Toward automated and real-time 3D PTV measurements for microfluidics

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The increasing use of microfluidics in industrial, biomedical, and clinical applications requires a more and more precise control of the microfluidic flows and suspended particles or cells. This leads to higher demands in three-dimensional and automated particle tracking methods, e.g. for use in feedback-control systems. In order to meet these demands, we present in this work an algorithm for performing General Defocusing Particle Tracking (GDPT) in a fast, versatile, and automated manner. GDPT is a 3D particle tracking method based on defocused particle images which is suitable for non-expert users and requires only standard laboratory equipment. The presented algorithm includes (i) an automatic identification of particles via the \emph{a priori} measured reference set of particle images, (ii) a quick depth coordinate determination through a prediction of expected particle image similarities, and (iii) an iterative approach for detection of overlapping particles. We show that the algorithm is versatile and can be applied to different types of images (darkfield and brightfield) and that it is not sensitive to background or illumination fluctuations. We use synthetic image sets of varying particle concentration to evaluate the performance of the algorithm in terms of detected depth coordinate uncertainty, particle detection rate, and processing time. The algorithm is especially suitable for applications where real-time feedback control is needed and we illustrate this by using synthetic images to simulate a real-time experiment of particles undergoing acoustophoresis in a microfluidic device. The simulated experiment showed that the processing time could be significantly reduced using the presented algorithm without compromising the reliability of the detection. Our results pave the road for real-time applications of GDPT and for its improvements in processing accuracy, precision, and time.

I. INTRODUCTION

The recent advancements in microfluidic devices, specially in fields like biology or medicine, require more and more precise and continuous measurements of microfluidic flow fields and suspended particles. In particular, two main needs are emerging in this domain: 3D-PTV tools that can effectively be operated by non-expert users like biologists or physicians, and automated, real-time methods suitable for active force and flow control (e.g. to allow single-cell manipulation [1]). Since the first application of microscopic PIV [2], about two decades ago, several methods have been proposed to track position and velocity of particles in microfluidics, both in 2D and 3D, using different principles such as defocusing [3, 4], astigmatic aberration [5, 6], evanescent waves [7], holography [8], Tomo-PTV [9]. However, most of these methods require complex calibration procedures as well as experienced users to properly perform a measurement and are therefore not suitable for quick or real-time applications [10].

One method with the potential to meet these needs is the General Defocusing Particle Tracking (GDPT) which was proposed by Barnkob \textit{et al}. [11] and is illustrated in Fig. 1. The only requirements for performing GDPT measurements is to have an optical system with sufficiently small depth of field (particle images must have different shapes depending on their depth positions) and a stack of calibration images that represents the particle image shapes at a finite set of depth positions. Both requirements are typically fulfilled in microfluidic applications, where large magnification objective lenses are used and where a calibration stack can easily be obtained by a systematic scanning of the microscope focus. Furthermore, GDPT can indifferently be used on brightfield, darkfield, or fluorescent images as long as the image contrast is sufficiently high and outliers (i.e. false positive) are automatically rejected based on a single similarity parameter that evaluates how well a target image is matched to the calibration image stack. Therefore, and due to its simplicity, GDPT is receiving an increasing interest in microfluidics and lab-on-a-chip communities, such as within the acoustic manipulation of microparticles, where information about the three-dimensional acoustophoretic behavior is crucial to further development [12–14] as well as for the translation to industrial and clinical use, where feedback control is essential to secure stable and viable conditions [15].

In this work, we present a new algorithm to perform GDPT measurements in a fast, automated and versatile fashion: (i) the particle images are identified automatically without an image segmentation step, (ii) a fast and robust estimation of the depth position is performed using only few cross-correlations, (iii) an iterative approach is used to refine the accuracy and resolve overlapping particles. The performance of the algorithm is evaluated using the benchmark dataset provided in Ref. 16 with

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FIG. 1. An example of 3D particle tracking performed by GDPT using a conventional microscope with brightfield illumination and a cylindrical lens introduced in front of the camera sensor. (a) The stack of reference calibration images of a spherical particle at known depth positions. (b) The raw image of the particles in the flow. The yellow contours indicate particles with identified 3D positions. (c) The resulting 3D particle positions corresponding to the identified particles.

The essential elements of the GDPT method are a look-up table (calibration stack) that maps defocused (or astigmatic) particle images with their respective depth position \( z \) and a function to compare the similarity between a target particle image and the reference particle images in the stack. In Refs. 11 and 16, and in this work, we use the normalized cross-correlation [19] to rate the similarity between target and calibration images, using the maximum peak value as the similarity coefficient, referred to as \( C_m \), see Fig. 2(d). The values of \( C_m \) can range from 0 to 1, with 1 corresponding to a perfect match between the target image and a calibration image.

Second, to evaluate a target image, we first perform normalized cross-correlations between the target image and the \( N_{\text{sub}} \) calibration images in the subset as shown in Fig. 2(b). The correlation maps have values between 0 and 1 and with peak values located in the center of the particle images with shapes similar to the corresponding calibration images. In this way, we automatically identify candidate target particles by looking at the local correlation peaks with magnitude larger than a certain threshold (normally 0.5). For each identified target particle, we have the in-plane position and a profile of \( C_m \) values for the \( N_{\text{sub}} \) number of \( z \) positions with no need of performing additional cross correlations. Each correlation profile can following be compared with the mapping of expected \( C_m \) profiles to obtain a robust guess of the \( z \) position, see Fig. 2.

With this approach we have three significant advantages:

II. FAST AND USER-FREE GENERAL DEFOCUSING PARTICLE TRACKING

The essential elements of the GDPT method are a look-up table (calibration stack) that maps defocused (or astigmatic) particle images with their respective depth position \( z \) and a function to compare the similarity between a target particle image and the reference particle images in the stack. In Refs. 11 and 16, and in this work, we use the normalized cross-correlation [19] to rate the similarity between target and calibration images, using the maximum peak value as the similarity coefficient, referred to as \( C_m \), see Fig. 2(d). The values of \( C_m \) can range from 0 to 1, with 1 corresponding to a perfect match between the target image and a calibration image.

The seminal approach for GDPT measurements consists of a segmentation step to identify candidate particles images and an optimized iterative procedure to identify the depth position \( z \) of each candidate particle [11]. This procedure is very accurate, however, it is relatively slow since it needs to compute a large number of cross-correlations (on average 6-10 cross-correlations for each particle image). Moreover, the segmentation procedure must be optimized for each image type and fails if the background or the illumination is not uniform, therefore a pre-processing step is often required. In addition, the seminal approach is not optimized for detecting the coordinates of overlapping particles.

In this work, we present a new algorithm to perform GDPT measurements based on two steps: One first step for a fast and automatic detection of particles with a robust estimation of their position and a second step using refinement to improve the measurement accuracy, especially with respect to overlapping particle images.

A. Automated determination of particle position

The proposed algorithm for the automated detection of particle positions is summarized in Fig. 2. First, we select a subset of \( N_{\text{sub}} \) calibration images, from the total \( N_{\text{cal}} \) images in the calibration stack, see Fig. 2(a). For each of the \( N_{\text{cal}} \) calibration images, we calculate an "expected" \( C_m \) profile by performing a normalized cross-correlation with the \( N_{\text{sub}} \) images in the subset. This gives an a priori mapping of the expected results which are used during the evaluation for a fast identification of the \( z \) positions. Second, to evaluate a target image, we first perform normalized cross-correlations between the target image and the \( N_{\text{sub}} \) calibration images in the subset as shown in Fig. 2(b). The correlation maps have values between 0 and 1 and with peak values located in the center of the particle images with shapes similar to the corresponding calibration images. In this way, we automatically identify candidate target particles by looking at the local correlation peaks with magnitude larger than a certain threshold (normally 0.5). For each identified target particle, we have the in-plane position and a profile of \( C_m \) values for the \( N_{\text{sub}} \) number of \( z \) positions with no need of performing additional cross correlations. Each correlation profile can following be compared with the mapping of expected \( C_m \) profiles to obtain a robust guess of the \( z \) position, see Fig. 2.

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FIG. 2. Evaluation method for automated and fast GDPT. (a) During the calibration process, a subset of $N_{\text{sub}}$ calibration images is extracted from the calibration stack and used to create a mapping of the estimated $C_m$ profiles for the given subset, across the entire measurement height. (b) In the evaluation process, a normalized cross-correlation between the experimental image and the subset of calibration images is performed, obtaining $N_{\text{sub}}$ correlation maps. From the maps (with values between 0 and 1), candidate particles are extracted and for each candidate the $C_m$ profile is calculated (c). The $z$ coordinate is identified from the comparison between the measured $C_m$ profile and the pre-determined expected $C_m$ profiles. (d) Example of normalized cross-correlation: the $C_m$ value represents the (local) peak value of the correlation map.

1. Automatic detection: The in-plane and out-of-plane positions of candidate particles are determined by setting a single parameter, namely $C_m$.

2. Fast detection: Only $N_{\text{sub}}$ cross-correlations is needed, regardless of the number of particles in the image.

3. Versatility: The same procedure can be applied for any type of particle images (fluorescent, brightfield, with non-uniform illumination, etc.) without any additional steps.

B. Refinement step for overlapping particle images

The first step already provides a complete measurement with the identification of the particles and their three-dimensional position. In addition, overlapping particles are, to a certain extent, also identified. As shown in Fig. 2(b), the cross-correlation already filters out particles based on their image shape, i.e. only particle images similar to the calibration image are highlighted. This allows to identify overlapping particles of different shapes. However, when the degree of overlapping between two particle images is too large, the height of the correlation peak decreases significantly and it is no longer possible to identify them.

A way to improve the detection in this case is to blank out one of the overlapping particle images. This idea is used in the presented refinement step. Practically, all the particles identified in the first step are first ordered according to their $C_m$ values, from larger to smaller. Then, a walking procedure is applied to the first particle image to find the best match with the images in the calibration stack, searching in positions close to the $z$ value predicted by the first step. When the refined $z$ position is obtained, the particle image is removed from the image by replacing its area with the intensity values of the background. The same procedure is repeated for the second particle and so forth. At the end of the refinement step, all the detected particles have been removed from the image as illustrated in Fig. 3(b). The remaining particles are the particles that were not identified in the first step. The processing (first basic step and second refinement step) can following be iterated to improve the number of detected particles, see Fig. 3.

The refinement step improves the accuracy and number of detected particles, however it consumes more computation time, therefore it might not be suitable for real-time applications. The relation between accuracy and computational time is investigated for a representative case in the next section.
III. PERFORMANCE ASSESSMENT OF THE PRESENTED ALGORITHM

The presented algorithm allows to automatically detect particle images and determine their 3D position from a suitable calibration image set. Only few parameters must be chosen from the user before starting the evaluation, namely:

- The number of images $N_{\text{cal}}$ in the calibration stack.
- The number of images $N_{\text{sub}}$ in the calibration subset.
- The threshold value of $C_m$ for identifying candidate particle images.
- The number of iteration $n_{\text{ref}}$ in the refinement step.

For the purpose of automated and real-time measurements, the most relevant parameters to evaluate are the uncertainty in the depth coordinate determination $\epsilon_z$, the relative number of valid detected particles $\phi_{\text{det}}$ (relative to the total number of particles in the image), and the overall processing time for each image. We do not consider here the uncertainty in the in-plane directions ($\epsilon_x$ and $\epsilon_y$), which are normally one or more orders of magnitude less than $\epsilon_z$. For a complete description of the assessment of GDPT methods, see Ref. 16.

In order to assess the effect of different parameter settings on the algorithm, we used a standardized dataset of synthetic defocused particle images presented in Ref. 16. The images are created using MicroSIG, a synthetic image generator for defocused and astigmatic particle images [18]. In particular, we used Dataset III, which contains sets of images of different particle image concentrations \cite{18}. Following Ref. 16, we define the particle image concentration $c_I$, as the number of particles in the image multiplied by the particle image area and divided by the total image area. A value $c_I = 1$ indicates that if the particle images were all packed side by side they would fill the entire image area. The dataset simulate measurements performed on 2-µm-diameter particles, with $10\times$ magnification over a measurement depth of 86 µm.

The results are presented in Fig. 4 for three different particle image concentrations ($c_I = 0.25, 1.49, 2.98$). Three parameter configurations have been investigated as a function of using different number of calibration images $N_{\text{cal}}$: Configuration 1 with $N_{\text{sub}} = 5$ and one refinement step (blue circles), Configuration 2 with $N_{\text{sub}} = 3$ and one refinement step (red squares), and Configuration 3 with $N_{\text{sub}} = 5$ and no refinement step (yellow diamonds). For all the configurations we used a similarity threshold value $C_m = 0.5$. In general, a use of $N_{\text{cal}}$ larger than 10 is required, however values larger than 20 do not improve the performance significantly. Also, the value of $N_{\text{cal}}$ minimally affect the processing time. The refinement step can decrease significantly the uncertainty, but to a cost of a significantly larger processing time, which increases proportionally with the particle image concentration. On the other hand, without the refinement step (Configuration 3), the processing time is basically independent of the particle image concentration. All the three configurations basically provide the same number of valid detected particles, which is however strongly affected by the particle image concentration. At $c_I = 2.98$, the relative number of detected particles drops to around 30 %. It is possible to increase the number of valid detected particles by adding more iterations of the refinement step as shown in Fig. 5. In particular, a second iteration brings this value up to 50 %, whereas a third iteration does not improve it much further. The cost of more iterations in terms of processing time is however significant.

\footnote{The dataset can be downloaded following this link.}
FIG. 4. Parametric assessment of the presented algorithm in terms of error in the depth coordinate determination $\epsilon_z$, the relative number of valid detected particles $\phi_{\text{det}}$, and the processing time, as a function of the number of images in the calibration stack $N_{\text{cal}}$, the number of images in the calibration subset $N_{\text{sub}}$, with or without the refinement step. The assessment is performed on synthetic reference images for three values of the particle image concentration: (a) $c_I = 0.25$, (b) $c_I = 1.49$, and (c) $c_I = 2.98$ [16].

FIG. 5. Effect of using more iterations of the refinement step on (a) the error in the depth coordinate determination $\epsilon_z$, (b) the relative number of valid detected particles $\phi_{\text{det}}$, and (c) the processing time. The analysis was performed for one parameter setting with $N_{\text{cal}} = 21$, $N_{\text{sub}} = 3$, and for one, two, and three refinement step iterations, and as a function of the particle image concentration $c_I$.

IV. REAL-TIME APPLICATION OF GDPT TO SIMULATED EXPERIMENT

In order to test real-time application of GDPT and our new algorithm, we set up a simulated experiment of particles undergoing acoustically-driven motion in a microfluidic channel. The particle motion is based on well-known and validated analytical predictions and turned into synthetic images resembling experimental acquisition. The images are processed with GDPT in a real-time fashion allowing for an objective assessment of the performance of GDPT in real time.

The use of acoustics in microfluidics is receiving increasing interest, e.g. due to its use in biomedical engineering and medicine for label-free and gentle manipulation of cells and biological particles [20]. Several new biomedical applications has been reported, including enrichment of extracellular vesicles [21] and creation of tumor spheroids [20, 22], which has led to an increasing demand in device reproducibility and control to reach industrial and clinical application, e.g. by the use of real-time feedback control [15]. This demand, and the intrinsically-involved complex and three-dimensional
FIG. 6. (a) Schematic of the simulated acoustophoretic experiment: an acoustofluidic device is used to focus particles in the center height of a rectangular microchannel. Real-time GDPT measurements are used to monitor the position of the particles inside the channel and to identify the time $t_{h/3}$, when 90% of the particles are inside a vertical region of thickness $h/3$ (indicated by red horizontal lines in (c)). (b) Illustration of the percentage of particles that has reached the $h/3$ vertical region as a function of time. The points indicate the real number of particles inside the region, while the circles indicate the corresponding GDPT measurements with lower temporal resolution from the computation time and fluctuations due to the measurement uncertainty. (c) Cross-sectional view of the simulated particle position in the measurement region for different time instants when assuming a Poiseuille flow with flow rate $Q = 3 \mu l/h$ and acoustic energy density $E_{ac} = 0.3 J/m^3$.

particle motion, makes acoustophoresis an interesting technology for application of real-time GDPT.

A. Acoustofluidic focusing experiment

We set up a simulation of a typical acoustofluidic experiment for separation or focusing of particles or cells [23, 24]. The simulated experiment is sketched in Fig. 6(a) and consists of suspended microparticles transported through a microchannel while being focused acoustically to the vertical center plane of the channel by acoustic radiation forces. The particles are polystyrene spheres with diameter $2a = 5 \mu m$ suspended in water at 25 °C and the microchannel has a rectangular cross-section of width $w = 200 \mu m$ and height $h = 100 \mu m$.

The particle suspension is passing through the microchannel by an externally-driven flow and the resulting stream-wise particle velocities $u_x^{\text{flow}}(y, z)$ are calculated from the analytical solution of a Poiseuille flow in a rectangular channel with flow rate $Q = 3 \mu l/h$ [25].

The microchannel is acoustically actuated by an attached piezo-electric transducer to induce a vertical half-wave standing acoustic waves of frequency $f_z = c_{wa}/(2h) = 7.49 \text{ MHz}$ across the channel height. The acoustic actuation leads to two primary second-order effects, namely an acoustic streaming through the viscous attenuation of the acoustic waves and acoustic radiation forces on the suspended particles due the waves scattering off the particles. We limit ourselves to the study of large-particle radiation-dominated particle motion which is a good approximation for particles of diameter larger than a few micrometers [26] and consequently we neglect any viscous drag from the acoustic streaming. The streaming-driven motion acoustic motion is typically of slower and more homogeneous character and does not contribute to harder demands in the 3D measurement compared to the radiation-dominated motion which additionally results in locally-increasing in-homogeneous particle concentrations. In addition, we neglect any hydrodynamic or acoustic particle interactions as well as wall-corrections to the viscous drag. As a result, treating the waves independently and through balance of the viscous drag force and the acoustic radiation force (neglecting thermoviscous corrections) [27], we can calculate analytically the cross-sectional acoustophoretic particle velocities as

$$u_x^{\text{rad}}(z) = \frac{2\pi a^2}{3} \frac{\Phi}{h \eta} E_{ac} \sin \left[ 2\pi \left( \frac{z}{h} + \frac{1}{2} \right) \right],$$

where $\eta$ is the fluid viscosity, $\Phi$ the acoustic contrast...
between particle and suspending fluid, and $E_{ac}$ is the acoustic energy density. We used an acoustic energy density $E_{ac} = 0.3 \text{ J/m}^3$, which is a low but realistic value corresponding to maximum acoustic particle velocities of approximately 8 $\mu$m/s [17, 28].

Before the onset of the experiment, the particles are randomly distributed inside the channel and the resulting cross-sectional particle positions over a time of 8 s are shown in Fig. 6(c).

B. Generation of synthetic images

The experimental acquisition of the acoustofluidic model system is simulated by use of synthetic images using MicroSIG [18]. We simulate 5-$\mu$m-diameter monodisperse spheres observed with a 10$\times$/0.3 objective lens plus astigmatic aberration on a 512$\times$512 pixels sensor (pixel size of 6.5 $\mu$m). The use of astigmatic aberration is used in many experimental setups to encode more efficiently the defocusing information by breaking the symmetry of defocusing patterns [5, 29]. In order to simulate experimentally-resembling images, we add a Gaussian noise resulting in signal-to-noise ratio SNR for individual particle images ranging from 30 to 240. A typical synthetic image is shown in Fig. 7(a), which corresponds to a classical darkfield image used in PIV setups (i.e. fluorescent particles observed with an epi-fluorescent microscope). Additionally, we used two other types of images: Brightfield images obtained by inverting the values of the darkfield images (Fig. 7(b)) and brightfield images with an added intensity disturbance, introduced to simulate non-uniform backgrounds or non-uniform illumination (Fig. 7(c)). The disturbance consists of a 2D sinusoidal pattern.

C. Simulation of real-time GDPT measurement

An ad hoc Matlab routine was written to simulate real-time GDPT measurements with a camera frame rate of 25 fps in a section of the microchannel as illustrated in Fig. 6(a). The objective of the simulation is to identify the "trigger" time $t_{h/3}$ for which 90 % of the particles have been focused in a vertical center region of thickness $h/3$ (marked with red horizontal lines in Fig. 6(c)).

There are mainly three parameters to consider for the assessment of real-time GDPT measurements: (1) the computational time of a single evaluation, (2) the accuracy of the measurement, and (3) the number of detected particles (this strongly depends on the particle concentration, since it is more difficult to process overlapping particles, see Section III). Improving points (2) and (3) leads to longer computational times therefore an optimal balance must be found. The computational time sets also the temporal resolution of the real-time measurement, therefore a random delay time proportional to the temporal resolution is expected. This is shown in Fig. 6(b), where the percentage of particles that has reached the $h/3$ vertical region is plotted as a function of time. The points indicate the real number of particles inside the region and the red vertical line indicates the real "trigger time" $t_{h/3}$. The circles show the corresponding GDPT measurements, which have lower temporal resolution due to the computational time of each frame. The blue vertical line marks the corresponding GDPT "trigger time" $t'_{h/3}$. In the hypothetical case of perfect GDPT measurements, the delay time $t'_{h/3} - t_{h/3}$ is between one and two times the temporal resolution. However, due to the GDPT measurement uncertainty (vertical fluctuations of the circles around the points), a smaller or larger delay time can occur. As an example, if the delay time is negative, GDPT detects that 90 % of the particles have reached the region before they actual have.

D. Results of real-time GDPT measurement

In a first set of experiments, we tested the performance of real-time GDPT measurements on the fluorescent images. As discussed in Section III, we test different strate-
FIG. 8. Real-time application of GDPT on a simulated microparticle acoustophoresis experiment as shown in Fig. 6. The results are shown for different algorithm settings and for (a-b) using different number of images in the calibration stack $N_{\text{cal}}$ and (c-d) for different types of images with $N_{\text{cal}} = 51$. (a,c) Measured detection delay time $t_{b/3} - t'_{b/3}$ as a function of the mean evaluation time where the grey area represents the random delay expected for the given evaluation time. (b,d) Average percentage of detected number of particles as a function of time.

We have presented a new algorithm for performing 3D particle tracking using GDPT that does not need preliminary pre-processing or segmentation steps. The presented algorithm needs to compute only few cross-correlations in comparison with other iterative approaches and is therefore suitable for a fast evaluation time. In addition, the algorithm allows for the detection of overlapping particles. The performance of the algorithm was tested on synthetic image sets of varying particle concentration in terms of uncertainty in the depth determination, detection of valid particles, and processing time. The algorithm was tested for real-time application by setting up a framework for real-time simulation of
an acoustophoretic experiment, created using synthetic images and an ad hoc Matlab routine. The real-time simulations show, that even without refinement steps, the presented algorithm is able to perform automated control-tasks and that work robustly on different types of images (darkfield or brightfield) also with fluctuations of the background intensity. The presented algorithm and simulation framework set the base for use of GDPT in real-time applications as well as for the further development and improvement hereof.

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