Unparalleled approaches to understand cytoskeletal processes

Cytoskeletal structure and transport
Application note – C-Trap® Edge

2022

LUMICKS

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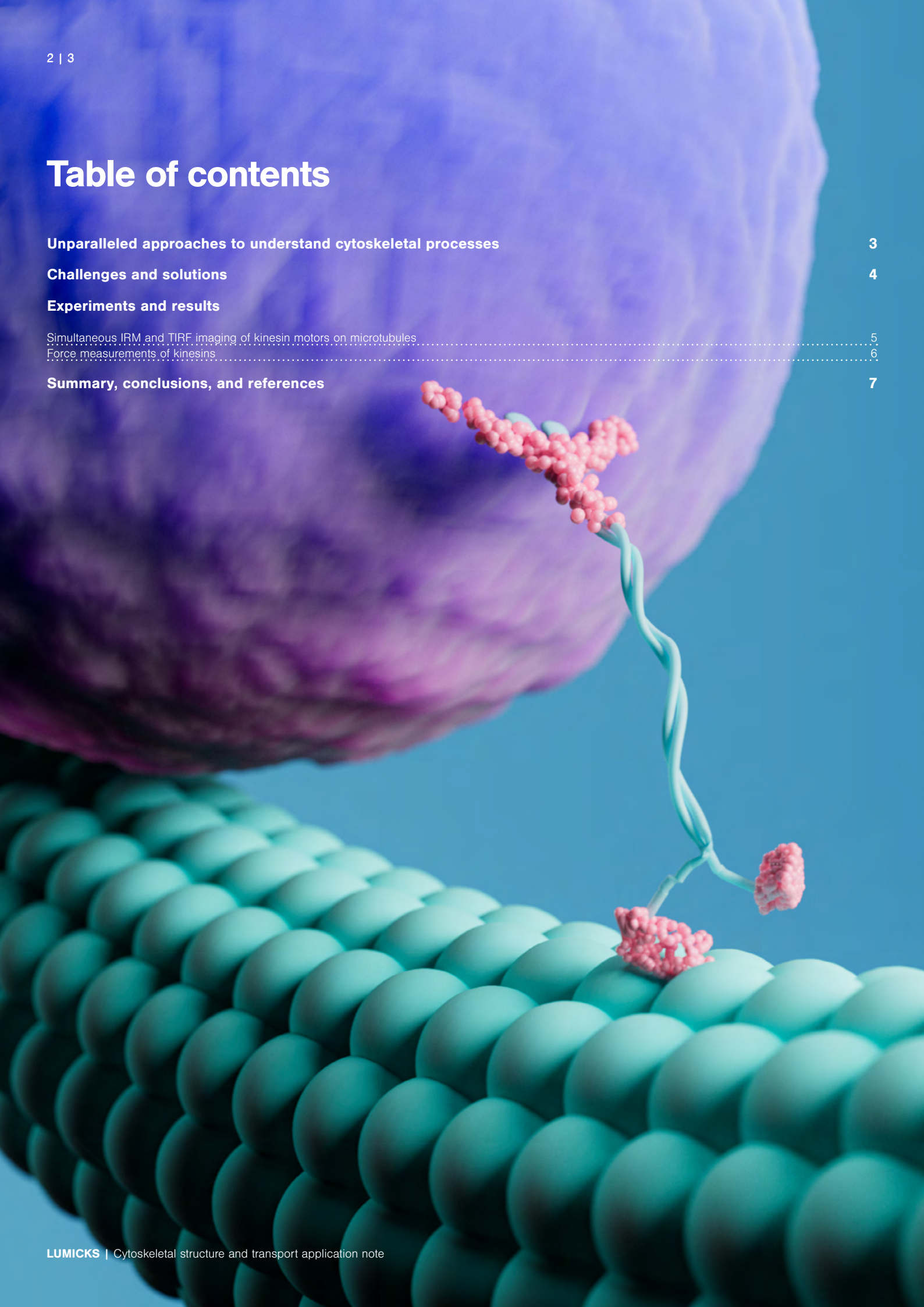
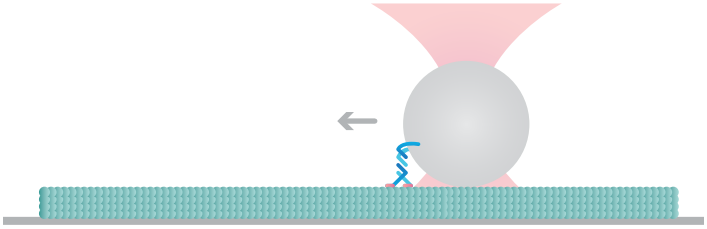


Unparalleled approaches to understand cytoskeletal processes

The cytoskeleton is a critical cell component by that regulates cell shape, cell migration, and intracellular organization. It consists primarily of microtubules, intermediate filaments, and actin filaments, each of which play unique roles and contribute to the cytoskeleton's diverse functionality. Certain cytoskeletal functions, such as intracellular transport, also require the activity of molecular motors – proteins that catalyze chemical energy into mechanical energy. Alterations in these cytoskeletal components can cause a variety of diseases, ranging from neurological disorders to muscular dystrophies, highlighting a need to better understand the cytoskeleton [1].

Two commonalities of the cytoskeleton are that (1) its activity is spatio-temporally dynamic, and (2) it's subject to and regulated by mechanical forces. Currently, separate tools exist for understanding either cytoskeletal dynamics or mechanics. Simultaneously studying the organization, dynamics, and mechanics of filaments and the interaction of filaments with force-generating motor proteins is, however, crucial to fully understanding the mechanisms involved in emergent cellular processes and associated pathophysiologies, such as multiple sclerosis, Charcot-Marie-Tooth syndrome, or hearing loss/deafness [**1,2**]. Therefore, researchers seek a comprehensive tool that can directly correlate intramolecular dynamics with mechanics.



1 Illustration of standard surface assay for studying cytoskeletal motor proteins with optical tweezers.

In this application note

This **application note introduces you** to a revolutionary single-molecule approach that enables you to simulatenously study the dynamics and mechanics of cytoskeletal processes. Specifically, we highlight the general workflow and results from experiments geared toward understanding how molecular motors interact with cytoskeletal filaments.

This application note shows how you can use correlated force and displacement measurements with fluorescence (total internal reflection fluorescence, TIRF) and label-free (interference reflection microscopy, IRM) microscopy to achieve an unparalleled look at the cytoskeleton with single-molecule resolution.

We introduce you to a surface assay-based approach for studying kinesin motors walking along microtubules. Using this approach, you can measure – for long periods of time, and in an extremely user-friendly setup – fluorescence and force measurements of:

- Velocity of motor proteins with a highly-sensitive and fast TIRF camera and a high resolution 2D force clamp.
- Step size and stepping rate of motor proteins by having the ultimate distance resolution.
- Stall force of motor proteins with high force resolution and stability.

User insights

Prof. Taekjip Ha
John Hopkins University

"In optical tweezers measurements, it is as if you are closing your eyes and using your hands to manipulate and measure the response of an object, whereas in fluorescence measurements, you have your hands tied in back and make passive observations with your eyes. By combining the two, we can hope to sample the best of both worlds."

Challenges and solutions

The challenges

The filaments composing the cellular cytoskeleton are small, ranging in diameter from roughly 5 to 25 nanometers. Kinesins have been found to take step sizes of roughly 8 nm and at rates up to 100 Hz [**3,4**]. Additionally, kinesins were found to exert forces up to 8 pN [**5**]. At the molecular level of the cytoskeleton, these step sizes, dynamics, and forces are the standard, thus limiting researchers in studying its biological properties

Imaging techniques allow the visualization of filaments and motor proteins with good spatial resolution, enabling researchers to track and analyze their activity. For instance, imaging can be used to study (de)polymerization of filaments, co-localization of proteins, and translocation of motor proteins. Data obtained by TIRF microscopy typically reveals motor dynamics occurring at 10Hz. However, these approaches lack the temporal resolution, force measurements, and manipulation needed to completely understand the molecular activity, which can be faster and involve the action of forces (e.g., kinesins stepping at 100 Hz). Thus, single-molecule force spectroscopy techniques such as optical tweezers are used to measure molecular activity and mechanical forces, at rates in the kilo-to-mega Hz range that cannot be determined from any other technique. For example, optical tweezers have been used to measure step sizes, stepping rates, and stall forces of various motor proteins, such as kinesins, dyneins, and myosins. In addition, by combining these force measurements with fluorescence microscopy one can, for example, study how different subunits of the motor move while the motor is translocating (by using FRET or multi-color imaging).

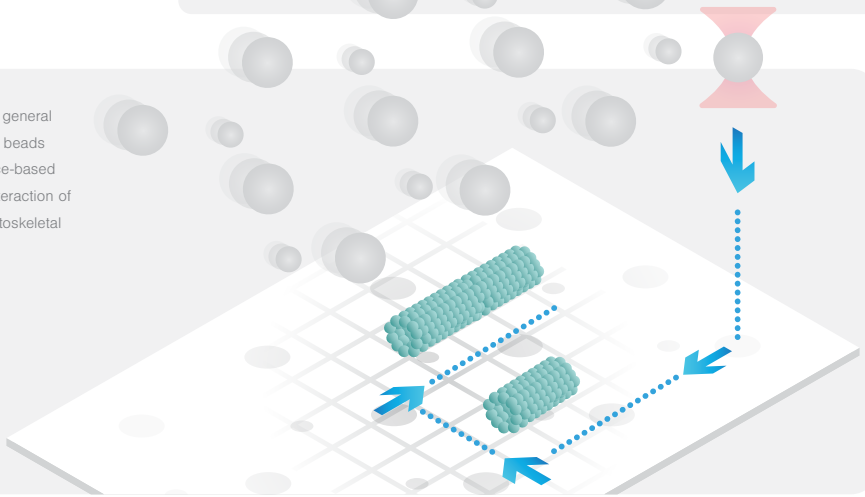
The solutions

Here, we present a unique and versatile instrument, the C-Trap® Edge, that enables unprecedented studies of biological processes with high spatial and temporal resolution at the molecular level. The system combines multiple imaging modalities and optical tweezers. For cytoskeletal applications, the C-Trap integrates label-free IRM and three-color TIRF imaging. This approach allows for high contrast and high temporal resolution imaging of label-free filaments, such as microtubules, while measuring and manipulating the forces that different binding proteins, such as kinesin or dynein, exert on them. Unlike traditional home-built systems, our instrument is an all-in-one, easy-to-use, turn-key system with truly correlated data.

The C-Trap's ease-of-use enables researchers of all expertise, not just a handful of the most experienced biophysics labs who have difficult to operate home-built systems, to catapult their research forward. Overall, this technology provides researchers with the freedom to test any hypothesis in real time and capture the smallest and most transient events.

How do you do it?

Flow cells are assembled by adhering a KOH-cleaned coverslip to a microscope slide with double-sided tape. The surface is functionalized by coating with biotinylated bovine serum albumin and avidin. To enable stable surface adhesion and fluorescence visualization, GMPCPP-stabilized microtubules are first labeled with biotin and Hilyte647 and then flushed in the channel, where they bind the avidin-coated coverslip [**3**]. Finally, the unanchored microtubules are washed away, and the final solution is flushed in.
Depending on the experiment, the final solution is also supplemented with kinesin motors or kinesin-coated beads. The beads are labeled with anti-GFP antibody, so they can bind GFP-labeled kinesins.



2 An illustration of the general workflow for capturing beads and conducting surface-based assays to study the interaction of motor proteins with cytoskeletal filaments.

The functionalized slide is then placed on the C-Trap's stage to begin observing motor protein activity in real time. LUMICKS' intuitive Bluelake software allows the user to simultaneously visualize and record TIRF and IRM images (**Figure 3**). To reduce background and increase signal-to-noise of the label-free images, the software uses a real-time background subtraction method. To avoid surface drift, active surface stabilization automatically corrects for long term drift to keep the image in focus.

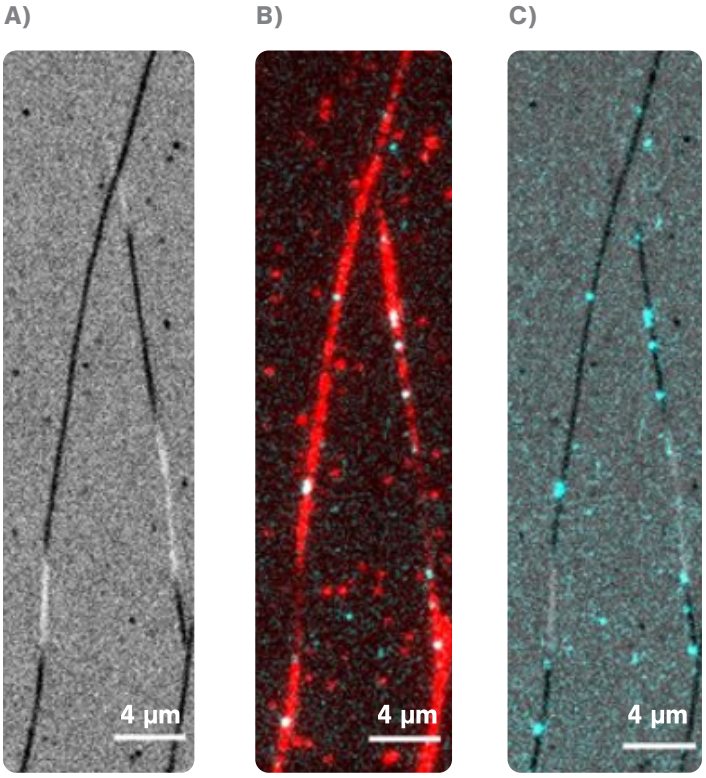
For force-measurement experiments, beads are caught above the surface and then moved closer to the surface to engage with the microtubule (**Figure 2**). Movements between different positions (for example, in solution to trap beads or next to a microtubule) are accomplished with a single click. When the motor engages with the microtubule and starts walking, the force can be monitored in real time and later exported for subsequent analysis of the desired force-displacement-time trace. Additionally, a 2D force feedback feature can be used to exert constant forces and follow the step-by-step motion of the motor at high spatiotemporal resolution. Virtually every aspect of the workflow can be automated in Bluelake.

Experiments and results

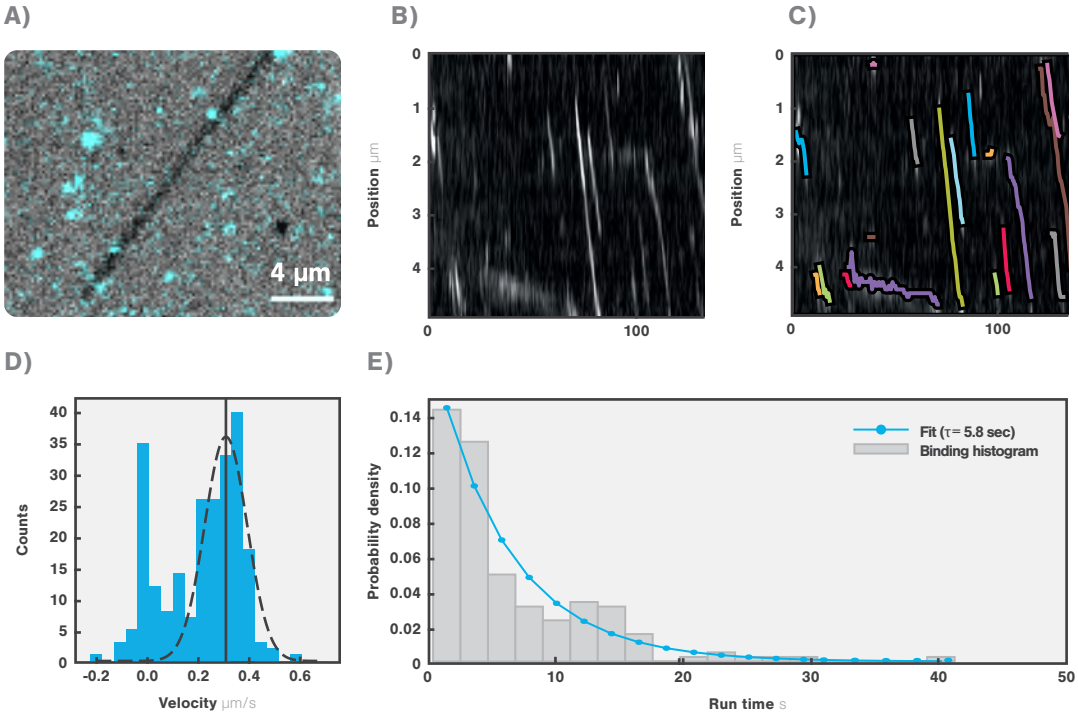
Simultaneous IRM and TIRF imaging of kinesin motors on microtubules

Using correlative IRM/TIRF technology, microtubules were imaged on the surface using IRM, while motility of GFP-labeled kinesin motors on microtubules were measured using TIRF (**Figure 3**). To keep the assay in focus for extended timelapse imaging, surface stabilization was enabled (< 3nm drift over 2 minutes). These images show how correlative imaging techniques enable the study of dynamic cytoskeletal interactions at the single-molecule level with high contrast and spatial resolution. It is important to note that thanks to the label-free capacity of IRM microscopy there is no need to label the microtubules. This reduces sample preparation costs, abolishes photobleaching of the filament, and prevents potential undesired effects of labels affecting the behavior of the motors on the filaments.

To characterize the velocity of kinesins, the timelapse movies of GFP-labeled kinesins were analyzed, by generating time traces (kymographs) of kinesins on individual microtubule filaments (**Figure 4a**). Kymographs were analyzed using a modified version of Kymotracker (see Harbor) adapted to TIRF images. Kymotracker identifies individual events of kinesins walking along the microtubule. From this data, the population of velocities can be calculated automatically (**Figure 4c**). We modelled this distribution as a combination of two Gaussian distributions. One for moving motors and one for static ones. The moving motors had an average velocity of 299±82 nm/sec. Note that some motors appeared to have a negative velocity, which is probably due to a crossing microtubule having motors moving in the opposite direction. Similarly, a histogram of the residence time of the motor can be calculated. A single exponential can be fitted, which leads to a mean lifetime of 5.8 seconds, with 95% confidence intervals [4.9, 6.7] (**Figure 4d**). This example shows how the C-Trap is a perfect toolbox to easily and quickly obtain speed and residence times of different motors under different conditions.



3 Example images of **A** unlabeled microtubules visualized with IRM (note: microtubules near surface are dark while non-adherent regions are lighter), **B** overlayed microtubules (red, labeled with Hilyte647) with GFP-labeled kinesins (cyan), and **C** overlay of IRM images with GFP-labeled kinesins.



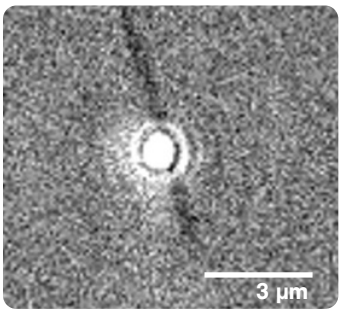
4 Identifying single kinesin excursions and calculating motor protein velocity and run time. **A** Example of a microtubule seen by IRM (black) with a motor walking on it (cyan). **B** Single-molecule traces of kinesin motors walking along the microtubule. **C** Automated identification and characterization of individual kinesins identified with a modified version of the Kymotracker (Harbor, LUMICKS) adapted to TIRF images. **D** Quantification of kinesin velocity from a number of traces (N=234) . **E** Dwelltime distribution of the moving motors (gray bins). An exponential distribution was fitted to the raw data using Maximum Likelihood Estimation [**ref 7**] (shown in orange) using Pylake (**https://doi.org/10.5281/zenodo.4280788**). This resulted in a time constant of 5.8 seconds (95% bootstrap confidence interval between 5.0 and 6.7 seconds). Tracked kymograph lines with a speed below 50 nm/sec were excluded from the analysis.

Experiments and results

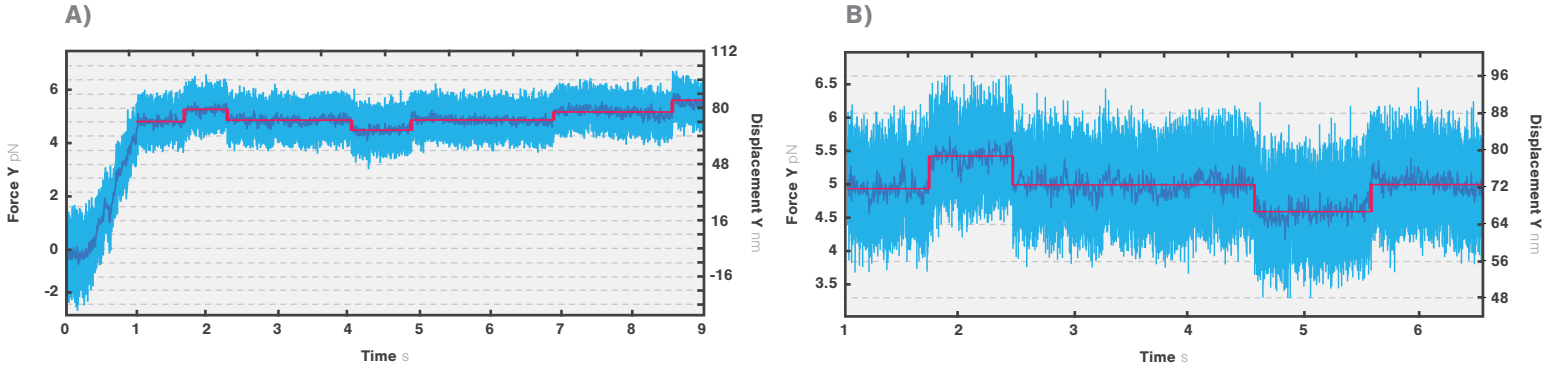
Force measurements of kinesins

In addition to passively observing kinesin motion, the C-Trap can be used to calculate step size and force generation of the kinesin motor protein in real time. This is done by conjugating kinesin motors to a bead. In this case, 520 nm-sized polystyrene beads were coated with anti-GFP antibodies, such that GFP-labeled kinesin can bind specifically. Once trapped, the kinesin-labeled bead can be brought into close proximity of a microtubule. The microtubule is visualized by IRM, which does not require fluorescent labeling of the microtubule (**Figure 5**).

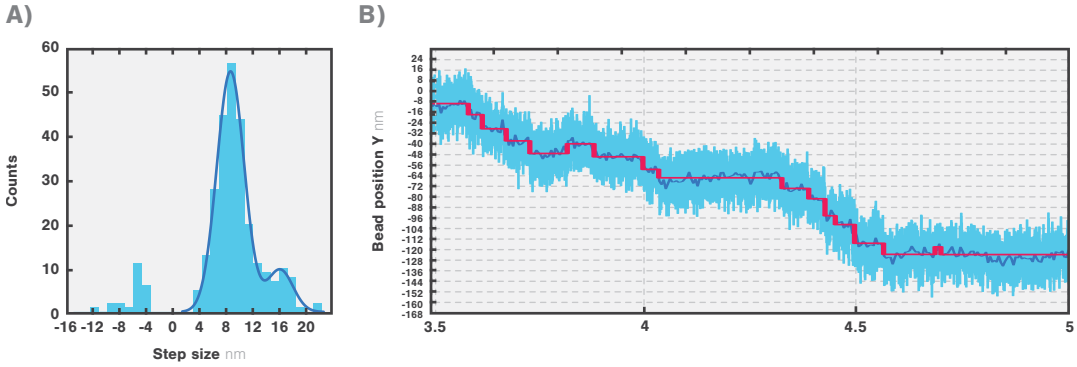
The stall force is an important parameter to understand, because it provides information on the kind of cargo a motor can translocate within a cell, or how different cofactors might affect the physical behavior of the motors. On the C-Trap, this can be easily determined by holding the trap static. Since the optical trap behaves as a Hookean spring (i.e., force is directly proportional to bead displacement), the force increases as the motor attempts to translocate. In this example, the trap was held static as a kinesin attempted to translocate (**Figure 6a**). Force and displacement data was recorded at 78 kHz (light blue) and noise was reduced by down-sampling to 200 Hz (dark blue). Stall forces were observed at approximately 5-6 pN (**Figure 6a**), consistent with previous literature [**6**]. Additionally, at the stalling force, the data also shows the forward and backward stepping of the motor with a step size of 8 nm (**Figure 6b**).



5 A sample screenshot from IRM timelapse imaging of a kinesin-coated microsphere walking along a single microtubule filament.



6 Stepping of a single bead-bound kinesin against a static trap. **A** Force vs. Time trace of the motor showing a stalling force at around 5pN. **B** Forward and backward 8-nm steps can be observed. Steps were found with an automatic step finder (red line, Jachowski, T.J. (2019). Stepfinder: A Python package to find steps in one dimensional data with low SNR. GitHub repository: **https://github.com/tobiasjj/stepfinder**). This graph shows the stalling force region of the curve on A. Trap stiffness was 0.069 pN/nm.



7 2D force clamp experiment following the motion of the motor. **A** During the clamping, the trap pulls on the kinesin motor with a backward load of 2.4 pN. The motor walks at a speed of 60 nm/s by resolvable 8-nm steps. Beads were 520 nm in diameter and the trap stiffness was 0.053 pN/nm. **B** Histogram showing the step size found with a step finder algorithm, revealing 8-nm (and 16-nm) steps. N=285, (mean ± SEM) = 8.4 ± 0.4 nm. Gaussian center: 8.84 ± 0.08 and 16.2 ±0.4. Steps were found with a Python package to find steps in one dimensional data with low SNR (red line). GitHub repository: **https://github.com/tobiasjj/stepfinder**

Furthermore, to study the force that individual motors can exert, the C-Trap enables 2D force feedback experiments by following the motion of the motor with high spatiotemporal resolution along any axes. In this example, the motor was walking against a constant, backward force of ~2pN applied by the trap and its motion followed (**Figure 7a**). This approach reveals how the motor walks with 8-nm steps (**Figure 7b**). The data also provides information on the number and duration of pauses or the instantaneous speed at which the motor walks. The force clamp can follow kinesin speeds of 200 nm/s and simultaneously resolve 8-nm steps.

Summary, conclusions, and references

Summary

Most research on cytoskeletal motors depend on passive observation of the motors' translocation. A complete understanding, however, relies on directly measuring and probing the force generation and step sizes of these motor proteins. Here, we showed how the C-Trap can be used to drive a mechanistic understanding of cytoskeletal motor proteins by:

- Direct observation of translocation for long periods.
- Unparalleled measurement of molecular forces and step sizes.
- Measuring motor translocation at constant forward or backward loads.

Conclusions

The C-Trap Edge is a revolutionary instrument that enables mechanistic understanding of cytoskeletal processes in previously unattainable ways. Here, the C-Trap's powerful capabilities were used to characterize the activity of individual kinesin motors. Notably, this technology can be applied to virtually any molecular motor, including dyneins and myosins, or cytoskeletal system. This approach will allow researchers to further understand the behavior of these motors whose malfunctions are linked to diseases such as multiple sclerosis, Charcot-Marie-Tooth syndrome or hearing loss/deafness, among others [**6,7**].

While this technology was previously reserved for a handful of the world's most specialized biophysics labs capable of building their own instrumentation, it can now be leveraged and operated by any researcher in the world providing a complete workflow and correlated data collection. Furthermore, it seamlessly integrates highly dynamic, yet sensitive, biophysical measurements with cutting-edge microscopy, enabling you to feel and visualize the activity of motor proteins. All these characteristics, make the C-Trap® Edge a powerful tool to complement and expand your current toolkit and capacity for high-impact scientific discoveries.

Recent C-Trap papers

Budaitis BG, Jariwala S, Rao L, Yue Y, Sept D, Verhey KJ, Gennerich A. **Pathogenic Mutations in the Kinesin-3 Motor KIF1A Diminish Force Generation and Movement Through Allosteric Mechanisms.** Journal of Cell Biology 2021

Lam AJ, Rao L, Anazawa Y, Okada K, Chiba K, Dacy M, Niwa S, Gennerich A, Nowakowski DW, McKenney RJ. **A Highly Conserved 310-Helix Within the Kinesin Motor Domain is Critical for Kinesin Function and Human Health.** Science Advances 2021

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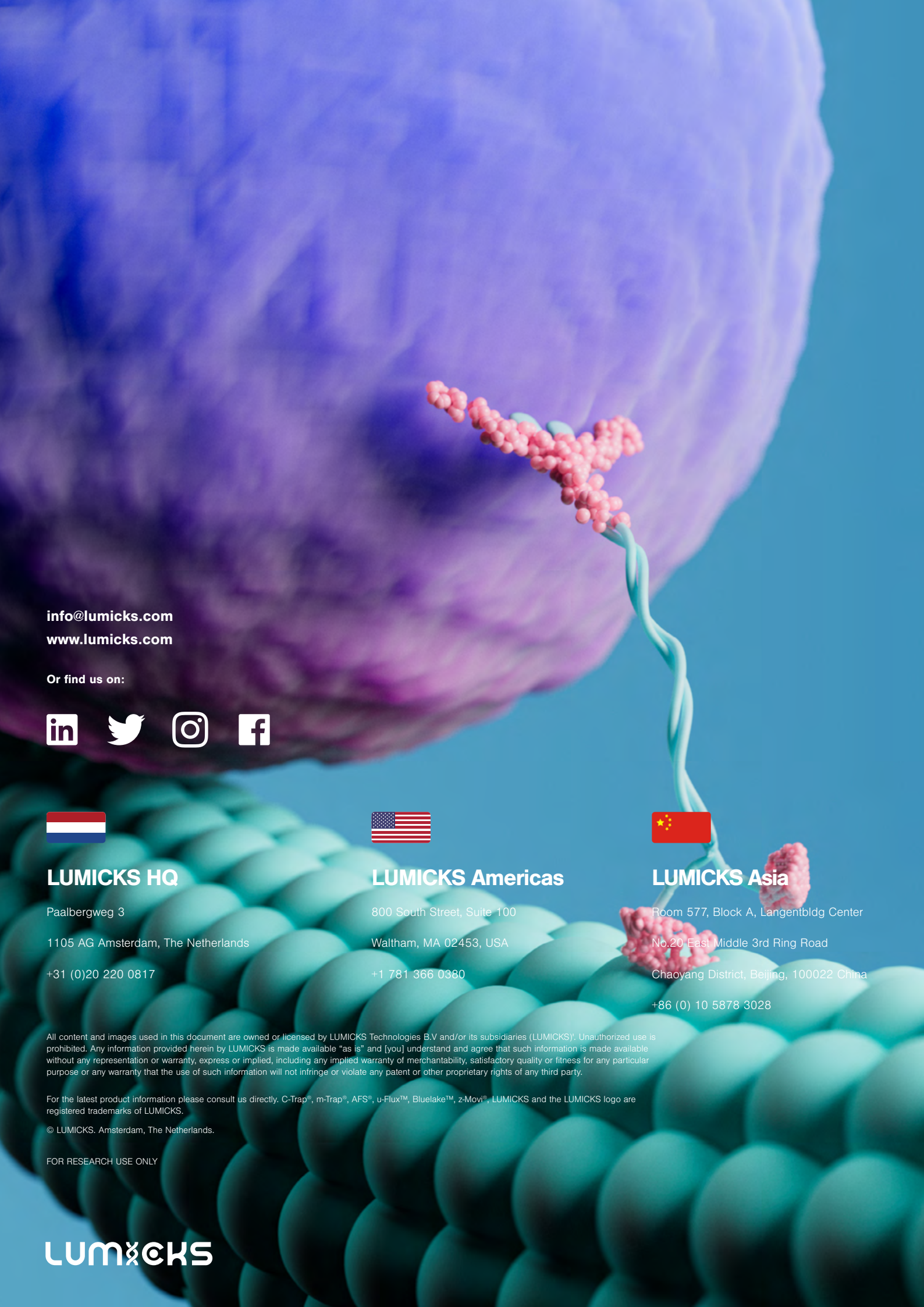

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