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Three-dimensional Flow Mapping in Microfluidic Channels with Widefield Cross-correlation Microscopy

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Abstract

Widefield cross-correlation microscopy enables large area measurements of flow vectors in microfluidic devices. Extending fluorescence cross-correlation spectroscopy, all points within each image give flow maps from image stacks acquired on a spinning-disk confocal microscope. The greater noise tolerance and efficient data collection for large volumes of interest enable even motion of single antibodies to be measured. A three-dimensional flow map for a typical microfluidic channel is evaluated, yielding full flow vector information. The method presented is general towards the input data, so applications concerning not only flow in microstructures, but biological transport and other microscopic motion phenomena are possible.

INTRODUCTION

Microfluidic devices have become the basis for automated sensors and miniaturized chemical reactors in a wide range of applications.^{1–3} To accommodate high throughput, even with low sample volume, these devices are becoming increasingly intricate, with additional functionalities packed into ever smaller designs. Often solution handling, processing, and sensitive detection are all to be performed on the same microscale device. The advent of “nanofluidics”, in which surface effects and fabrication processes play a large role in device performance, and the common need to direct analyte to the surface for sensing, make imaging the resulting flow inside devices more difficult.⁴ Most flow imaging techniques rely on tracking many particles moving with the flow stream to determine their motion through consecutive images. Such methods require following large numbers of individual particles, but may still ineffectively map flow through all areas of the channel.⁵

Brightly fluorescent tracer particles are often utilized to enable high signal, but the high concentrations necessary to rapidly map out flow leads to high background from out-of-focus tracer particles. Such single particle imaging requires a high number of photons per frame, coupled with efficient background rejection and tedious analysis to map out only a subset of flow vectors within the channel. Following rapidly flowing particles requires high frame rates and therefore bright particles (e.g., fluorescent beads of several hundred nanometers diameter). As microfluidic devices shrink, tracer particles must also become smaller, thereby limiting brightness. Below a certain brightness threshold it becomes impossible to localize the individual particles in the image, precluding particle tracking algorithms from determining the flow speeds in the system.⁶

Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Cross-Correlation Spectroscopy (FCCS) have both been successfully applied to measure flow within

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microfluidic channels.^{7–11} Such methods offer efficient background rejection due to imaging of fluorescence fluctuations resulting from dye or tracer traversal through individual (FCS) or pairs (FCCS) of diffraction-limited volumes^{12–15} While background is efficiently rejected by means of collection through a pinhole, the spatial resolution is severely limited, making the task of mapping the flow vectors in the entire channel arduous.¹⁶

FCCS uses two focal volumes and the cross-correlation of the two signals from the individual focal volumes to improve flow measurements over that possible with FCS.^{17–19} The cross-correlation that is generated has a maximum value whose position is dependent on the flow speed, distance, and angle between the flow vector and the vector connecting the two focal volumes (see Theory, below). Cross-correlation analysis has some important advantages over autocorrelation techniques—the cross-correlation is more tolerant of non-ideal focal volume shape and saturation effects, while simultaneously being more robust at measuring directional flow.^{20,21} Most importantly, FCS measures fluctuations in a single symmetrical focal spot, precluding the determination of direction in flow while the pair of spots in FCCS allows both speed and direction to be measured.

Combining the particle averaging advantages of FCCS and the large-area imaging of particle tracking would be beneficial in mapping flow. To reduce out-of-focus fluorescence, a Spinning Disk Confocal Scanning Unit (CSU) enables visualization of fluorescence from a single image plane. Here, we develop optically-sectioned fluorescence cross-correlation imaging for rapidly measuring steady-state flow throughout the entire channel.

The Yokogawa CSU unit consists of a pair of spinning disks, one with a series of pinholes, the other with aligned micro lenses, that trace out illumination over a wide area as the disks spin. The resulting emission is collected through the same set of pinholes, which rejects light from other focal planes and constrains the field of view in the axial (z) direction. The resulting confocal images are captured at high frame rates on an EMCCD camera. With each pixel in the CCD acting as a focal volume, cross-correlations can be calculated by correlating the fluorescence vs. time trace between any two arbitrary pairs of pixels throughout the focal plane.^{22,23} Changing the focal plane in the z direction yields a map of the cross-correlation parameters through the entire volume of interest. Such a setup has previously been used with FCS to measure diffusion in aqueous solutions and collagen matrices.²⁴

The technique presented here provides a method to measure the amplitude and directional flow of particles through a large volume of interest. FCS and FCCS have been employed to measure flow speeds^{8,18,19} or directions²⁵ in microfluidic devices. By repositioning the microscope objective, some have extended this to imaging beyond individual spots or pairs of spots to three dimensions as well.^{9,16,26} However, to image an entire volume, these methods rely on taking individual intensity vs. time traces at each position. Here, data for all points in a single focal plane are recorded at once. From this, the individual time traces are reconstructed later, greatly reducing the total acquisition time and allowing for a corresponding increase in resolution. At a single focal plane, the same data set can be used to also reconstruct the total flow vector, eliminating the need for repositioning the detection volume during data acquisition.

The nature of the steady-state analysis can determine flow velocities with probe brightnesses much lower than can be handled by particle-tracking algorithms. Because of the generality of this method, the technique could also be applied to motion or transport along cellular structures. In addition, because the original data are recorded as images, other desirable variables can be extracted from the same data set.

THEORY

Fluorescence correlation spectroscopy uses the fluctuations of the fluorescence signal at time t about the mean fluorescence intensity, given by

$$\delta F(t) = F(t) - \langle F \rangle \quad (1)$$

where the brackets indicate an average over time. The normalized cross-correlation function, $G_{cc}(\tau)$, resulting between the fluorescence signals F_1 and F_2 of two focal volumes, V_1 at $\vec{r}$ and V_2 at $\vec{r}'$, is given by

$$G_{cc}(\tau) = \frac{\langle F_1(t, \vec{r}) F_2(t+\tau, \vec{r}') \rangle}{\langle F_1 \rangle \langle F_2 \rangle} \quad (2)$$

in which the brackets indicate an average over-all time t . From eq 2 and the equations appearing in ref ¹⁸, the cross-correlation function for a system experiencing directional flow is given as

$$G_{cc}(\tau) = \left[N \left(1 + \frac{\tau}{\tau_D} \right) \sqrt{1 + \frac{\omega_0^2 \tau^2}{z_0^2 \tau_D^2}} \right]^{-1} \cdot \exp \left\{ \frac{-R^2}{\omega_0^2} \frac{1}{1 + \frac{\tau}{\tau_D}} \left(\frac{\tau^2}{\tau_{Flow}^2} + 1 - 2 \frac{\tau}{\tau_{Flow}} \cos \alpha \right) \right\} \quad (3)$$

where τ_D and τ_{Flow} are the time scales for diffusion and flow, respectively. The two detection volumes are assumed to be equal volume 3-D Gaussians with $1/e^2$ radii ω_0 and z_0 the in the xy and z directions, respectively, and separated in the xy plane by the “spacing” vector, with length R and in-plane angle α relative to the vector of flow. In this work the spacing vector is that $\vec{R}$ connecting the center of each pixel pair chosen by the algorithm for generating each cross-correlation, the set of which yield the flow direction at each point.

The applicability of the cross-correlation analysis requires flow to be constant in speed and direction within the analysis volume and measurement acquisition time. In addition, the flow can be assumed to be much faster than diffusion, such that $\tau_{Flow} \ll \tau_D$, which simplifies eq 3 to

$$G_{cc}(\tau) = \frac{1}{N} \exp \left\{ \frac{-R^2}{\omega_0^2} \left(\frac{\tau^2}{\tau_{Flow}^2} + 1 - 2 \frac{\tau}{\tau_{Flow}} \cos \alpha \right) \right\} \quad (4)$$

which has a maximum value at $\tau = \tau_{Flow} \cos \alpha$. For flow of unknown angle, the flow vector can be determined by exploiting the cosine dependence between the flow vector and the spacing vector, $\vec{R}$ (Fig. 1). The vector-variant flow analysis is described in more detail in section Flow Angle Fitting.

For flow in a single direction over the entire volume of interest, the form of the cross-correlation can be further simplified to reduce computational time. By choosing a spacing vector that is parallel to the flow vector, the $\cos \alpha$ term is reduced to 1, reducing eq 4 to

$$G_{cc}(\tau) = \frac{1}{N} \exp \left\{ \frac{-R^2}{\omega_0^2} \left(\frac{\tau^2}{\tau_{Flow}^2} + 1 - 2 \frac{\tau}{\tau_{Flow}} \right) \right\} \quad (5)$$

Here the maximum of the cross-correlation function is simply at τ_{Flow} , and since R is known, the flow speed at that point is given by

$$V = \frac{R}{\tau_{Flow}} \quad (6)$$

The details of the flow analysis algorithm are covered in more detail in sections 3-D Flow Mapping and Flow Angle Fitting. For constant-angle flow, pixel pairs separated by a constant spacing of distance R and parallel to the flow vector are iteratively repositioned throughout the image space, and the resulting cross-correlations are fit to eq 5. The τ_{Flow} value at the location of the pixel pair is used with eq 6 to map the flow speed in the image space. Varying or unknown flow requires that the flow angle first be determined. For this, a central pixel is chosen and cross-correlations between this and a set of surrounding pixels are taken. From either the amplitudes or measured τ values of these cross-correlations as a function of position, the flow angle, and subsequently the velocity can be determined.

The derivation of eq 3 invokes Gaussians to describe the shape of the focal volumes. Pixels of a CCD have definite non-Gaussian character, and previous works have defined the relevant physical parameters in terms of the pixel dimensions²⁷ or introduced corrections to ω_0 to account for this.²⁴ While these corrections were necessary for autocorrelations, the comparative robustness of the cross-correlation to the non-Gaussian character of the detection volumes makes these corrections unnecessary in this work.²⁰

EXPERIMENTAL

Experimental Setup

The experimental setup was based on a spinning disk confocal scanner (Yokogawa CSU 10) mounted on an inverted microscope (Olympus IX 70). Imaging was achieved with a 1.2 NA 60× water immersion objective. Microfluidic channels ($50 \times 50 \mu\text{m}$ cross section) were molded in poly(dimethylsiloxane) (PDMS) and sealed on a glass cover-slip.

Flow was achieved by having a solution of fluorescent beads or dye loaded into the inlet tube on one side of the channel and gravity-fed through the device or by syringe pump. The 496 nm line from an Ar⁺ laser (Coherent Innova 90) was aligned into a 3.5 μm optical fiber, coupled into the scanner, and routed through the side port of the microscope. The excitation was directed through the microscope objective, exciting the tracer particles (fluorescein-impregnated polystyrene fluorescent beads of 100 nm or 20 nm nominal diameter or Alexa 488-labeled anti-mouse IgG). The emission was collected through the same objective, side port, and scanner, through the dichroic and emission filters inside the scanner, before detection with a back-illuminated EMCCD camera (Andor iXon DV887).

To increase the frame rate of the camera, only a limited area of the CCD was used and pixels were binned together (4×4 bins). With these accommodations, an area of 100×512 pixels with 4×4 pixel bins could achieve over 1000 frames per second and full-frame images of 512×512 with 4×4 binning (creating 128×128 superpixels) up to 150 frames per second. The finite number of pinholes of the spinning disk requires that the frame rate of the camera

be synchronized to the rotation of the disk, so the frame rates used were the fastest possible that achieved the necessary synchronization with sufficient exposure times.

All chemicals were used as received. Alexa Fluor 488 and Alexa Fluor 488-conjugated anti-mouse IgG and 100 nm FluoroSpheres fluorescent microspheres were obtained from Invitrogen (Carlsbad, California, USA) and Polyethylene Glycol 8,000 (PEG 8K) was obtained from Teknova (Hollister, California, USA).

Data Analysis

A series of images, typically 1,000–50,000 frames, in a single focal plane were collected. A collection of these image stacks taken at a sequence of z -positions constituted a data set. Data sets were exported as ASCII files and analyzed in MATLAB on a PC.

Flow Simulations

Imaging of flow was simulated in MATLAB with initial particle locations being evenly distributed randomly in 3-D across a 100×100 pixel CCD image. The z position of the particle was used to simulate the effect of focal depth in the system. A pre-chosen parabolic flow function was used to translate the center positions of each of the points for the subsequent frame based on the current position. The flow function was a constant displacement along the x -axis of 0.5 pixels/frame (here, $50 \mu\text{m}/\text{sec}$) with additional 3-D diffusion appropriate for 100 nm beads. Simulations and experience indicate that 0.5 pixels/frame is an optimum flow rate for the cross-correlation algorithm described here (data not shown). From the (x, y, z) position of the particle centers, an image frame was generated by modeling the intensities as symmetric 2-D Gaussian distributions.

$$I = I_0 \exp\left(\frac{-[(x - x_0) + (y - y_0)]^2}{2\sigma_{xy}^2}\right) \exp\left(\frac{-z^2}{2\sigma_z^2}\right) \quad (7)$$

Here, σ_{xy} and σ_z represent the e^{-2} radii of the experimentally-determined PSF for the system. To simulate the pixelation from the CCD, the function was only evaluated in integer steps in the xy direction. The zero for the z -axis was taken as the focal plane and the distance above or below resulted in a change in intensity projected on the image.

Typically 10,000 frames were simulated, and the requisite number used to determine the image noise tolerance of the cross-correlation algorithm. To simulate Poisson noise inherent in CCD-based fluorescence imaging, the images generated as above were first scaled to a maximum intensity per particle from $10^{0.25}$ to 10^3 (1.77 to 1000) counts per frame. To this was added a constant background of 10 counts per frame. The value for the final intensity at each pixel was taken as a Poisson-distributed random number (generated with MATLAB's `poissrnd` function) with a mean equal to the raw intensity (the sum of the scaled signal and constant background).

The S/N ratio for these images were determined by

$$S/N = \frac{I}{\sqrt{\sigma_{bgd}^2 + \sigma_{obj}^2}} \quad (8)$$

with σ_{obj}^2 and σ_{bgd}^2 as the variances of the signal and background, respectively. Here the noise is Poissonian, so these variances are also equal to the mean raw intensity values used

to generate the simulated images. Substitution for the variance with the respective raw intensity yields the easily calculable eq 9.

$$S/N = \frac{\lambda}{\sqrt{10 + \lambda}} \quad (9)$$

Images were generated in this fashion with identical time-dependent particle locations, but with peak S/N values ranging from 0.8716 to 31.47.

The RMS deviation between the model and expected flow maps were defined as

$$Error = \sqrt{\langle Expected - Fit \rangle^2} \quad (10)$$

The expected values were taken from the initial displacements used (here, 1 pixel/frame in the center of the channel, 0 at the walls) to build the simulated image stacks, and the fits measured to determine the analysis algorithm's ability to return the expected values in the presence of varying amounts of Poisson noise. Because the flow speed was made to vary with the position along the x-axis, the fit values in a single column from the flow speed map were averaged in the determination of the error. Increasing number of frames for simulations generated with varying S/N ratios were taken when generating the cross-correlation.

3-D Flow Mapping

To create a map of flow velocities in 3-D, a series of flow maps in 2-D are first determined and later reassembled to form the flow map for the entire volume. For a 2-D flow map, the analysis algorithm chooses a pair of pixels in the image stack separated by constant spacing vector, $\vec{R}$, which has length R and is parallel to the flow vector.

This vector is initially set in a distance in pixels in the image stack to be analyzed, but this is easily converted to units of distance (e.g., μm) upon calibrating the effective object-space pixel dimensions. The data for each of the pixels in the pair through the entire image stack are transformed from intensity vs. position plots to a pair of intensity vs. time traces. The cross-correlation of the intensity vs. time traces is fitted to eq 5, yielding τ_{Flow} , and then velocity (V) from eq 6. The resulting velocity is mapped back onto the position of the upstream pixel of the pair. Iterating the position of the pixel pair with constant spacing vector throughout the entire xy image results in a 2-D flow map, a series of which can be stacked at known positions along the z axis to form a 3-D flow volume map.

Flow Angle Fitting

While straight sections of a microfluidic channel operating under laminar flow conditions rarely see any significant deviation from a single flow direction, there are many areas surrounding bends, obstructions, or other inconsistencies in a channel that alter both the magnitude and direction of flow. To determine both components of the flow vector in such a case, the dependence of the cross-correlation function on $\cos \alpha$, as seen in eq 4, is exploited. For a single cross-correlation, the apparent τ from the fit will follow the form

$$\tau = \tau_{Flow} \cos \alpha \quad (11)$$

in which α is the angle between the spacing vector and the flow vector. By choosing a series of pixels about a central pixel and taking the cross-correlations between the central and

surrounding pixels, a plot of the measured τ/R values vs. β will result in a cosine curve (Fig. 2) with an amplitude τ_{Flow} and phase Ψ . To produce a full 2π range of angles, both the forward and reverse cross-correlations are performed for each pair of pixels. The cross-correlation that fits best and yields the greatest τ value of the two is chosen as the data point for that β , with those taken from reverse cross-correlations taken as the negative.

In practice, it is challenging to take advantage of the cosine dependence because of the highly directional flow. A more effective method involves mapping the maximum amplitude of the cross-correlation between a central and surrounding pixels onto the position of the surrounding pixel. The cross-correlation amplitude function can be found by setting the derivative of the angle-dependent cross-correlation function (eq 4) equal to zero, yielding eq 12:

$$G(\tau_{\max}) = S \frac{1}{N} \exp \left[\left(\frac{R^2}{\omega_0^2} \right) \sin^2 \alpha \right] \quad (12)$$

To remove the ambiguity in the angle resulting from the identity $\sin^2(\alpha) = \sin^2(-\alpha)$ an additional parametric term, S , is introduced (eq 13):

$$S = \begin{cases} 1: -\frac{\pi}{2} \leq \alpha \leq \frac{\pi}{2} \\ -1: -\frac{\pi}{2} > \alpha > \frac{\pi}{2} \end{cases} \quad (13)$$

As before, forward cross-correlations between the center pixel and surrounding pixel are taken as positive values and the reverse cross-correlation as the negative. Once the value for the angle is known, the velocity can be determined using eq 11 with pixel pairs most closely colinear with the determined flow angle. While eq 11 relies on values returned from the fitting of cross-correlations, eq 12 determines angles when using the maximum of the measured cross-correlation, removing the need for an accurate fitting to a potentially noisy data set. This shortcut does not allow for the highest precision when mapping maximum correlation amplitudes, but this lack of precision is of little consequence when averaged out in the subsequent fitting of the cross-correlation map.

Pixel pairs that are far from the flow vector will return poor cross-correlations of low intensity. Fitting the peak position rather than the amplitude weights these pixels equally with those closer to the flow vector and so a fit to a noisy correlation would obscure the expected cosine dependence. To avoid such an issue, strict constraints must be placed on the acceptable τ values returned by the algorithm. In addition, very large (upwards of 50,000 frames) data sets are required in order to improve the quality of the cross-correlations and therefore accuracy of the returned τ value. With the amplitude-mapping algorithm those poor cross-correlations have low amplitudes and pixel pairs closer to the flow vector will yield cross-correlations of higher amplitude, effectively weighting the angle fit in favor of directions of higher-quality cross-correlations and closer to the true flow angle. This results in an order of magnitude decrease in the length of the necessary data set and an increase in accuracy in the flow vector measurements over the method relying on measured delays.

RESULTS AND DISCUSSION

Noise Tolerance

Cross-correlations have the distinct advantage of being able to accurately measure steady-state properties by trading precision in time for the often more important tolerance to noise. While particle-tracking algorithms can more precisely determine the translation of a particle

between one frame and the next, it comes at the cost of having to have particles bright enough to locate in every frame, requiring a S/N of around 4 to determine individual particle displacements with an average error of less than 0.1 pixels/frame.⁶

The advantage goes to correlation analysis when the average across a larger time range is acceptable and steady-state values are desired. Only the intensity fluctuations corresponding to actual physical events are correlated—those due to noise are uncorrelated. In addition, fluctuations from particles making the transit through only one of the two focal volumes in a cross-correlation yield no contribution to the resulting correlation function. In particle tracking, out-of-plane diffusion seriously limits trajectory lengths.

An improvement in S/N of each image will improve the quality of the analysis in both correlation and particle tracking methods. For the most difficult case of fast-moving, dim particles, one can have longer exposure times or take more frames of data, both of which benefit correlation analysis, while for particle tracking, only the former is an available option. Because the particles must be located in each individual frame, more frames with too low a S/N will be of no benefit.

The resulting error in measuring flow speeds demonstrates the ability of the algorithm to tolerate a low S/N in the input image data. Simulations indicate that by sampling 10,000 frames, the maximum tested, a S/N as low as 1.4 will generate fits as good as any higher S/N value (see Fig. 3). At these intensities, it is very difficult to find the particle locations, rendering particle tracking even more difficult, if not impossible. Single frames representing identical bead positions at S/N values of 31.4 or 1.4 are shown in Fig. 4. With a sufficient number of frames in the data stack either S/N values can be used to generate an accurate flow map.

While particle tracking does allow one to know the exact positions of the imaged particles in each frame of data, this information does come at the cost of necessary S/N and often is unnecessary, especially for the application described here. For situations where the locations of the particles is unimportant but rather the average motion of the particles is of concern, the use of cross-correlation flow mapping allows the use of data with lower S/N with improved accuracy.

3-D Flow Mapping

As described previously, 3-D flow mapping is achieved by stacking the 2-D flow maps determined at the known axial distances at which the image stacks were recorded. From this determination of the flow volume, slices along any arbitrary vector or axis can be taken. The resulting plots of a microfluidic device show a radially symmetric cross section, with flow fastest in the center. To our knowledge, this is the first time the flow for an entire microfluidic device has been imaged using a correlation method. The obvious applications of this method include the straight-forward manner that devices can be screened for asymmetries and anomalies inherent in device manufacture.

The 3-D flow maps measured for a rectangular channel appear in Fig. 5. Stacked correlation maps can be arbitrarily sliced to yield different views of the of the flow throughout the device volume. Here slices have been taken along three perpendicular axes. Flow speeds at different positions in the channel can be fit to the analytical solution for laminar flow in a rectangular channel.^{9,28}

$$v_x(y, z) = \frac{v'}{2} b^2 \cdot \left\{ 1 - \frac{z^2}{b^2} + 4 \sum_{n=1}^{\infty} \left[\frac{(-1)^n \cosh[\gamma_n(y/b)]}{\gamma_n^2 \cosh[\gamma_n(a/b)]} \cos\left(\gamma_n \frac{z}{b}\right) \right] \right\} \quad (14)$$

$$\gamma_n = \frac{\pi(2n-1)}{2} \quad (15)$$

in which a and b designate the half widths of the rectangular channel and v' is the characteristic velocity of the channel. The infinite series arises from the analytical solution for the correction of flow in a rectangular channel versus two infinitely-spaced parallel plates. Velocities were averaged along the flow direction at the desired positions to yield the data plotted in Fig. 6. As seen, the data fit the expected form for a rectangular channel very well along the in-plane axis (xy) while there is a reasonable fit along the axial (z) direction. This slight asymmetry is suspected to be due to nonlinearities in the transformation of the piezo nosepiece.

Experiments were commonly run at high enough concentrations of tracers to obscure the positions of the particles. At these higher concentrations the entire area of channel is occupied with multiple particles at once, avoiding any danger of missing subtle effects in the channel from lack of recorded particle paths.

Full Flow Vector Determination

The interesting parts of channels are rarely where things actually move in a straight line. Here, we have calculated the flow vectors near a partial obstruction (position indicated by circle) in a channel (Fig. 7). The flow vectors follow the expected path around the obstruction to recombine downstream with flow moving faster at positions away from the obstruction than closer.

Demand for computational time is much higher for this method for two main reasons: (1) this algorithm involves taking typically 10–25 pairs of pixels at each center instead of a single cross-correlation in the above flow-aligned method; (2) Each pixel pair requires two cross-correlations, not just one. Even with these additional needs the ability to measure flow vectors in a device without prior knowledge of the flow direction at a particular point in a channel is, in practice, more useful than measuring flows in a straight channel.

Reducing Fluorophore Size

Because of the advantages afforded by correlation analysis, the option to reduce the size, and therefore brightness, of the tracer particles becomes possible. While 100 nm beads were the typical tracer of choice, it was possible to image flow speeds with 20-nm beads and anti-mouse IgG antibodies conjugated to approximately 5–7 Alexa 488 dyes. Diffusion of the antibodies was retarded by adding 10% PEG 8K to the solution. FCS indicates the radius of hydration for these particles to be $R_h = 15$ nm (data not shown). A single image of antibodies in a microfluidic channel as well as the resulting flow map appear in Fig. 8.

Using even smaller particles—single Alexa488 dyes—as tracers in this system was attempted, but was unsuccessful. Even with very high concentrations of PEG, slow flow speeds, and a large number of frames (up to 100,000), we were unable to extract a significant cross-correlation. By inferring from the antibody data, for which a single point gave a signal of approximately 700 counts/frame, and assuming the manufacturer-specified

5–7 dyes/particle, a single dye under similar conditions should yield 100–120 counts/frame, which is close to the noise floor. With brighter dyes or a sufficient number of frames, it should be possible for this experimental scheme to map flows with single organic dyes as tracers, a feat that particle tracking would have a much harder time accomplishing. However, even using antibodies as tracers represents an order of magnitude improvement in tracer particle size over the typically used 100+ nm fluorescent beads.

CONCLUSIONS

Here we have found a novel method for determining flow vectors through pixel-pair cross-correlation analysis. In conjunction with a Nipkow disk confocal scanner, 3-D flow maps in a microfluidic device were determined as well as flow vectors in an area with inhomogeneous flow vectors. This method has also demonstrated a large tolerance for noise and low fluorescence intensity from the tracer particles. Because of the generality of the algorithm put forth here, the use of this method on systems other than microfluidic devices should be straight-forward. In addition, cross-correlations are not limited to flow phenomena. By fitting the cross-correlation to other models (diffusion, hindered diffusion, etc.) this method can be applied to a large number of other systems. With the use of brighter dyes, this method could well be applicable to measuring flow rates over large fields of view in three dimensions with single organic dyes as tracer particles.

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Fig. 1. Diagram of angles used in angle-fitting analysis. Here, α designates the angle between the flow vector and the spacing vector, $\vec{R}$, β the angle between $\vec{R}$ and the x-axis, and Ψ that between the flow vector and x-axis.

Fig. 2. Measured τ as a function of angle β from measured cross-correlations (circles) and fit (line). Those values not fitting within constraints are set at $\tau = 0$ and not included in the fit.

Fig. 3. Relative error of the flow map with simulated data as a function of S/N of individual frames (eq 9) and number of frames sampled. With 10,000 frames sampled a S/N as low as 1.4 is sufficient to obtain a correlation map comparable to higher S/N values.

Fig. 4. Pair of simulated images with identical bead positions with S/N ratios of 31.4 (a) and 1.4 (b) from a stack of 10,000 under a simulated parabolic flow between 0 and 1 pixels/frame towards the top of the image. With sufficient frames the flow map of both image stacks can be determined with an error less than 0.02 pixels/frame.

Fig. 5. Velocity profiles taken along axes perpendicular to flow. (a) Data (circles) taken for varying z positions from bottom of channel (bottom curve) to center (top curve). (b) Data taken along varying y positions from left (bottom curve) to center (top curve) of channel. Fits to eq 14 in black.

Fig. 6. Slices of 3-D flow volume taken along perpendicular axes. Flow in channel was parallel to x-axis.

Fig. 7. Flow vectors around a channel obstruction (circle). For reference, the highlighted arrow indicates a flow at -2.01 radians relative to the x-axis and a speed of $287 \mu\text{m}/\text{sec}$.

Fig. 8.

(a) Single frame of Alexa488-labeled IgG in 10% PEG solution flowing towards top of image. (b) Measured flow for channel with labeled IgG as tracers. The streaks in the flow map result from a less-than-ideal concentration of tracers in the channel.