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Ingenuity + Instrumentation: Creating A Novel 4D Microscopy Method

Source: Mad City Labs, Inc.

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Particle tracking is a fundamental technique applicable to a range of problems, including understanding viral infections and fluid flow. Tracking particles at the micro (10-6m) or nanoscale (10-9m) is inherently difficult due to the complexity of the systems studied, as well as the technical challenges: a blend of instrument design, photonics, modeling, and imaging.

A research group at Duke University, seeking to understand how viruses navigate the extracellular space, recently developed a real-time 4D microscopy method for single particle tracking when previous techniques proved incapable of supporting the observations they required. The group was led by Kevin Welsher, an Assistant Professor of Chemistry, and Courtney Johnson, then a Ph.D. student and Research Assistant at Duke, and currently a Postdoctoral Associate at Howard Hughes Medical Institute's (HHMI) Janelia Research Campus. Here, we examine the challenges they faced, how their method was implemented, and the instrumentation that makes it possible.

Particle Tracking Challenges

The key challenge of particle tracking is relatively simple to sum up: you are trying to observe something very small, moving fast, usually among other objects. These circumstances make it difficult to have any certainty even when observations can be achieved.

In terms of technical challenges, particle tracking can be likened to Eadweard Muybridge's 1878 feat of using a camera to capture a horse's gallop at every stage — providing undisputable evidence to settle a debate that had relied purely upon speculation to that point. Muybridge's achievement was driven by his ability to reduce the exposure time of his images from about two seconds to one-thousandth of a second.

In particle tracking, too, if a researcher is limited by the exposure time of a camera, things that move fast appear as a blur and individual motions cannot be identified. But, for Duke University researchers, overcoming this challenge was not a matter of increasing frame rate. It was a matter of circumventing frame rate to obviate the need for long exposure times. They sought to escape the constant battle caused by reliance on fluorescence. Specifically, the target must be labeled so it can be tracked, but because fluorescence provides a finite amount of photons, observation is a race against time before bleaching occurs. Thus, a sensitive method is required to track the movement of those particles in the extracellular space — in this case, the Duke team's approach is to use real-time, single-molecule, active feedback tracking microscopy.

Innovation and Thinking Inside the Box(es)

A whole suite of imaging approaches exists to examine the viral infection process, starting when the virus already is bound to the tissue. However, the Duke team wanted to explore the process at an earlier stage, understanding how viruses navigate the epithelial space, through mucus and the periciliary layer. Researchers set out to build a microscope capable of observing that journey, not directly in the lungs, but in a tissue culture model that closely replicates the lungs.

Other particle tracking methods — specifically, 2D and 3D particle tracking velocimetry (PTV) — were inadequate for this purpose not only because of the limits imposed by camera exposure time, but also the speed at which such images can be produced (i.e., because many images are required to generate even a single 3D image). The interlinked nature of these factors — field of view, volume size, and temporal resolution — dramatically limits the ability to capture a moving object in 3D.

A tight depth of field is required when tracking a single particle in 3D. While tissue is intrinsically three-dimensional — layers of cells that must be individually imaged to produce an accurate picture of the environment — only one plane is in focus at any given time. During 3D viewing, a tight depth of focus allows the observer to understand each particle's depth within a 3D volume. Each image is

subject to these limiting factors, rendering the tracking of fast objects in 3D exponentially more difficult. The Duke team's microscopy method decouples the observation from those limiting factors, enabling the monitoring of fast processes (e.g., extracellular diffusion) in three dimensions.

The resulting instrument comprises two key elements. The first, a microscope referred to as 3D-SMART, is designed to perform active feedback tracking. It utilizes optics that rapidly drive a laser spot, in three dimensions, to "dance" around the target molecule, causing it to emit photons. The instrument collects those photons at high speed, using their arrival times to determine the exact location of the fast-moving molecule. Used alone, this first element allows researchers to track viruses at high speed but provides no context. For example, particles can be observed bouncing around, but it could not be determined whether that was caused by interactions with other objects.

The instrument's second element is a volumetric imaging scope — enabling a technique called 3D-FASTR — built around the 3D-SMART microscope. 3D-FASTR is fundamentally similar to 3D-SMART, scanning a laser in three dimensions. However, the scan is executed over a much larger range and its findings are used not to make position estimates, but to build an image (Fig. 1).

At the center of the combined instrument is a Mad City Labs nanopositioning stage, which enables the high-speed tracking. Specifically, photons emitted by the virus allow observers to estimate the position of a single particle within a small region. Then, the stage permits researchers to move the entire sample to "lock" the particle in place for observation. Thus, as the virus particle quickly moves around, the nanopositioner uses active feedback from the tracking to reposition the sample in real time — keeping the molecule centered in the observer's field of view/lab frame.

> **"Mad City has a can-do attitude: What do you want to do? How can we help you? And for a scientist, that really feels good; you want to work with people like that. Every time we had a question, were worried we broke something – or we did break something – they have been really fast and eager to help us get back up and running."**
>
> Kevin Welsher,
> Duke University researcher

> **"I had a specific experience where we were seeing something and we were unsure whether or not it was a feature of the stage. I am a chemist by training, not an engineer, so I didn't come in with any knowledge of piezoelectric stages and how they work. Not only was [Mad City Labs] responsive, they were able to break it down in a way that took something very technical and made it very intuitive."**
>
> Courtney Johnson,
> Duke University researcher

Notably, software plays as critical a role in the system as the hardware. Since the tracking is done in real time, using a feedback algorithm, all system actions must be driven by the software. The stage controller's analog inputs and outputs connect directly into this feedback loop, wherein the software translates position data from the stage and returns commands based on the tracked particle's location. Duke researchers wrote the software code in-house (using a field programmable gate array [FPGA]) to seamlessly enable a feedback loop that includes the piezo stage, microscope, photon-counting detector, and the laser.

The result is comparable to a video game camera's point of view: the protagonist is centered on the screen all the time while the environment moves around them. 3D-SMART is the mechanism by which the camera remains centered on the target molecule (the protagonist); 3D-FASTR imaging provides data about everything occurring around the protagonist.

Combining Technical Acumen with Passion for the Science

Despite constructing a novel setup, the Duke University researchers did not need to order any custom components to build their apparatus. This is because Mad City Labs designs its products with an eye toward people who will use them in different ways.

For example, Mad City Labs' nanopositioning stages feature an embedded, closed-loop feedback controller that ensures both precise movement of the stage and the ability to return exactly to its original position if the user desires. However, the Duke researchers needed to lock onto a particle, rather than the stage's position, and they were able to take advantage of a switch on the Mad City Labs' stage allowing them to disable closed-loop control and operate in an open-loop system (as well as reengage closed-loop operation, if necessary).

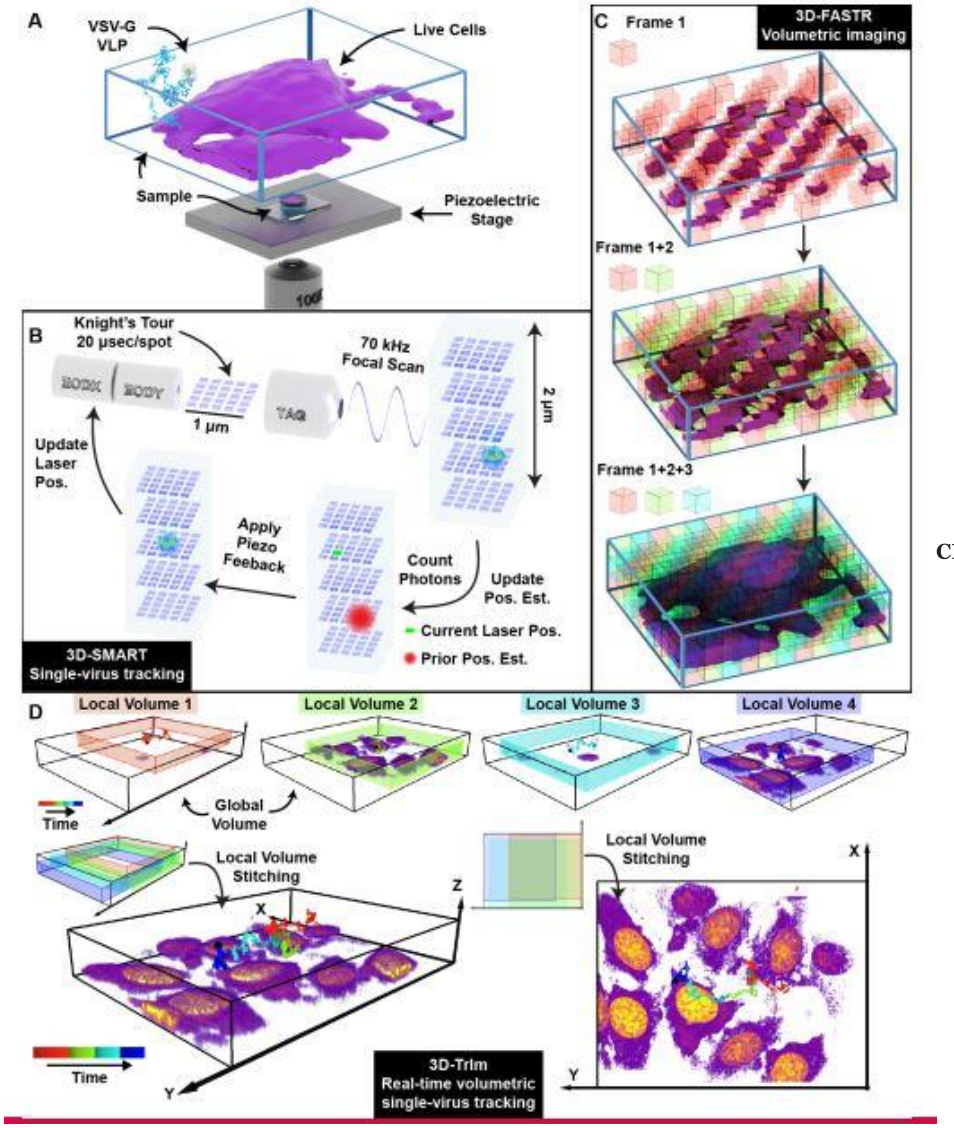

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Fig 1: 3D Tracking and Imaging (3D-TrIm) — (A) Experimental setup. Fluorescently labeled VLPs are added to live cells plated on a coverslip. The sample is placed on a heated sample holder mounted on a piezoelectric stage. (B) Overview of 3D-SMART tracking of single viruses. The electro-optic deflector (EOD) and tunable acoustic gradient (TAG) lens rapidly scan the local particle area. Photon arrival times and the current laser position are used to calculate the position of the virus within the scan area. Using the measured position, the piezoelectric stage moves to recenter the virus within the scan area. (C) Concept of 3D-FASTR volumetric imaging. By outfitting a traditional two-photon LSM with an ETL, a repeatable, tesselated 3D sampling pattern can be generated during each frame-time. Over a set number of frame-times, the entire volume is sampled. (D) Construction of global volumes in 3D-TrIm. As the virus diffuses, 3D-SMART moves the sample and the 3D-FASTR imaging system collects sequential volumes from different areas around the particle. These time-resolved local volumes can be used to generate an integrated global volume."

Additionally, Mad City Labs' open-platform microscopy stand makes it easy for users to add components. Again, this feature is designed to accommodate researchers developing their own unique instrumentation setups, users who do not simply build a microscope and point it at something, but need an open structure to integrate their own microscopy ideas and techniques.

Finally, the Duke researchers' work was aided by Mad City Labs' sales and technical support staff — who are one and the same. Working with the same individual from purchasing through building their instrument and conducting their experiments, the Duke team benefited from easy communication with someone who shared equal enthusiasm for their work.

Final Thoughts

The Duke University team's particle-tracking triumph serves as a testament to their ingenuity, as well as an example of the importance of designing and choosing the correct instrumentation to solve challenging problems. Collaboration across scientific disciplines, as well as between vendors and their customers, is vital to answering the innumerable questions remaining about our universe and its functions at the smallest scale. To learn more, contact the contributors and visit madcitylabs.com.

About The Contributors

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About Mad City Labs

Mad City Labs designs and manufactures a complete product line of high-precision piezo nanopositioners, micropositioners, atomic force microscopes, and single-molecule microscopes. We provide innovative instrument solutions from the micro- to pico-scale for leading industrial partners and academic researchers. Visit www.madcitylabs.com or email mclgen@madcitylabs.com for more information.