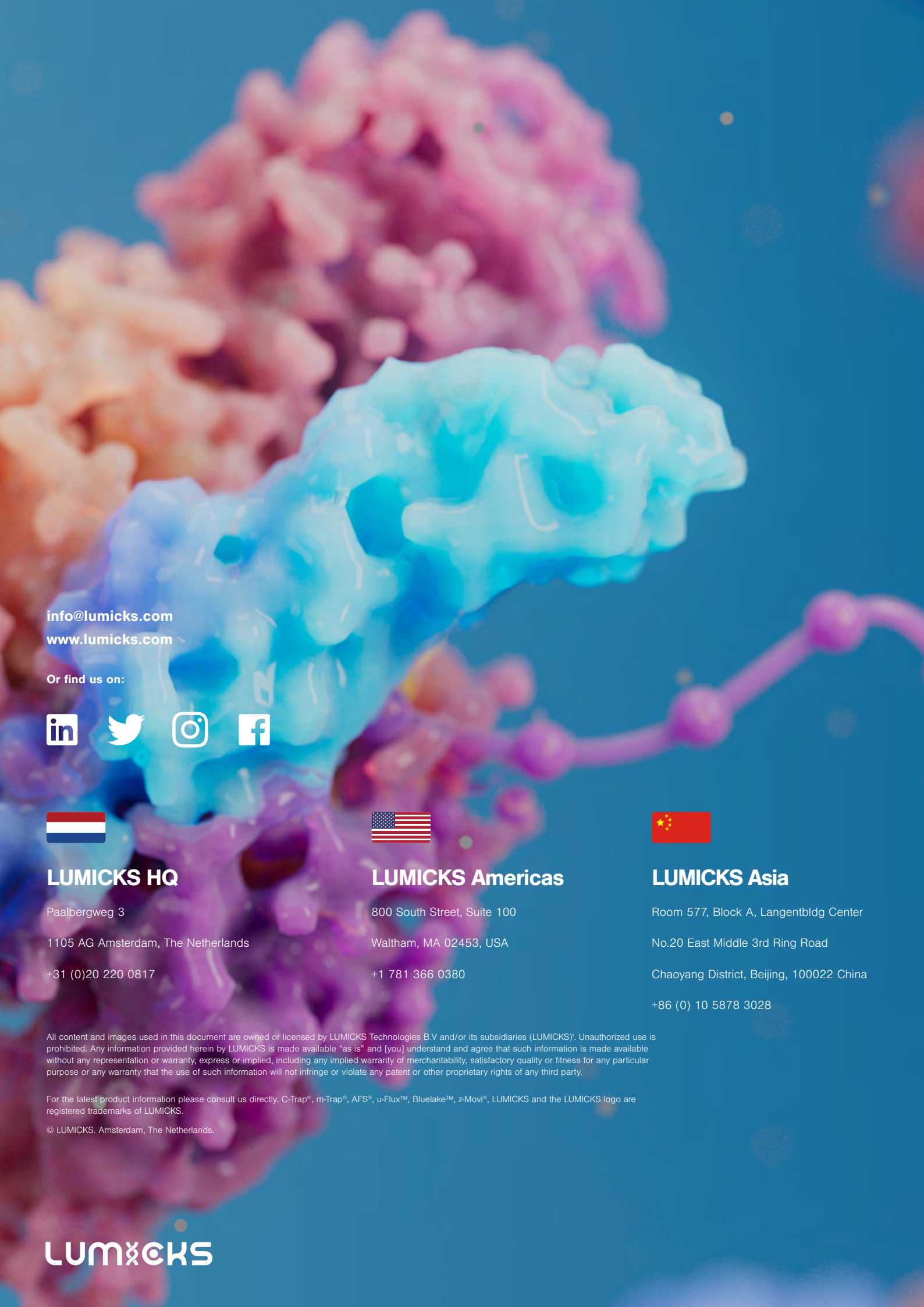
You can further expand and improve the outcome of conformational analyses by combining the C-Trap measurements with technologies that can highlight the structure and dynamics of specific protein domains. For example, single-molecule Förster resonance energy transfer (smFRET) microscopy relies on the energy transfer between two fluorophores and can assess conformational changes at distances between 10 and 100 Å (1–10 nm) [2]. Combining the two technologies into hybrid tools can provide us with additional insights into intramolecular or intermolecular dynamics of specific domains. These hybrid approaches can serve to reveal information where traditional structural biology approaches are limited.

Results achieved through dynamic single-molecule approaches like the C-Trap® optical tweezers will serve our appreciation of the intricacies of protein mechanics. They also may ultimately serve as a screening tool for the development of highly specific and effective therapeutic drugs against diseases.

References

[1] Pelz B, Žoldák G, Zeller F, Zacharias M, Rief M. Subnanometre enzyme mechanics probed by single-molecule force spectroscopy. Nat Commun. 2016.

[2] Holden SJ, Uphoff S, Hohlbein J, Yadin D, Le Reste L, Britton OJ, et al. Defining the limits of single-molecule FRET resolution in TIRF microscopy. Biophys J. 2010.



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