Accuracy improvement of centroid coordinates and particle identification in particle tracking technique

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Abstract We have applied an algorithm to improve the accuracy of particle identification and centroid coordinates for each particle image in particle tracking technique. The algorithm introduced two techniques; 1) cutting off by each threshold at the peak in the pixel intensity distribution for each image of local area around the particle, and 2) calculation of the centroid based on pixel intensities in the original image of the particle instead of binarized data. The former properly cuts the noise in the background for each particle which has large variety in level particle by particle due to fluctuating illuminations and out-of-focus particles in the image, and the latter avoids the loss of accuracy by the commonly used binarization. We have demonstrated that the algorithm significantly improves the accuracy in determination of centroid coordinates and the correctness in particle identification. We have also validated the advantage of the algorithm in accuracy by applying the algorithm to a sequence of confocal microscopy images of diffusing particles in a polysaccharide solution. This algorithm will be significantly useful in particle tracking technique for biological systems, especially for fluorescence microscopy observations with considerable obstructive stray fluorescent signals.

Keywords carrageenan, particle tracking, weighted centroid, micro-rheology

Abbreviations
\begin{itemize}
\item \(c(x_0, y_0)\) initial centroid coordinate of \(j^{th}\) particle
\item \(L_{org,j}\) local image of \(j^{th}\) particle
\item \(d_{s}\) number of pixels for one side of \(L_{org,j}\)
\item \(L_{sm,j}\) smoothed image of \(L_{org,j}\)
\item \(I_{sm,j}(m, n)\) pixel intensity indexed by \((m, n)\) on the \(x\) and \(y\) axis of \(L_{sm,j}\)
\item \(m = 1, \ldots, d_s\) pixel row index of \(L_{sm,j}\)
\item \(n = 1, \ldots, d_s\) pixel column index of \(L_{sm,j}\)
\item \(I_{cut,j}\) calculated cut off intensity of \(L_{sm,j}\)
\item \(I_{cut,j}(m, n)\) pixel intensity indexed by \((m, n)\) on the \(x\) and \(y\) axis of \(L_{cut,j}\)
\item \(C_{w,j}\) weighted centroid coordinate of \(L_{cut,j}\)
\end{itemize}

1. Introduction

Particle tracking has been used in numerous applications to study the mechanical and rheological properties of microscopic environments in polymer gels like Carbopol gels [1], in biological systems such as intercellular region of live cells [2, 3], and actin solutions and bundles [4] and in food gels, such as gelatin [5], β-glucan solutions [6], carrageenan systems [7, 8] and emulsion systems [9, 10] providing valuable information on the microenvironment rheology, that is, the local rheological parameters in the spatial range of the particle size [11, 12].

Several methods have been developed to track probe particles for the micro-rheological studies in different media. The commonly used algorithm to track particles utilizes the binarization at a threshold for the whole image of each frame, and then, the binarized image data are used to calculate the center of mass or the weighted centroid [13]. Because of its simplicity and fast processing, this method is widely used in image processing routines and
Particle tracking algorithm. However, the simplification by the binarization loses details of the image and leads to an inaccurate calculation of the centroid for each particle. Further, improper determination of the threshold can cause significant error in the centroid calculation especially for images with large noise, undesirable images of fluctuating illuminations and out-of-focus particles. There are some approaches to calculate the centroid without using the binarization, accordingly a cut off is obtained at a certain percentage from the maximum intensity and applied to all the particles in the frame [14–17]. Meanwhile no use of the threshold resulted in unsatisfactory accuracy due to the background pixel intensity with the noise [14]. Since particle tracking technique can contribute valuable insights in the micro-rheology, the improvement of accuracy in the calculation is extremely important.

In this study, we have applied an algorithm to improve the accuracy of centroid coordinates and particle identification in particle tracking technique. The algorithm introduced a cut-off threshold for each image of local area around the particle, and a calculation of the centroid based on the pixel intensity instead of binarized data. The former is expected to properly cut the noise in the background for each particle, and the latter avoids the loss of accuracy from the binarization. Improvements of the accuracy of centroid coordinates and the correctness in particle identification have been examined. The advantage of the algorithm has been validated by applying the algorithm to a sequence of confocal microscopy images of diffusing particles in a polysaccharide solution.

2. Materials and Methods

2.1 Particle tracking algorithm

Particle tracking algorithm was implemented in Mathematica 10 (Wolfram Research, Inc., Champaign, IL). Fig. 1 shows the flowchart of the particle tracking algorithm. The main steps of the algorithm included: Step 1) selection of candidate particles and determination of the initial centroids, Step 2) calculation of the weighted centroid coordinate of each candidate particle and Step 3) link the centroids in a series of frames into trajectories for each particles. In Step 1, the candidate particles were determined in each frame and the initial centroid coordinates for each particles were obtained. In Step 2, the local image of each particle was extracted, which may suffer from spiky noise due to background variations of in and out of focus particles and uneven illumination. The median filter was applied to the local image which removes spiky noise without affecting the particle image and Gaussian filter was applied to reduce the noise [18]. Cutting off by a threshold was performed to each local image to remove the effect of the background in the calculation of the weighted centroid. The weighted centroid coordinate was determined based on the pixel intensities of the particle. In Step 3, the particle was connected to the particle with the closest coordinate in the subsequent frame from the first to the end of the series of frames to obtain particle trajectory.

2.2 Experimental details

Carrageenan samples were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All carrageenan samples were dialyzed against NaCl solution and subsequently against deionized water, to obtain Na⁺ type carrageenan solutions. The samples for particle tracking experiments were prepared by diluting the dialyzed stock solution and KCl solution with deionized water separately, followed by heating at 70°C for 30 min under vigorous stirring. The prepared solutions were mixed to obtain a total carrageenan concentration of 1.5% w/w and KCl concentration of 10 mM and then heated at 90°C for 20 min. Subsequently, fluorescent labeled probe particles (0.1 μm, Green, Thermo Scientific) were added at a total solid concentration of ~0.01% (w/v). The sample solution was stirred and heated at 90°C for another 5 min to assure homogeneous dispersion of probe particles. The hot sample solution containing probe particles was placed in a custom-built sample chamber of a glass bottom dish (Matsunami Glass Inc., Ltd.) equipped with a temperature sensor for temperature monitoring and sealed with a cover glass and a silicone glue.

Particle tracking experiments were performed on a BZ-9000 microscope (Keyence), equipped with a PlanFlour 100× NA 1.30 oil-immersion objective lens (Nikon). Movies of the diffusing fluorescent labeled particles were recorded using a built-in 2/3 inch, 1.5 megapixel, 12-bit, monochrome cooled CCD camera (Keyence) at a frame rate of 7.5 frames per second. The video recording of diffusing probe particles was deconstructed into a series of frames using Virtualdub software wherein each frame has approximately 30-50 particles.

3. Results

The performance of the method was evaluated using a series of time lapse microscope frames. The approach was to determine the centroid coordinate of each particle based on the pixel intensities with proper cutting off method of the background intensity.

3.1 Calculation of weighted centroid coordinate

The series of frames were processed to track each probe particles by the particle tracking algorithm shown in Fig. 1. In Step 1-i, the candidate particles were determined by template-matching where a particle image was selected as a template and was used to find particles in each frame which
Fig. 1 Flowchart of the particle tracking algorithm.
have similar images with the template [19]. In Step 1-ii, the image of frame in Step 1-i was binarized to determine the candidate particles from the noisy background. In Step 1-iii, the initial centroid coordinate of the binarized image of the \( j \)th particle, \( c(x_0, y_0) \), was calculated using a built-in function in Mathematica.

In Step 2-i, the local image of the \( j \)th particle, \( L_{org,j} \), was clipped out from the raw frame as a square with the center of \( c(x_0, y_0) \), and a side of \( d_s \) pixels as shown in Fig. 2a. The \( d_s \) was set as the search diameter larger than the particle diameter and smaller than the average distance between particles. In microscopy experiments, the \( L_{org,j} \) suffers from a considerable imperfection including nonuniform contrast, random noise, and geometric distortion. In Step 2-ii, therefore, each \( L_{org,j} \) was treated by the 2-dimensional median filter and Gaussian filter. The former replaces each pixel intensity with the median value of the neighboring pixels and is effective to remove spiky noise without blurring the detail of particle image [17] and the later suppresses the random noise of the local image. As seen in Fig. 2b, the smoothed local image, \( L_{sm,j} \), showed a great improvement in the signal to noise ratio without affecting the details of the particle image.

The inclusion of the background intensities in the calculations of the weighted centroid coordinate leads to a larger bias and causes undesired inaccuracy [14]. Image thresholding was carried out for each \( L_{sm,j} \) to decouple the effect of the background by application of cut off intensity, \( I_T \). In the previous works [15–17], the \( I_T \) was set at a certain percentage of the maximum pixel intensity in the frame e.g. 30%–60%, which only include portion of the whole particle image. In our method, the cut off intensity for each \( L_{sm,j} \) \( I_{T,j} \) was calculated independently. In Step 2-iii, pixel intensities were ranked in ascending order as shown in Fig. 3, and \( I_{T,j} \) was calculated from the peak in the second derivative of the ranking. The peak indicates the steepest change in the frequency of pixel intensity in the \( L_{sm,j} \). This method extracts the whole intensity profile of the particle. In Step 2-iv, the cut off image \( I_{T,j} \) was applied to each \( L_{sm,j} \) to obtain the local image, \( L_{cut,j} \) using Eq. 1.

\[
I_{cut,j}(m,n) = \begin{cases} 
I_{sm,j}(m,n) & \text{if } I_{sm,j}(m,n) > I_{T,j} \\
0 & \text{otherwise}
\end{cases}
\]

where \( I_{sm,j}(m,n) \) is the pixel intensity in the \( L_{sm,j} \) indexed by integer \( m = 1, ..., d_s \) as the pixel row index and \( n = 1, ..., d_s \) as the pixel column index and \( I_{cut,j}(m,n) \) is the pixel intensity of the \( L_{cut,j} \). In Eq. 1, pixel intensity greater than the \( I_{T,j} \) are unaltered while pixel intensity lower than the \( I_{T,j} \) are set to zero.

It is expected that the intensity weighted centroid could provide sub pixel resolution for the particle tracking [15–17]. In Step 2-v, the weighted centroid coordinates of the \( L_{cut,j} \) \( C_{w,j} \) was calculated by

\[
C_{w,j} = c(x_0, y_0) + \frac{\sum_{m=1}^{d_s} \sum_{n=1}^{d_s} (c(m,n) \times I_{cut,j}(m,n))}{\sum_{m=1}^{d_s} \sum_{n=1}^{d_s} I_{cut,j}(m,n)}
\]

where \( c(m,n) \) is the integer coordinate of \( I_{cut,j}(m,n) \). The \( C_{w,j} \) is marked by white cross in Fig. 2c. The result on the calculation of the \( C_{w,j} \) showed efficacy on the determination of the centroid of particle.

### 3.2 Improvement of accuracy in weighted centroid coordinate for a geometrically distorted \( L_{org,j} \)

Fig. 4a shows the surface plot of \( L_{org,j} \) with geometric distortions and asymmetry. The geometric distortions of particles are usually caused by the optical aberrations in the microscope optics, uneven illumination and digitization of frames. High \( I_{T,j} \) (upper plane) and low \( I_{T,j} \) (lower plane) are also indicated. Fig. 4b shows the \( L_{org,j} \) with the outlines of cut off images using low \( I_{T,j} \) (thin) and high \( I_{T,j} \) (thick),

![Fig. 2](image) Local images of a candidate particle in a 21 × 21-array of pixel intensities \((d_s = 21)\). a) \( L_{org,j} \) b) \( L_{sm,j} \) c) Zoomed image of \( L_{cut,j} \) with the centroid coordinate (white cross).

![Fig. 3](image) Pixel intensity as a function of pixel index. The arrow indicates the steepest change in the frequency of pixel intensity.
which are corresponding to lower and higher planes in Fig. 4a, respectively.

The binarized images of the particle after cutting off with the low and high \( I_{T,j} \) are shown in Fig. 4c. The centroid coordinates from the binarized images with the low \( I_{T,j} \) and high \( I_{T,j} \) are indicated by white cross and “×”, respectively. A large difference between the centroid coordinates was observed. This indicates that a small change in \( I_{T,j} \) can cause a large shift on the centroid coordinate. The change in \( I_{T,j} \) comes from the randomly flickering noise in the background. As a consequence, the calculations for the particle movements, such as the mean square displacement, are affected by the flickering noise which frequently appears in the fluorescence microscopy observation.

On the other hand, the weighted centroid calculation is expected to improve the accuracy. Fig. 4d shows the \( L_{org,j} \) of the particle using the low and high \( I_{T,j} \) with the weighted centroid coordinates \( C_{w,j} \) calculated by Eq. (2) indicated by white cross and “×”, respectively. The results of the centroid calculation with different \( I_{T,j} \) did not show a significant difference in the centroid coordinate. This indicates that the centroid is not significantly affected by the level of the applied \( I_{T,j} \). In the calculation of the weighted centroid coordinate using the image intensity of the particle, each pixel intensity has a different value which mitigate the change of \( I_{T,j} \) by fluctuating noise level. Additionally, the distribution of the values of pixel intensity provides the information on the actual centroid coordinate of the particle. Finally, the algorithm showed improved accuracy on the determination of centroid coordinate even for geometrically distorted particles.

3.3 Link the particle centroids

The last part of the algorithm (Step 3) links the weighted centroid coordinates of each particle in a subsequent frame to construct particle trajectories. The algorithm was accomplished by identifying the same particle in the series frames using the nearest neighbor calculation, where, each particle was connected to the closest particle in the subsequent frame by calculating the inverse of square distance. Particles connected in the next frame were used in the next iteration while those particles which were not connected with any particle in the next frame were not used in further calculations. Finally, the trajectory of Brownian motion of fluorescent particles in a polysaccharide solution were obtained and used to extract the information on the local physical property (micro-rheology) of the media, such as mean square displacement (MSD) [20, 21].

4. Conclusion

In this report, we have demonstrated an improvement in the accuracy of particle tracking algorithm which identifies each probe particle with different threshold intensity in one source frame. The particle tracking algorithm facilitates to recognize, locate and track particles simultaneously from the first to the end for a series of frames. The algorithm was applied to systems with fluorescent probe particles in a variety of background intensities and considerable overlapping fluorescent signals of in-and-out-of-focus particles. The algorithm allows us to determine the centroid coordinates and track the movement of each particle with improved precision. With the proposed algorithm, the diffusive motion of fluorescent probe particle was determined and tracked with sub-pixel resolution. We believe that the algorithm presented here provides an improvement on the determination of the centroid coordinates which provide great importance in studying the microrheological property of the different complex fluids.

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