Preprints of the 21st IFAC World Congress (Virtual)
Berlin, Germany, July 12-17, 2020

Feedforward Control for Single Particle Tracking Synthetic Motion

Nicholas A. Vickers * **Sean B. Andersson** *,**

* *Department of Mechanical Engineering*
** *Division of Systems Engineering*
Boston University, Boston, MA 02155 USA
e-mail: nvickers, sbanderss@bu.edu

Abstract: Single particle tracking (SPT) is a method to study the transport of biomolecules with nanometer resolution. Unfortunately, recent reports show that systematic errors in position localization and uncertainty in model parameter estimates limits the utility of these techniques in studying biological processes. There is a need for an experimental method with a known ground-truth that tests the total SPT system (sample, microscope, algorithm) on both localization and estimation of model parameters. Synthetic motion is a known ground-truth method that moves a particle along a trajectory. This trajectory is a realization of a Markovian stochastic process that represents models of biomolecular transport. Here we describe a platform for creating synthetic motion using common equipment and well-known, simple methods that can be easily adopted by the biophysics community. In this paper we describe the synthetic motion system and calibration to achieve nanometer accuracy and precision. Steady state input-output characteristics are analyzed with both line scans and grid scans. The resulting relationship is described by an affine transformation, which is inverted and used as a prefilter. Model inverse feed forward control is used to increase the system bandwidth. The system model was identified from frequency response function measurements using an integrated stepped-sine with coherent demodulation built into the FPGA controller. Zero magnitude error tracking controller method was used to invert non-minimum phase zeros to achieve a stable discrete time feed forward filter.

Keywords: Non-minimum phase systems, Model Approximation, Inverse transfer function, Feedforward control, Brownian motion

1. INTRODUCTION

Single particle tracking (SPT) combines optics, controls, signal processing, and estimation to measure the dynamic processes of subcellular transport with unrivaled precision and accuracy (Levi and Gratton, 2007). It is now commonplace to track biological molecules and structures inside cells and tissues with nanometer accuracy and tens to hundreds of millisecond temporal resolution. SPT has enabled breakthrough discoveries in biology and biophysics including understanding viral infection pathways (Brandenburg and Zhuang, 2007), drug delivery (Schuster et al., 2015), and dynein motor motion (Reck-Peterson et al., 2006). However successful the application of SPT has been in understanding subcellular biophysics, there are still many challenges in the development of SPT microscopes and algorithms that need to be overcome to continue advances in this arena. In this work, we focus on one such challenge: the need for methods that allow biophysicists to characterize and test their microscope systems to validate their experimental findings. Different methods have been developed to address this gap for particle localization (Thevathasan et al., 2019; von Diezmann et al., 2015), however, there is currently no known ground-truth method for testing the estimation of motion model parameters used in SPT experiments in the context of the total system (sample, microscope, and algorithm).

At a high level, the goal of an SPT experiment is to localize an individual particle (such as virions, protein complexes, lipid vesicles, and other biomolecules) over time and understand the dynamics of its motion. As these particles, with sizes on the order of 1-100 nm, are smaller than the diffraction limit of optical wavelengths they cannot be viewed using standard microscopy. The first step in the experiment is to label the target particles, either with a scattering label such as gold nanoparticles, or with a fluorescent label such as quantum dots or fluorescent protein (Rodriguez et al., 2017). Once labeled, the sample is placed under the SPT microscope and observed, generating a time series of observations, typically using either a widefield modality (in which case the data takes the form of a sequence of camera images) or a confocal modality (in which case data takes the form of a time series of intensity measurements and the spatial locations of those measurements). The resulting data sets are then analyzed with an algorithm which estimates the particles positions over time and its motion model parameters. Thousands of particles may be observed in a single experiment uncovering particle dynamics, distributions and transport mechanisms.

Each class of microscope has its benefits and draw backs. Widefield imaging is the more common approach. It can

⋆ This work was supported in part by NIH through grant NIGMS 5R01GM117039-02.

measure multiple particles in the same field of view, though at the cost of lower acquisition rates, and can give the cellular context in which the particle is being studied. The series of camera images is analyzed to identify particles in the image (segmentation), localize particles in each frame (localization), and connect those locations into trajectories (linking). These trajectories are then analyzed to infer parameters of the motion model describing the dynamics. These steps may occur sequentially (e.g. applying a Gaussian fit (Thompson et al., 2002) followed by a mean square displacement analysis (Saxton and Jacobson, 1997)) or simultaneously (e.g. through applying nonlinear filtering and maximum likelihood estimation (Ashley and Andersson, 2015)). The resolution of these approaches is ultimately limited by the details of the point spread function (PSF) of the instrument and the number of collected photons (Chao et al., 2016). Because of the wealth of problems that can be addressed using SPT methods, much attention has been given to algorithm development and software implementation. Comparisons between developed algorithms (Cheezum et al. (2001), Chenouard et al. (2014)) and software packages (Holden and Sage (2016), Sage et al. (2019)), using simulated data as ground-truth, show that many simplistic algorithms do not perform well. Even of the best performing algorithms, there is no universally best technique, with some algorithms performing well for only a small range of operating conditions and all of them having their own pros and cons. It is then important to understand the applicability of a particular algorithm to specific experimental conditions. Furthermore, it is important for a biophysicist to understand the applicability of the total system to a particular set of experimental conditions.

Although widefield microscopes are more commonly used in SPT experiments, laser scanning confocal microscopes have the advantage of higher acquisition rates. Common frame rates for widefield SPT are 1-10 Hz, slow enough to yield artefacts such as motion blur and the loss of ability to accurately capture fast or transient processes. In contrast, confocal tracking microscopes can track particles with time resolution on the order of 1 kHz. These microscopes focus a laser into the sample and use the intensity measurements in real time to track the particle. Typical scan patterns are orbital in nature where the particle is located inside the circumference of the scan and a feedback controller applied to keep the particle centered in the scan (Lanzanò and Gratton, 2014). An alternative approach uses extremum seeking, moving the confocal measurement volume towards the peak intensity found at the particle location (Ashley et al., 2016). Unfortunately, there has not been a systematic comparison for confocal tracking microscopes, nor a comparison between confocal tracking and widefield techniques.

SPT development is not only limited to algorithms, but also includes the microscopes used in data collection. Recently the boundaries of SPT measurement capabilities have pushed past the traditional constraints of 2D imaging and moved into 3D super resolution microscopy (von Diezmann et al., 2017) allowing for better quality measurements of biological processes throughout the volume of the cell. At the heart of these technologies are engineered PSF microscopes which allow for the simultaneous measurement of both the lateral position (x,y) and the axial position (z) of a labeled particle. Unfortunately, it has been recently reported that this class of microscope have errors on the order of 100 nm in both lateral and axial localization (Li et al. (2019), Rehman et al. (2018), McGorty et al. (2014), Cabriel et al. (2018)). This necessitates the need of a sample dependent calibration that can be error prone and can introduce unwanted complexity into the experimental procedure. Additionally, there is no way to guarantee that this calibration method will give good results due to the lack of an experimental known ground-truth test. It is clear that the biophysics community needs a known ground-truth method to test the total SPT microscope system. While this community is by its nature interdisciplinary, it is not common to find a deep knowledge of systems and control theory as their expertise often lies in the field of molecular biology. As a result, any system must be reasonably straightforward to implement.

One potential approach for testing the entire system is the use of synthetic motion (described in section 2). This can provide repeatable ground-truth motion of the sample paths of stochastic processes typically encountered in SPT experiments. Previous work has showed through simulations that band limited synthetic Brownian motion was accurate in reproducing both position and diffusion coefficient for a range of sampling rates and system time constants (Vickers and Andersson, 2019). Additionally, the diffusion coefficient was accurate in the limit as the sampling rate increased well beyond the bandwidth of the system, establishing the potential utility of this scheme for adoption in a biophysics lab with moderately performing equipment. In this paper we establish synthetic motion as a basis for testing SPT microscope systems through describing the creation of a synthetic motion platform. Here we focus particularly on creating a system that can be easily adopted by the biophysics community, that is, by a user who is typically not trained in advanced control systems, but is familiar with topics of classical, single-input, single-output control.

2. SPT SYSTEM TESTING AND SYNTHETIC MOTION

While SPT is an extremely common technique in the study of biophysics, there is no formalized procedure to test algorithms on the specific systems of individual researchers. The most commonly used technique is to carefully prepare a solution of fluorescent particles such as microspheres or quantum dots into a viscous fluid. By choosing a particular diameter of the particles and viscosity of the fluid, one can create a system with a specific diffusion constant that can be calculated using the Stokes-Einstein equation. This value can then be compared to the experimental results. In practice, however, the "known" value of the diffusion coefficient is typically only good to an order of magnitude precision due to uncertainty in the particle size, particle shape, and local viscosity of the fluid. Additionally, it only allows for calibration on Brownian motion and excludes testing other stochastic models relevant in biophysics. Furthermore, the particle trajectories are not known, making any characterization of accuracy and precision impossible. There has been work on developing methods which can provide a control independent of the biological experiment to verify findings.

In these methods, engineered particles (Saxton, 2014), and engineered substrates (Saxton, 2012) establish the basis for controlling the motion model and its parameters. Unfortunately, these methods do not provide a ground truth, nor are they immune to experimental uncertainties and noise. There is still a need for a repeatable, known ground-truth method of testing the complete SPT system.

Synthetic motion, first described by Michael Saxton (Saxton, 2012), is a method of generating repeatable particle motion with a known ground-truth as a basis for testing SPT microscopes. The particle, typically a fluorescent or scattering particle, is fixed to a microscope slide. The slide is then moved by the microscope stage along a specified trajectory that is a realization of a stochastic process that are representative of biomolecular transport. Many realizations can be generated and saved to create a dictionary for repeated use in experiments across different microscopes and across time on the same microscope. To achieve the nanometer position accuracy and precision needed for state-of-the-art SPT algorithms, piezo-actuated microscope stages are used. These are common in many biophysics labs. However, to achieve the high resolution and repeatability needed to use them to assess the accuracy of an instrument and an algorithm, it is vital to understand the complexities of the system, from the impact of noise to limitations arising from system nonlinearities.

3. SYSTEM DESCRIPTION

As illustrated in Fig. 1, our synthetic motion platform consists of four distinct parts. First a computer creates realizations of stochastic motion models and coordinates the microscope, microscope stage, image acquisition, and data analysis, all integrated with a graphical user interface for ease of use. A National Instruments cRIO FPGA controller handles control signal generation, signal processing and data acquisition. The control signals feed into the amplifier/controller of a Mad City Labs piezo actuated microscope stage. This amplifier has the option to use a factory set closed-loop mode or to run the piezo stage in open loop. Designing feedback controllers for these piezo actuators can be complex due to the highly resonant character of the system as well as potential nonlinear behavior. We envision biophysicists would implement synthetic motion using the built-in closed-loop feedback controller. Finally the microscope used for observations is a Zeiss Axiovert 200 inverted microscope set up for both confocal and widefield imaging of fluorescently tagged particles, and phase contrast microscopy for imaging the cellular context.

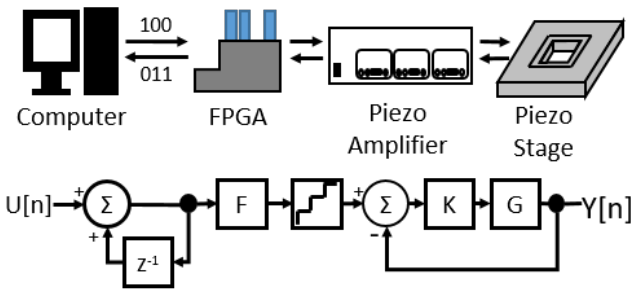


Fig. 1. System illustration showing the four main components and the resulting block diagram.

There are two distinct modes of operation for synthetic motion, each illustrated in Fig. 2. In the first, called 'single position per image', the illumination and camera image acquisition are triggered after the piezo stage has settled on the next position in the trajectory. While image acquisition rate in this scheme is limited by the bandwidth of the stage, no error exists between the trajectory and the stage position (up to the accuracy and precision of the stage), allowing for the realization of any Markovian stochastic process sampled at a rate equal to the image acquisition rate. Imaging rates of SPT experiments are typically between 1-10 Hz, matching well with bandwidths of many commercially available piezoelectric stages. In the second method, termed 'multiple positions per image', the stage moves to multiple positions per acquisition for a closer approximation to a continuous time stochastic process, allowing us to capture the effect of motion blur (where the particle moves appreciably during the image capture). The difficulty of this mode is that the bandwidth of the stage may accumulate error causing the stage's trajectory to diverge from the intended trajectory, leading to effective parameters for the underlying stochastic process that differ from those defined by the original trajectory (Vickers and Andersson, 2019). This can be mitigated by ensuring the bandwidth of the controlled system is as high as possible. In this work we do this through the addition of feedforward control to increase system bandwidth (described in Sec. 5).

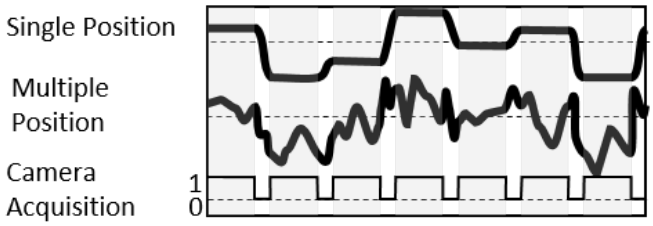
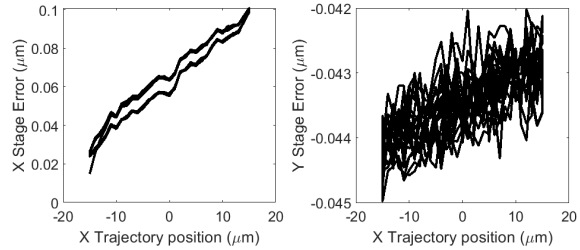


Fig. 2. Illustration of the two modes of operation of a synthetic motion platform. Top shows single position per acquisition. Bottom shows multiple positions per acquisition enabling the system to approximate more closely continuous time stochastic processes.

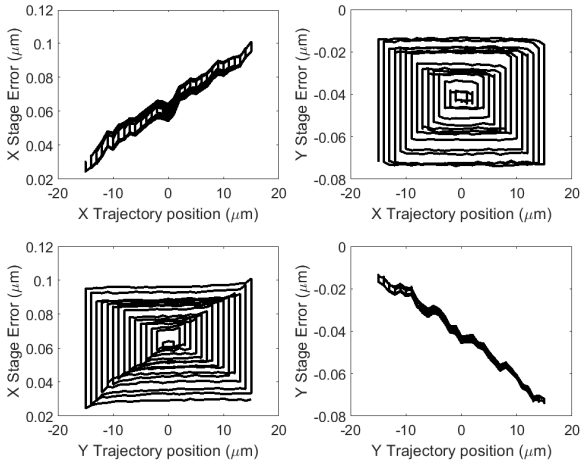
Piezo actuated nano-positioning microscope stages form the core of the synthetic motion platform and are a common and popular tool in the biophysics lab. These stages come in a few different configurations. One common configuration is a direct drive 2-axis or 3-axis stage, allowing for nano-positioning with accuracy and precision of 15 nm or better. Unfortunately, real world actuators all have their limitations. Piezo actuators often exhibit bandwidth-limits with repeated resonant peaks at higher frequencies (Adriaens et al., 2000), non-minimum phase zeros, hysteresis, slew rate limits, saturation, actuator coupling and drift. Often times these issues are addressed with the closed loop controller developed by the equipment manufacturer and provided with the piezo stage amplifier. However, it is very important to characterize your system for both steady state and dynamic performance in order to get the best results and to understand its limitations.

4. STEADY STATE SYSTEM CHARACTERIZATION

Although many piezo systems are well characterized and tuned when initially purchased, system parameters can vary with temperature, loading characteristics, and aging, necessitating re-calibration. Two useful testing methods are line scans and grid scans to assess the steady state input-output characteristics over the desired range of motion. The line scan is a point-to-point trajectory along an axis of motion. This is used to assess line straightness and reproducibility. It can be that the resulting path the stage takes is curved, exhibits hysteresis, or is coupled with other axes of the system. Grid scanning is another point-to-point trajectory that moves to all points in a square or cubic grid. This method uncovers actuator coupling, grid distortion, and offsets in the position input-output relationship. Once the (static) input-output relationship is known it can be inverted and applied as a prefilter.



(a) System error from a repeated line scan



(b) System error from a grid scan using a spiral trajectory

Fig. 3. Experimental results on the steady-state input-output characteristics of a Mad City Labs 3-axis nanopositioning stage.

We applied both line scans and grid scans to understand input-output behavior of the Mad City Labs piezo system in our lab. Fig. 3a shows the results of the line scan exhibiting both position dependent error and hysteresis. Fig. 3b shows the resulting error for a grid scan along a spiral trajectory. The input-output characteristics clearly show a combination of hysteresis and an affine mapping. While inverting hysteresis is certainly possible (see, e.g. (Leang and Devasia, 2007)), the tools for doing so are likely not familiar to a biophysicist. As a result, and since the effect of hysteresis is often minimized by the built in feedback controller, we chose to invert only the affine portion. This results in an augmented transformation matrix which describes the input output characteristics of our system,

$$T = \left[\begin{array}{c|c} GL(2,\mathbb{R}) & \mathbb{R}^2 \\ \hline 0 & 1 \end{array}\right], \tag{1}$$

where $GL(x,\mathbb{R})$ is the generalized linear group of 2×2 invertible matrices over the set of real numbers. A least squares regression was used on the results of the grid scan to get the particular values of T for our specific system given by

$$T = \begin{bmatrix} 1.00221 & 4.32623 \times 10^{-5} & 0.0646743 \\ 3.6661 \times 10^{-5} & 0.997938 & -0.0420928 \\ 0 & 0 & 1 \end{bmatrix} \tag{2}$$

The inverse of this matrix is used as a steady state feedforward prefilter applied to the input trajectory before and model inverse feedforward is applied.

5. SYSTEM IDENTIFICATION AND FEEDFORWARD CONTROLLER DESIGN

The dynamic performance is as important as the steady-state characteristics as the bandwidth of the system will act as a low pass filter on the input trajectory, causing error in the resulting motion (Vickers and Andersson, 2019). As one would expect, a faster actuator results in better trajectory following which is particularly important for the multiple positions per image operation mode of synthetic motion. In the single position per image mode, the motion of the stage occurs in the time period between image acquisitions allowing for a motionless stage during acquisition and thus the speed of trajectory tracking is not relevant. However, the total experiment time depends on the settling time. Since many fluorescent labels used in SPT have very limited lifetimes, it is important to minimize "wasted" time and thus an increased bandwidth is important in the single position per image mode as well.

We expect that most biophysicists would hesitate on designing their own feedback controller and opt to use the closed-loop mode provided by the equipment manufacturer due to concerns about robustness and stability. Feedforward control is a viable option that can be straightforward to design and easily adopted. Of course, feedforward model inverse design require a good model of the system.

Good models of real systems start with high quality measurements of system behavior. This is especially important for high-Q resonant systems such as piezo actuators. The Frequency Response Function (FRF) of our microscope stage was measured using a stepped-sine scheme implemented on the FPGA of the controller for real time magnitude and phase measurements. This was done by generating a sine wave from the FPGA and employing coherent demodulation on the measured signal as shown in Fig. 4 (Abramovitch, 2015). The magnitude and phase was measured at 250 frequencies per decade from 5 Hz to 5 kHz. To avoid errors from transients, the measurement was not started until after the system had settled. Time domain measurements were done at a 100 kHz sampling rate with an adequate number of periods sampled to ensure good signal to noise. The resulting FRF measurements for one axis of our system is shown in Fig. 5. It is important

to note that the quality of FRF measurements really affect the resulting identified model. This is particularly true of the sharp resonant peaks characteristic of piezo actuators and clearly present in the higher frequency band of our system. Careful consideration of this is necessary especially when using a model inverse as a controller.

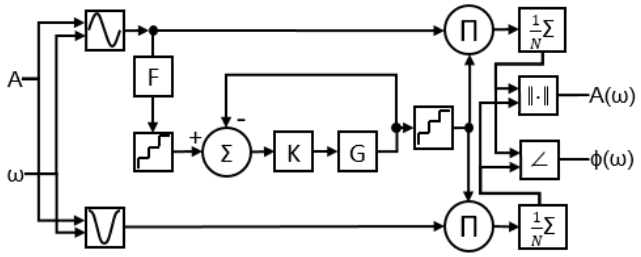


Fig. 4. Block diagram showing the implementation of coherent demodulation for built-in FRF measurements.

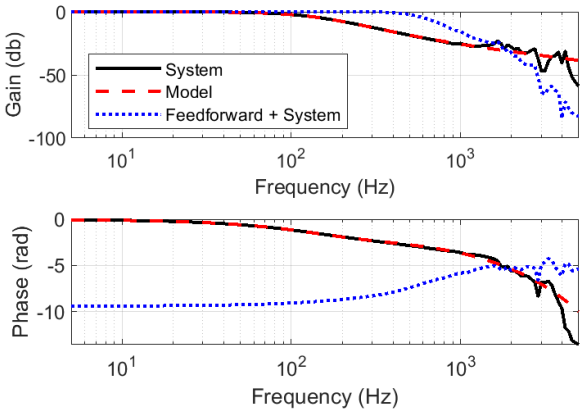


Fig. 5. Bode plots showing both magnitude (top) and phase (bottom) for system FRF measurements, the identified system model, and the resulting system performance with feedforward.

After acquiring the FRF measurements, system identification was done using this data to build a model of the system. We started with a candidate model with the number of poles and zeros as well as the input-output delay selected based on the data and prior experience. A weighting function was used to prioritize the fit to low frequency portion of the FRF measurement and to define the frequency range in which the model was fit. Model parameters were estimated in Matlab (MathWorks) using a subspace Gauss-Newton method with constraints to enforce stability. Models resulting in a low fit quality were modified and re-analyzed to iteratively move to a good model fit. The resulting model consisted of a low pass filter with non-minimum phase (NMP) zeros and alternating double poles and double zeros typical of piezo electric actuators employing high-gain PI feedback control.

The identified model can be inverted (in the appropriate frequency range) to filter the input signal. The identified system was converted from continuous to discrete time and then inverted to find the corresponding discrete time infinite impulse response (IIR) filter. This inversion process was done one actuator at a time, using a zero magnitude error tracking controller (ZMETC) method to ensure that inversion of the NMP zeros did not result in an unstable feed forward controller (Butterworth et al., 2012). The motivation for inverting one actuator at a time (rather than considering a full multiple-input, multiple output system to account for cross-coupling) was two-fold. First, we envision that many biophysicists would be willing to invert a single transfer function but that the higher complexity of a transfer function matrix might pose a significant barrier. Secondly, the results of a full identification for our system showed that the coupling between the axes was low, with a typical peak gain of -28 dB.

Finally a lowpass filter was employed using a Chebyshev type II filter to reduce the impact of unmodeled higher frequency dynamics. This lowpass filter was chosen as a compromise between phase dispersion and high frequency attenuation in order to minimize the settling time of the system. The resulting step response shown in Fig. 6 exhibits a large improvement in both rise time and settling time relative to the manufacturer-provided closed-loop controller, allowing for the system to perform synthetic motion at faster acquisition rates and with less error.

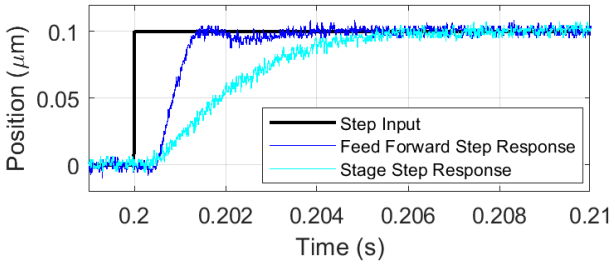


Fig. 6. Step response of our system with (dark blue) and without (cyan) feedforward applied.

6. CONCLUSION

Single particle tracking is an invaluable tool in the study of subcellular biology. There is a need, however, for methods for calibrating and characterizing ones own SPT equipment as well as a basis for developing microscopes and algorithms. Synthetic motion is a ground-truth method that can be used with many stochastic processes which describe biomolecular transport. We have described a method of characterizing and calibrating a system for synthetic motion, focusing on keeping the technique simple enough so that a biophysicist with limited controls experience can replicate and use it. While this comes at a cost of achievable performance, we feel the tradeoff is necessary to encourage adoption. The basic approach is to: 1) characterize the steady state input-output relationship and invert it. In many cases this transformation can be represented by an affine transformation represented by an augmented matrix; 2) Characterize the dynamic performance through a high resolution FRF measurement. Piezo actuators are high-Q dynamic systems, so fine frequency sampling in the FRF measurement is necessary for model inversion; 3) Apply model inverse feedforward control to increase the bandwidth of the piezo stage. This increase in bandwidth enables faster acquisition rates for the multiple position per image operation mode and reduces the total experiment time in the single position per image mode. All of this can be accomplished on modest equipment which

includes a computer, a controller, and a piezo actuated microscope stage with matching amplifier.

ACKNOWLEDGEMENTS

The authors would like to thank Boris Godoy Torres and Samuel Pinto for insightful conversation and interesting suggestions throughout the duration of this work.

REFERENCES

Abramovitch, D.Y. (2015). Built-in stepped-sine measurements for digital control systems. In *2015 IEEE Conference on Control Applications (CCA)*, 145–150. IEEE.

Adriaens, H., De Koning, W.L., and Banning, R. (2000). Modeling piezoelectric actuators. *IEEE/ASME Transactions on Mechatronics*, 5(4), 331–341.

Ashley, T.T. and Andersson, S.B. (2015). Method for simultaneous localization and parameter estimation in particle tracking experiments. *Physical Review E*, 92(5), 052707.

Ashley, T.T., Gan, E.L., Pan, J., and Andersson, S.B. (2016). Tracking single fluorescent particles in three dimensions via extremum seeking. *Biomedical Optics Express*, 7(9), 3355–3376.

Brandenburg, B. and Zhuang, X. (2007). Virus trafficking–learning from single-virus tracking. *Nature Reviews Microbiology*, 5(3), 197.

Butterworth, J., Pao, L., and Abramovitch, D. (2012). Analysis and comparison of three discrete-time feedforward model-inverse control techniques for nonminimum-phase systems. *Mechatronics*, 22(5), 577–587.

Cabriel, C., Bourg, N., Dupuis, G., and Lévêque-Fort, S. (2018). Aberration-accounting calibration for 3d single-molecule localization microscopy. *Optics Letters*, 43(2), 174–177.

Chao, J., Ward, E.S., and Ober, R.J. (2016). Fisher information theory for parameter estimation in single molecule microscopy: tutorial. *Journal of the Optical Society of America A*, 33(7), B36–B57.

Cheezum, M.K., Walker, W.F., and Guilford, W.H. (2001). Quantitative comparison of algorithms for tracking single fluorescent particles. *Biophysical Journal*, 81(4), 2378–2388.

Chenouard, N., Smal, I., De Chaumont, F., Maška, M., Sbalzarini, I.F., Gong, Y., Cardinale, J., Carthel, C., Coraluppi, S., Winter, M., et al. (2014). Objective comparison of particle tracking methods. *Nature Methods*, 11(3), 281.

Holden, S. and Sage, D. (2016). Imaging: super-resolution fight club. *Nature Photonics*, 10(3), 152.

Lanzanò, L. and Gratton, E. (2014). Orbital single particle tracking on a commercial confocal microscope using piezoelectric stage feedback. *Methods and Applications in Fluorescence*, 2(2), 024010.

Leang, K.K. and Devasia, S. (2007). Feedback-linearized inverse feedforward for creep, hysteresis, and vibration compensation in AFM piezoactuators. *IEEE Transactions on Control Systems Technology*, 15(5), 927–935.

Levi, V. and Gratton, E. (2007). Exploring dynamics in living cells by tracking single particles. *Cell Biochemistry and Biophysics*, 48(1), 1–15.

Li, Y., Wu, Y.L., Hoess, P., Mund, M., and Ries, J. (2019). Depth-dependent psf calibration and aberration correction for 3d single-molecule localization. *Biomedical Optics Express*, 10(6), 2708–2718.

McGorty, R., Schnitzbauer, J., Zhang, W., and Huang, B. (2014). Correction of depth-dependent aberrations in 3d single-molecule localization and super-resolution microscopy. *Optics Letters*, 39(2), 275–278.

Reck-Peterson, S.L., Yildiz, A., Carter, A.P., Gennerich, A., Zhang, N., and Vale, R.D. (2006). Single-molecule analysis of dynein processivity and stepping behavior. *Cell*, 126(2), 335–348.

Rehman, S.A., Carr, A.R., Lenz, M.O., Lee, S.F., and O'Holleran, K. (2018). Maximizing the field of view and accuracy in 3d single molecule localization microscopy. *Optics Express*, 26(4), 4631–4637.

Rodriguez, E.A., Campbell, R.E., Lin, J.Y., Lin, M.Z., Miyawaki, A., Palmer, A.E., Shu, X., Zhang, J., and Tsien, R.Y. (2017). The growing and glowing toolbox of fluorescent and photoactive proteins. *Trends in Biochemical Sciences*, 42(2), 111–129.

Sage, D., Pham, T.A., Babcock, H., Lukes, T., Pengo, T., Chao, J., Velmurugan, R., Herbert, A., Agrawal, A., Colabrese, S., et al. (2019). Super-resolution fight club: assessment of 2d and 3d single-molecule localization microscopy software. *Nature Methods*, 16(5), 387.

Saxton, M.J. (2012). Wanted: a positive control for anomalous subdiffusion. *Biophysical Journal*, 103(12), 2411–2422.

Saxton, M.J. (2014). Wanted: scalable tracers for diffusion measurements. *The Journal of Physical Chemistry B*, 118(45), 12805–12817.

Saxton, M.J. and Jacobson, K. (1997). Single-particle tracking: applications to membrane dynamics. *Annual Review of Biophysics and Biomolecular Structure*, 26(1), 373–399.

Schuster, B.S., Ensign, L.M., Allan, D.B., Suk, J.S., and Hanes, J. (2015). Particle tracking in drug and gene delivery research: State-of-the-art applications and methods. *Advanced Drug Delivery Reviews*, 91, 70–91.

Thevathasan, J.V., Matti, U., Kahnwald, M., Peneti, S.K., Nijmeijer, B., Kueblbeck, M., Ellenberg, J., and Ries, J. (2019). Nuclear pores as universal reference standards for quantitative microscopy. *Biophysical Journal*, 116(3), 137a.

Thompson, R.E., Larson, D.R., and Webb, W.W. (2002). Precise nanometer localization analysis for individual fluorescent probes. *Biophysical Journal*.

Vickers, N.A. and Andersson, S.B. (2019). Monte carlo simulation of Brownian motion using a piezo-actuated microscope stage. In *2019 American Control Conference (ACC)*, 567–572.

von Diezmann, A., Lee, M.Y., Lew, M.D., and Moerner, W.E. (2015). Correcting field-dependent aberrations with nanoscale accuracy in three-dimensional single-molecule localization microscopy. *Optica*, 2(11), 985–993.

von Diezmann, A., Shechtman, Y., and Moerner, W. (2017). Three-dimensional localization of single molecules for super-resolution imaging and single-particle tracking. *Chemical Reviews*, 117(11), 7244–7275.