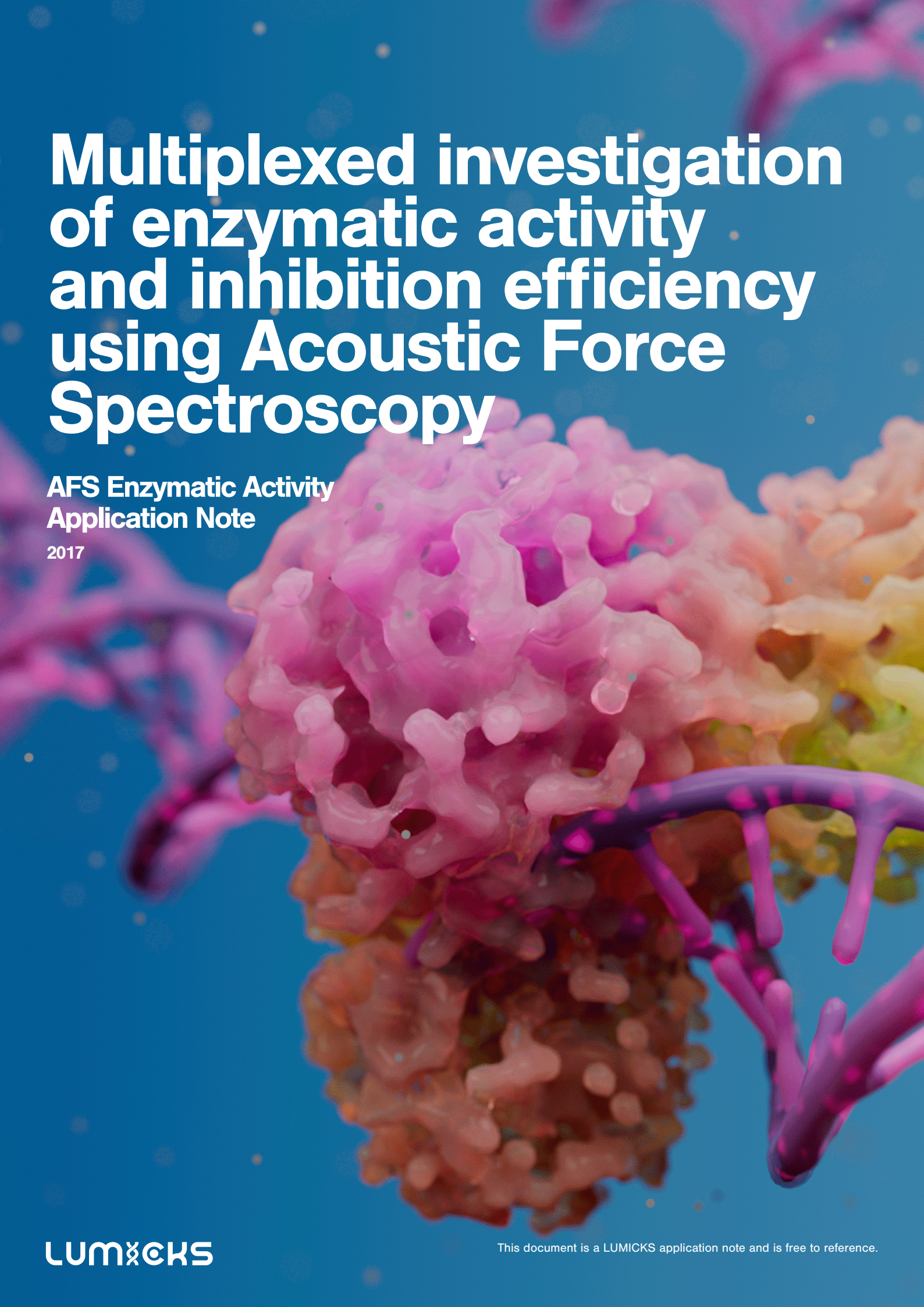
Multiplexed investigation of enzymatic activity and inhibition efficiency using Acoustic Force Spectroscopy

AFS Enzymatic Activity Application Note

2017



LUMICKS

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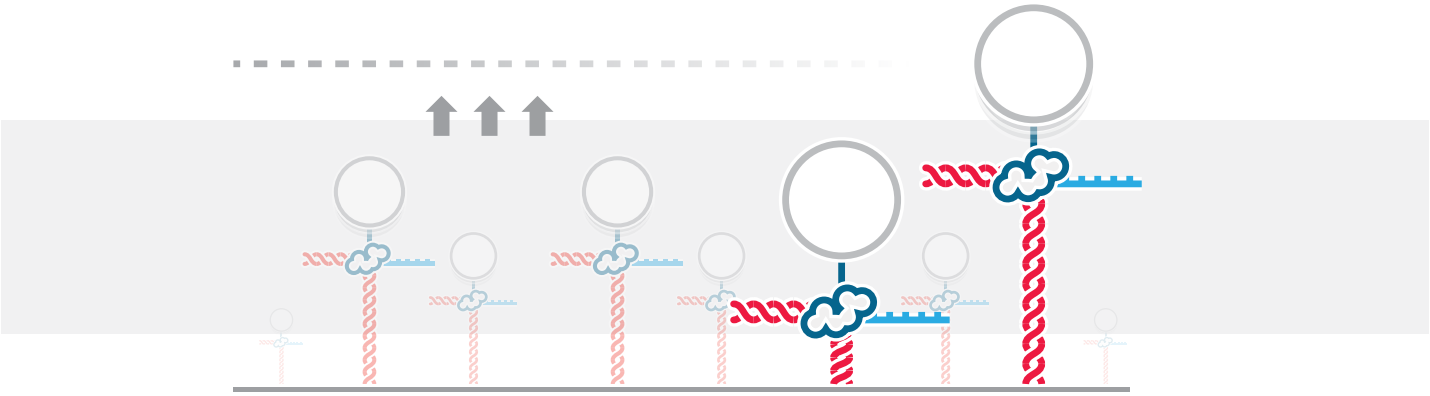


1 A schematic representation of an experiment using AFS. Multiple beads are tethered to a glass surface via a DNA-RNAP complex. By stretching the DNA-protein complex at a constant force while measuring the position of the bead, it is possible to measure the activity of the RNA polymerase, as the length of the DNA between the surface and the protein changes.

Study Enzymatic Activity using Acoustics

The characterization of the enzymatic activity of proteins involved in the DNA machinery and the identification of their inhibitors are key for the development of a variety of treatments. Understanding the mechanisms underlying these processes at the molecular scale and distinguishing rare events from intrinsic stochasticity is vital for creating an efficient and a holistic method for novel drug design.

Using acoustic force spectroscopy to manipulate and track the activity of enzymes on hundreds of individual biomolecules in parallel represents an ideal approach for obtaining high data throughput, while still detecting and quantifying unique-occuring behavior at the single molecule level.

An observation of these states allows the discovery of compounds capable of inhibiting these rare and transient steps. The benefit of this is twofold:

1. inhibitors can be found that target key phases in biochemical processes, e.g., transcription initiation; and
2. the total number of drug targets increases since biochemical processes consist of many transient steps, e.g., transcription promoter binding, initiation, elongation, and termination.

In this application note we will discuss how LUMICKS' Acoustic Force Spectroscopy (AFS™) technology can be employed for the study of DNA transcription and the detection of novel transcription elongation inhibitors.

High-throughput *in singulo* characterization of enzymatic activity.

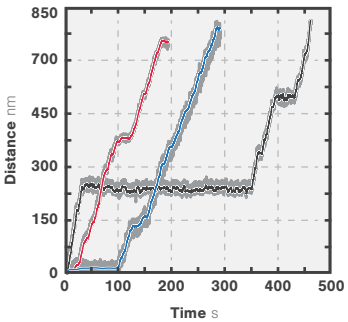
When investigating the enzymatic activity of proteins it is essential to collect statistically-relevant data. This is typically achieved by using enzymatic assays designed for high-throughput screening applications.

However, while these assays provide valuable insights into the bulk characteristics of enzymes, they suffer from the limitation that the unique behavior of each molecule is averaged out. Often, the root cause of a disease can be traced back to a single step in a biomolecular process that was carried out abnormally. The AFS provides you with the unique ability to measure the activity of hundreds of enzymes in parallel, while retaining valuable information, such as the **processivity**, **rate** and **pausing steps** of each individual enzyme.

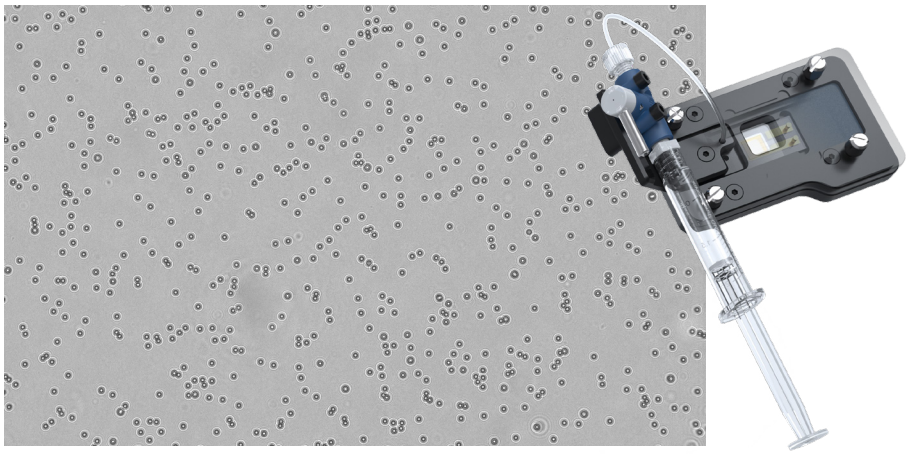
Figure 2 shows measurement data from an experiment involving three DNA molecules with stalled E. coli RNA polymerase (RNAP) complexes. By stretching the DNA-RNAP complex with a constant force while measuring the position of the bead over time, we can precisely monitor the activity of many individual RNAP molecules as they transcribe each DNA scaffold in real time (**Figure 1**). The graph shows the typical complex nature of the protein activity: stochastically-occurring elongation is frequently interrupted by pausing events of different nature.

In order to effectively track the behavior of each RNAP, these types of measurements require to maintain a constant force over a long period of time with high stability. The AFS enables you to determine the enzymatic activity and capture all mechanistic properties of each molecule with accuracy below 10 nanometers and provides hours-long static force stability.

In this proof-of-concept experiment, Anatoly Arseniev et al. carried out 3 independent RNAP activity measurements simultaneously. Measurements such as the one presented here can be done with up to **hundreds of single-molecules** in parallel. **Figure 3** shows a typical AFS field of view, displaying the



2 Activity bursts of multiple RNA polymerase proteins.



3 Typical field of view (FOV) displaying the simultaneously imaging of 4.5 µm polystyrene beads that are tethered to the surface with single protein-DNA molecules. The image is recorded with a 20x magnification objective. The smart flow cells contain onboard electronics to interface with the AFS, and provide basic onboard fluidics for quick and stable flow, preventing shockwaves, air bubbles and other annoyances while enabling rapid buffer exchange. The flow cells can be securely inserted and removed with ease.

simultaneous imaging of 4.5 µm polystyrene beads that are tethered to the surface of a flow cell using single DNA-protein complexes. In a common field of view, hundreds of microspheres can be tracked in real-time and manipulated in parallel resulting to the collection of a large dataset that provides insights into the stochastic and heterogeneous behavior of individual biomolecules.

Identification of novel inhibitors and determination of inhibition efficiency.

Next, the effect of two novel peptides, acinetodin and klebsidin, was investigated with respect to transcription elongation generated by RNA polymerase[1]. The configuration of this experiment consisted of a single DNA tether attached at one end to the surface of the AFS chip and at the other to a polystyrene bead through RNAP stalled on the DNA template. Constant force was applied to detect the activity of the RNAP in the **presence of different known and unknown inhibitor molecules**. The detection of transcription elongation was determined by the presence of varying concentrations of acinetodin, klebsidin and microcin J25; the last peptide being a known transcription inhibitor.

The kinetic analysis shows that — just as microsin J25 — both acinetodin and klebsidin inhibit transcript elongation by E. coli RNA polymerase, with the inhibitor activity of klebsidin being comparable to that of microcin J25 and more active than the activity of acinetodin (**Figure 4**).

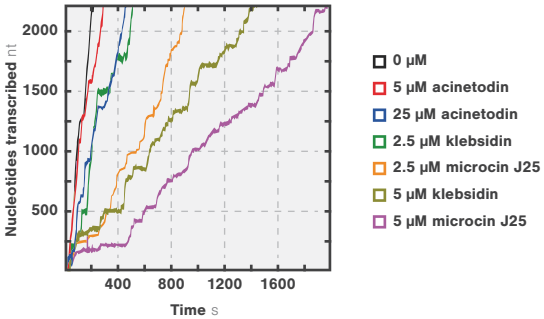
By utilizing the rapid buffer exchange feature of the AFS built-in fluidics system, the tethered DNA-protein complexes can be promptly tested in the presence of different small molecules of varying concentrations or in different buffers. The unique benefit of this feature is that it allows one to easily measure the same molecule under different conditions, thus achieving high experimental reliability by eliminating intermolecular variations.

Furthermore, by exchanging the loading buffer (in which the enzyme can be loaded to the template while being inactive) to a buffer that contains chemicals which activate the enzyme, such as nucleotides or ATP, the rapid buffer exchange allows to precisely control the starting point of the enzyme's activity. In contrast to many bulk assays where the reaction has already started when the measurement is performed, this feature allows one to conduct experiments with the highest reliability.

This application note demonstrates how the AFS technology can be utilized to study the enzymatic activity of RNAP, a DNA processing enzyme, and characterize the RNAP

inhibition efficacy of two newly-characterized lasso peptides.

The same experiments can be done with many single-molecules in parallel allowing you to collect properties such as the rate and pausing events of each RNAP enzyme *in singulo* and map their collective distribution in histograms to identify the processivity properties of RNAP in the presence of different molecules. Future studies in this direction could relate structural information with specific inhibitor binding by repeating the same experiment using different RNAP mutants.



4 Inhibition of RNAP in a single-molecule AFS experiment. Representative elongation profiles for individual RNAP's for various concentrations of microcin J25, klebsidin, and acinetodin and in the absence of inhibitors plotted as nucleotides transcribed over time. Data were filtered by a 0.5 Hz low pass filter. Reprinted with permission from ACS Chem. Bio., 2017, 12 (3), pp 814-824. Copyright 2018 American Chemical Society.

1 Metelev *et. al*. ACS Chemical Biology (2017)

Data courtesy of Anatoly Arseniev, Georgii Pobegalov & Mikhail Khodorkovskii at Peter the Great St. Petersburg Polytechnic University, Russia.

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