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Mechanical Vibration Compensation Method for 3D+t Multi-Particle Tracking in Microscopic Volumes.

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Abstract— **The acquisition and analysis of data in microscopic systems with spatiotemporal evolution is a very relevant topic. In this work, we describe a method to optimize an experimental setup for acquiring and processing spatiotemporal (3D+t) data in microscopic systems. The method is applied to a three-dimensional multi-tracking and analysis system of free-swimming sperm trajectories previously developed. The experimental set uses a piezoelectric device making oscillate a large focal-distance objective mounted on an inverted microscope (over its optical axis) to acquire stacks of images at a high frame rate over a depth on the order of 250 microns. A problem arise when the piezoelectric device oscillates, in such a way that a vibration is transmitted to the whole microscope, inducing undesirable 3D vibrations to the whole set. For this reason, as a first step, the biological preparation was isolated from the body of the microscope to avoid modifying the free swimming pattern of the microorganism due to the transmission of these vibrations. Nevertheless, as the image capturing device is mechanically attached to the "vibrating" microscope, the resulting acquired data are contaminated with an undesirable 3D movement that biases the original trajectory of these high speed moving cells. The proposed optimization method determines the functional form of these 3D oscillations to neutralize them from the original acquired data set. Given the spatial scale of the system, the added correction increases significantly the data accuracy. The optimized system may be very useful in a wide variety of 3D+t applications using moving optical devices.**

I. INTRODUCTION

Automated tracking and analysis of moving objects in image sequences has been and continues to be a major theme in digital image analysis research. Within this field, one important and relatively novel area with applications in several knowledge fields is the study of microscope based systems with spatiotemporal evolution (3D+t). Results in this area are promoting important social and economic impact, specially concerning the human reproduction field. Achieving robustness and high accuracy in tracking and motion analysis in images is hampered by factors that limits the spatial resolution of the microscope, the signal noise embedded in the measurements and the large variability of the systems image data attributed to the intrinsic heterogeneity of the systems under study [1].

Despite many challenges remaining, there are notable technical advances in 3D+t data acquisition. As an example, an interesting case is the work of Göbel *et al* [2] that introduces an experimental system based in two photon microscopy that permits fast fluorescence measurements from several hundred cells distributed in a 3D space. They combined sinusoidal vibration of the microscope objective at 10 Hz with movements of galvanometric x-y scanners to repeatedly scan the laser focus along a closed 3D trajectory. Likewise, in their work, Cang *et al* [3] introduces an experimental device based on confocal microscopy that uses a near-infrared laser and a dark-field condenser for illumination of a gold nanoparticle. By monitoring the scattered light from the nanoparticle with the use of a piezoelectric stage, the system was able to continuously bring the diffusive particle in a glycerol/water solution back to the focal volume with spatial resolution and response time of less than 210 nm and a millisecond, respectively.

More recently, we have developed a new image acquisition approach for 3D multi-tracking of spermatozoa using a single camera and a single optical microscope [4], [5]. In this system, a piezoelectric device was mounted between the long distance objective of the microscope and the turret. This device was driven with an amplified ramp signal to make it oscillate at up to 70 cycles per second, while acquiring synchronously at different focal planes (in a depth of 100 μm) the images of free-swimming spermatozoa at a rate of 4,200 images per second. This produced the necessary data to track their trajectories in three dimensions. In several other experimental systems for analysis of the systems 3D+t, the piezoelectric is employed to achieve nano-metric displacements of the microscope objective, [6], [7], [8], [9], [10], [11].

Nevertheless, when the piezoelectric oscillation reaches high frequencies (with high loads as the microscope objectives), the mechanical vibration transmitted to the microscope could become an important factor into the noise sources for the data. One work that take into account this factor and evaluate the total error of the tracking procedure in the determination of the particle position is presented by Levi *et al* in [7], where a method for three-dimensional fluorescent particle tracking using a two-photon excitation microscope is proposed. The standard deviation of the positions of the particles is estimated by measuring the trajectory of 500 nm fluorescent beads, nevertheless, no correction is proposed.

In this work we introduce an original and useful method to determine and eliminate the artificial data component induced by the piezoelectric device through mechanical vibrations in optical microscope-based systems for three-dimensional scanning in micro volumes.

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II. EXPERIMENTAL DEVICE.

The system setup is presented in Fig. 1. A piezoelectric device P-725 (Physik Instruments, MA, USA) was mounted between a 40X 0.60 NA long-working distance objective (Olympus Ph 2 FL N) and the inverted microscope (Olympus IX71). This piezoelectric device was controlled by a servo-controller E-501 via a high current amplifier E-505 (Physik Instruments, MA, USA). The servo-controller was excited with a ramp signal from the E-506 function generator. A synchronizing TTL pulse coming from the servo-controller was used for triggering the high speed camera Motion-Pro HS4 (RedLake, AZ, USA) with 4 Gigabyte RAM (for grabbing up to 8 seconds image sequences of 512 x 512 pixels; in our case 2,000 images per second). The microscope was mounted on an optical table (TMC) to isolate the system from external vibrations. The system includes a thermal cooling module (Warner Instruments TCM/CL-100) to preserves the optimal conditions of biological preparations. The imaging chamber containing the target to measure was mounted on an external support inserted between the microscope objective and the light output to isolate it from the vibrations produced by the piezoelectric device (see Fig. 1). Data acquisition and tracking was achieved with a Pentium IV PC (1.8 GHz) with the Image-Pro Plus ver 5.2 image analyzer. With this method, whole 3D paths could be visualized and measured (see more details in Corkidi *et al* 2008, [5]).

III. THE CORRECTION METHOD

In 3D+t piezoelectric focal based systems with relative high temporal resolution requirements, it is common that the oscillating device works at high frequencies; given its mechanical coupling to the microscope, this vibration is transmitted and reflected throughout the composite structure, causing undesirable 3D displacements of the microscope objective. In this work, we study the 3D vibrations pattern in the mechanical coupled system (MCS) microscope/piezoelectric/objective and determine a simple and general method to obtain the non-nominal displacement of the objective.

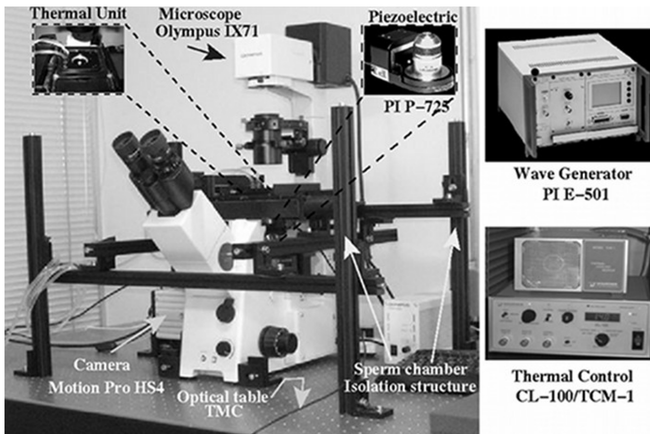


Fig. 1. Image acquisition system. The piezoelectric device, attached to the microscope long working distance objective, is controlled by the Piezo-nano-positioning wave generator. The piezoelectric displacement is synchronized with the camera acquisition sequence. The microscope was mounted on an optical table to isolate the system from external vibrations. The system includes a thermal cooling module to preserves the biological preparations.

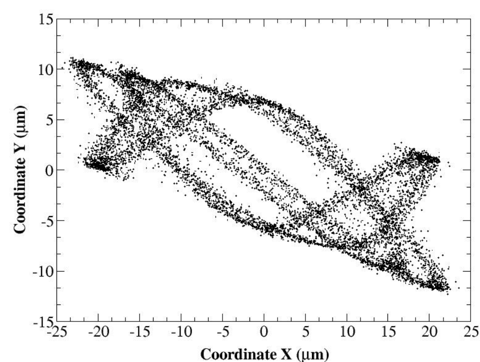
The MCS design has intrinsically a strong mechanical coupling, validating the supposition that its vibration pattern could be defined in terms of three main components; the oscillation defined by the piezoelectric signal itself (principal), the transmitted vibration (secondary) and the noise. To analyze the entire vibrating system, we assume the following facts:

1) The vibration of the objective along the optical axis (defined by the Z axis) is a forced oscillation driven by the piezoelectric control, meaning that the mechanical response of the MCS in Z is dominated by the forced movement induced by the piezoelectric device. In consequence, the Z axis component of the displacement is very close to the nominal oscillation defined by the input wave function generator.
2) The MCS mechanical response (secondary component) to the forced oscillation, induces vibrations of the microscope objective in the perpendicular optical axis plane (X-Y plane). The magnitude and frequency of this response depends on the amplitude and frequency of the piezoelectric oscillation and on the MCS physical properties (weight, geometry, density, etc).
3) The noise, usually associated to external noise and microscope mobile parts, is given by the stochastic vibrations in the system.

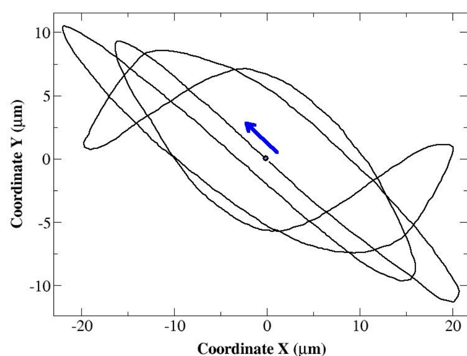
Assuming these facts, to obtain the entire vibration pattern of the observation device, we analyze and determine the contribution that the secondary vibrations component has on the movement. To achieve this goal, we have included a fix target: a micro-hole with an approximate size of 20 microns or 5 times bigger than the mean sperm head diameter. This size of micro-hole enabled its visualization over the whole volume depth of analysis along the Z axis. The micro-hole was drilled in a thin aluminium plate that is placed instead of the biological preparation into the sperm swimming chamber before acquiring the sperm images. In typical experimental conditions we acquire a complete sequence of images of the target; under the normal illumination, this target appears in all single images of the 3D+t stacks, despite the focus change on time due to the Z displacement of the objective. Consequently, the center of mass of the target can be tracked in the whole image sequence upon depth of the experimental volume. This procedure allows to obtain the global displacements induced by the measurement device into the images which can be subsequently filtered from the experimental data.

IV. RESULTS.

By tracking the micro hole over an entire acquired sequence (18,000 frames at 2,000 frames/second, microscope objective at 40X), we have obtained the 3D displacement of the microscope objective for a triangular wave of 30 Hz and 250 μm in amplitude driving the piezoelectric device.



(a)



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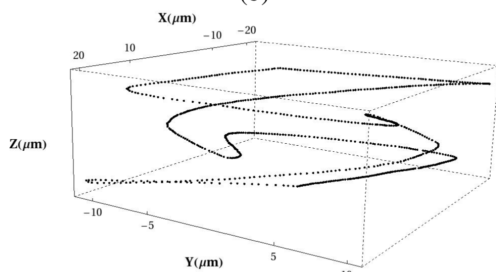
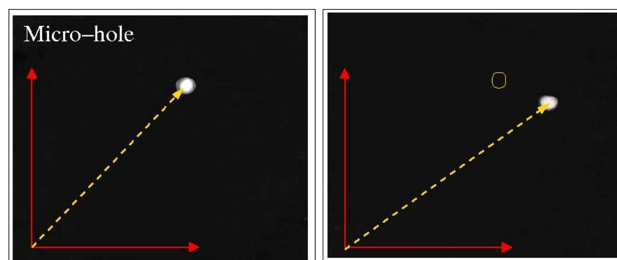


(c)

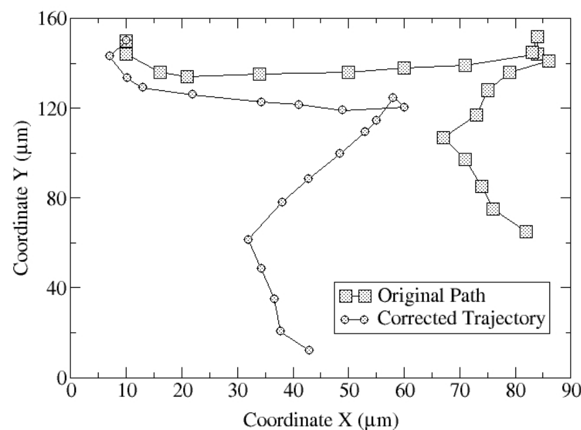
Fig. 2. Tracking of the microscope objective 3D displacement. **a)** Temporal evolution of the objective displacement after the transitory of $\sim$ 0.25 s (stationary state). The points corresponds to the tracked positions of the micro-hole mass center. **b)** Average trajectory of the objective displacement (standard deviation of 1.76 μm). **c)** Three-dimensional curve that corresponds to the the average $X-Y-Z$ displacements obtained from the micro-hole tracking and the Z displacement produced by the piezoelectric device. This trajectory is a cyclic curve with 30 Hz period.

In the Fig. 2.a) we show hundreds of tracked trajectories corresponding to the micro-hole center of mass displacements. Then, we have determined the average objective displacement (Fig. 2.b). The 3D temporal evolution of the total displacement (composed by the planar component X-Y of the vibration and the Z displacement controlled by the piezoelectric device) on a complete period is shown in Fig. 2.c).

We have estimated the average dispersion of the trajectories in the perpendicular direction to the average path and determined that in a stationary state, the error of the vibration trajectory is within 1.76 microns. The average displacement curve is used to eliminate the vibration from the original image sequence and to obtain the real trajectory described by the tracked micro-organisms. To achieve this, the experimental images sequence is registered with the 3D displacement data by using the micro-hole extreme positions. For this purpose, the phase of the tracked vibration is matched with the phase of the experimental set of images. This is achieved by labelling the maxima of the averaged vibration data as a starting point and matching it with the same reference in the experimental data. This last reference can be obtained by extracting it from the collective movement pattern described by all the objects which globally follows this vibration pattern (this can be done since over a single vibration cycle, the swimming sperm movement is negligible). To illustrate the correction, the Fig. 3 shows an example of a corrected trajectory of free-swimming sea urchin.



(a)



(b)

Fig. 3. **a)** Example of two fields within different Z focal planes showing the micro-hole used for tracking the vibration. It can be appreciated that the fix micro-hole appears in different X,Y positions in the images due the undesirable vibration transmitted by the piezoelectric device to the microscope. **b)** Comparison between a biased trajectory of the sea urchin sperms and a corrected one using the average displacement curve.

Our results shows that this procedure is enough to correct the artificial displacements and consequently obtain real trajectories of the sperm swim. Given the scale, this micro-correction is very important to achieve accurate tracking data.

V. CONCLUSIONS.

We have developed an original method to subtract the vibrations induced by the movement of a piezoelectric device mounted into an optical microscope, used to focus video sequences with spatiotemporal activity 3D+t. While the piezoelectric device is used to drive the oscillations of a microscope objective in the Z axis (while a camera acquire 2D images of different focal plane (X-Y)), undesirable mechanical vibrations are transmitted to the whole microscope, producing biased data when tracking the trajectories of these high speed free swimming cells as sperm. The proposed method determines the mean shape of the 3D displacements (determined to be a closed and cyclic trajectory with temporal period equal to that of the piezoelectric device), permitting to subtract it from the original video sequence. By this way, we could estimate with μm precision ($\pm 2\mu m$) the necessary compensating displacement to eliminate the undesired vibrations in the 3D+t data set. The proposed method can be applied applied to several experimental devices to determine the induced oscillations and eliminate the induced undesirable displacements. The described results correspond to the particular case where a triangular waveform with a period of 30 Hz and amplitude of 250 μm drives the piezoelectric device. However, the described method can be applied to any other experimental system where the undesired vibrations induced by the piezoelectric device need to be subtracted from the acquired spatiotemporal information of microscopical particles activity.

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