

AUTOMATION OF PARTICLE DETECTION AND TRACKING OF MOTOR
PROTEINS

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[Murti Salapaka]

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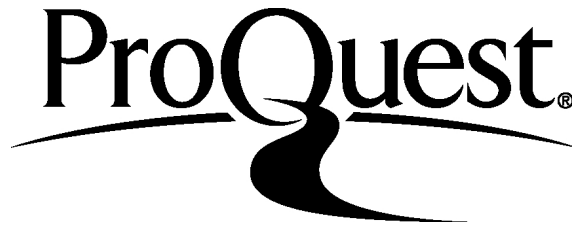
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Dedication

This thesis is dedicated to my parents, Shivanna P and Mayura S and my brother Chandrajith Kodi Shivanna.

Abstract

Cells are the basic structural, functional and biological functional unit in living organisms. Cells are usually called “fundamental building blocks of life” due to their ability to replicate independently. The study of cells and cell processes is imperative in the study of living organisms. Particle detection and tracking plays a vital role in understanding the processes that occur in living cells. Biological processes involve complex and dynamic machinery which makes it very difficult to analyze and draw conclusions from the observations. Technological advancements have significantly improved the quality and quantity of data that can be collected: particles with nanometer resolution can now be imaged with intricate details over a significant interval of time, thus providing us access to information about the biological processes at a cellular and molecular level. The extensive use of fluorescent probes sheds light on the different particles and their roles in the various processes. However, there are a lot of factors which affect the processes that are not under our control thereby inhibiting us from successfully detecting and tracking particles. The presence of a plethora of particles which vary in size, nature, density of occurrence, fluorescence, nature of motion etc. makes it impossible to have a unified detection and tracking algorithm that can provide us with the most accurate results. The presence of a wide number of independent parameters some of which are mentioned above makes it hard to simulate a process and hinders the understanding of processes and drawing conclusions from them. This study mainly focuses on summarizing some of the most popular detection and tracking algorithms. Towards the end, the developed detection and tracking algorithm is applied to study bidirectional axonal cargo transport.

A video containing the result of the tracking algorithm has been submitted as Supplementary Video 1.

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Chapter 1

INTRODUCTION

The various developments in technology over the last two decades has greatly increased our ability to conduct experiments and obtain data at the cellular and molecular levels. This growth can be attributed to the development of a range of fluorescent probes and the milestones that have been achieved in the field of optical microscopy. The improvements in optical microscopy provide us the insight that is necessary to understand biological processes at the molecular and cellular level. The availability of a wide range of fluorescent probes provides us more control over the visualizations of experiments and helps us focus on particles of interest. These advancements provide us access to a plethora of experimental data; however being able to separate data of interest from the entire set is still a challenge.

Particle tracking plays a major role in studying the movement and behavior of proteins along the cell membrane, aids us in studying the nature of the motion of particles at the cellular level. Knowledge of the nature of motion of particles equips us to better understand biological processes and cellular kinetics. Motor proteins are responsible for the transport of cellular cargo and malfunctions in their behavior are linked to multiple human diseases [3]. The particles of interest are usually labelled with the help of an optical label or a fluorescent tag, which helps distinguish these from others that may also be present in the medium. Once these particles are easily identifiable the next step in the process is to track them.

Tracking a particle involves observing the motion of particles in the medium by estimating the positions of the particles during this time. Tracking of particles is typically a very laborious process since it is complicated by the fact that there are number of factors which are not under our control. The diversity in the type, size and shapes of particles found in the abundant biological processes makes it hard to have a uniform detection scheme. In case of 2D imaging, particles tend to move in and out of the plane of focus which leads to temporary disappearance and reappearance of particles;

depending on the nature of the process itself particles may merge or split; typically the density of the particles of interest is also not under the control of the experimental set-up. The above mentioned constraints make it hard to have a universal solution that detects and tracks particles for all, or even most of the applications under our interest. In order to accurately detect and track particles, algorithms will have to be developed for the specific application that is being investigated.

This thesis focusses on developing and validating an algorithm for tracking motor proteins moving along the axons. A detection algorithm was developed and Linear Assignment Problem (LAP) [1] based tracking algorithm was used to track motor proteins. A comparative study of some of the existing algorithms was also performed as a part of this study [2].

Chapter 2

MOTIVATION

Manual tracking involves observation of particle motion in a series of images that are obtained as a part of the experimental set up.

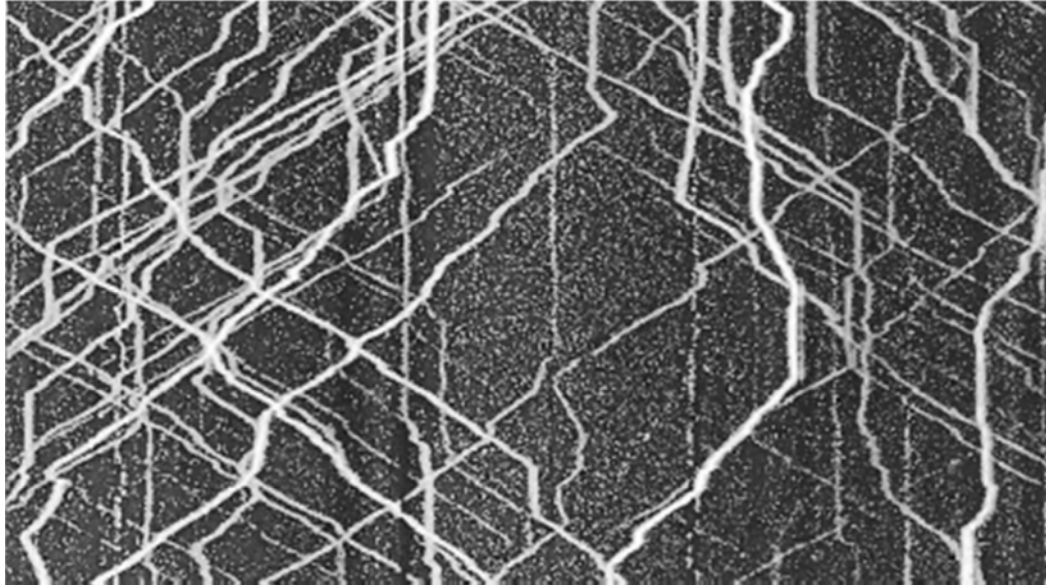


Figure 1: Kymograph

Currently, once the images have been obtained, kymograph of the series of images is generated. A kymograph is a graph which contains the estimates of the position of the particles versus time. Once the kymograph is generated, the required statistics are calculated manually by observing the kymograph. The number of particles, the mean velocity of the particles in the anterograde direction, standard deviation of the particles in the anterograde direction, the mean velocity of the particles in the retrograde direction and the standard deviation of the particles in the retrograde direction is calculated by calculating the slopes of the particle trajectories from the obtained kymograph. This is a laborious and time consuming process. Also, there is no way to validate the results as the results rely on observation and are likely to be subjective. This is the motivation behind working on this topic as part of the dissertation.

The main objective of this study is to ease the difficulties associated with the manual tracking process and to come up with a suitable algorithm to successfully track the particles and obtain results that are as accurate as or more accurate than the results obtained by manual tracking. The goal of this project is to automate the detection and tracking of particles and generate the required statistics as was being generated by the manual tracking. The relevant results that had to be generated were same as the ones generated from manual tracking.

Chapter 3

GENERAL APPROACH

The general approach for detection and tracking of particles usually consists primarily of two independent steps: detection and tracking. Once the data, which typically consists of sequence of images, has been obtained, the next step is to distinguish and separate the particles of interest from the rest of the data. This is the detection stage and typically comprises of image processing algorithms. Successful detection of the particles is followed by the tracking stage. The input to this tracking stage is the output of the detection stage. Tracking essentially involves linking the detected particles in space and time for the entire set of data consisting of the detected particles. It now becomes clear that in order to detect and track particles in a sequence of images, both detection and tracking have to be accurate independent of the each other.

At the detection stage tradeoffs have to be made based on the constraints present which in turn depend on the application that is being studied. At the detection stage attributes like SNR, suppressing the background noise, determining the region of interest, image enhancement, particle filtering and other factors determine the robustness of the detection technique. The detection algorithm must be robust enough to deal with the changes in the detection parameters. Attributes of the particles of interest like shape and size of the particles can be exploited when developing the detection technique.

Once the particles of interest have been separated from the plethora of data, the next step involves linking of the particles in space and time along the entire sequence of images. This step is usually complicated by a series of factors, some of them being: number of particles is not constant and not under our control which makes tracking a large number of particles computationally expensive; particles undergo merging (two particles approaching each other within resolution limit) and splitting (unresolved particles diverging to resolvable distances) [4] [5] based on the biological process being considered; particles also tend to move in and out of the plane of focus which results in

temporary disappearance and reappearance of particles; nature of the motion of particles of interest also varies depending on the particle itself.

The following chapters cover the details of the detection and tracking algorithm that is implemented to study the bidirectional movement of motor proteins.

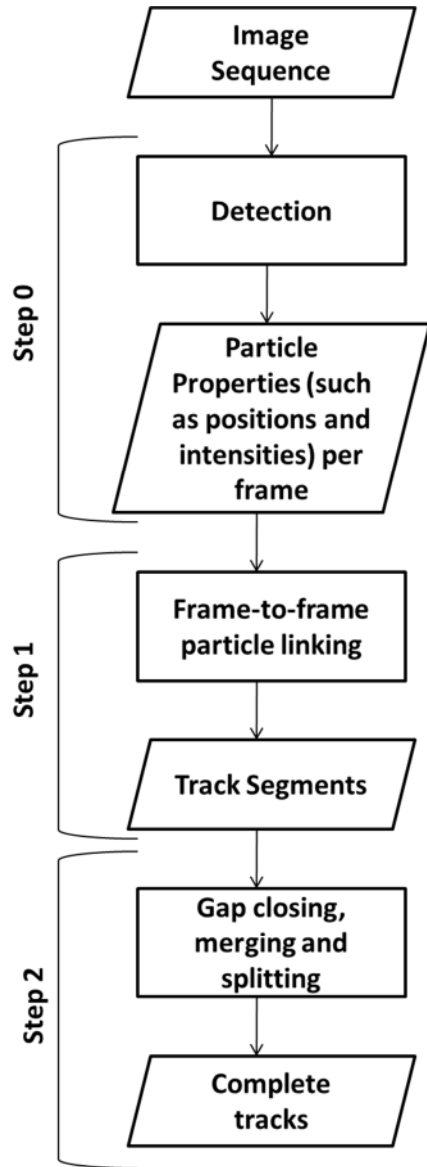


Figure 2: Particle tracking flowchart [1]

Chapter 4

DETECTION ALGORITHM

The first step in the development of a tracking algorithm is to successfully separate the particles of interest from the rest of the data that is acquired during image acquisition. This process is unique depending on the nature of the images acquired.

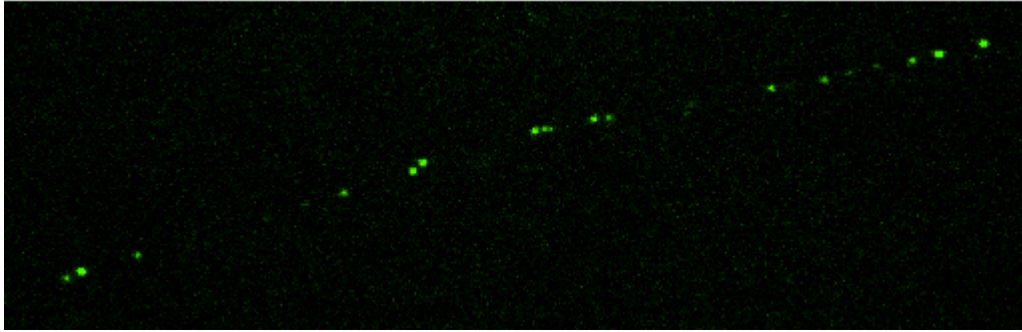


Figure 3: Raw image before detection

The following steps are a part of the detection algorithm developed for the application under consideration:

- Top hat transform resulting in increased disparity between the particles of interest and background.
- Image segmentation resulting in a binary image containing the objects.
- Opening of the binary image resulting from image segmentation.

These steps result in the separating of the particles from the raw image data.

The different steps involved in detection are explained in detail in the following sections. Top-hat transform and opening of an image are based on morphological image processing techniques. The next section explains the basics of morphological image processing and it is followed with the steps implemented as a part of the detection algorithm.

4.1. Morphological image processing

Morphological image processing were originally described for binary images and eventually extended to gray-scale images. The concepts of morphological image processing are explained for binary images in the section that follows and its extension to gray scale images is explained at the end of the section.

Morphological image processing techniques consist of non-linear operations based on shapes or morphology of features present in an image. Morphological operations probe an image with a small shape or template called a structuring element. The structuring element is moved to every pixel in the input image and it is compared to the corresponding neighborhood of pixels. This morphological operation results in an output image containing non-zero values only at the pixels that passed the test.

The morphological operations are typically based on set theory and rely on the fact that the objects in an image can be represented as points belonging to a set.

4.1.1. Binary Morphology

An object in a binary image can be represented in terms of a set depending on presence of absence of a certain property. In case of a binary image, this property can be the intensity value associated with the pixels.

Binary morphology consists of two primary components.

- Structural element: Consists of a simple pre-defined shape which is used to probe an image.
- Operators: The different operators that constitute binary morphology are erosion, dilation, opening and closing. The operations erosion, dilation and opening form the basis of the detection technique implemented and are explained in detail.

Erosion

The erosion [28] of a binary image A by a structuring element B is defined as:

$$A \ominus B = \{z | (B)z \subseteq A\} \dots(1)$$

Where A and B are sets in Z^2 . The above equation indicates that the erosion of A by B consists of all points Z such that B, translated by z, is contained in A. From equation

(1) it is clear that B is a subset of A and this leads to the following equivalent definition of erosion:

$$A \ominus B = \{z | (B)_z \cap A^c = \Phi\} \dots (2)$$

Where A^c the complement of A and Φ is an empty set.

As the structural element is translated in the binary image, the value of the output pixel is the minimum value of all the pixels in the input pixel's neighborhood. This neighborhood is defined by the structuring element.

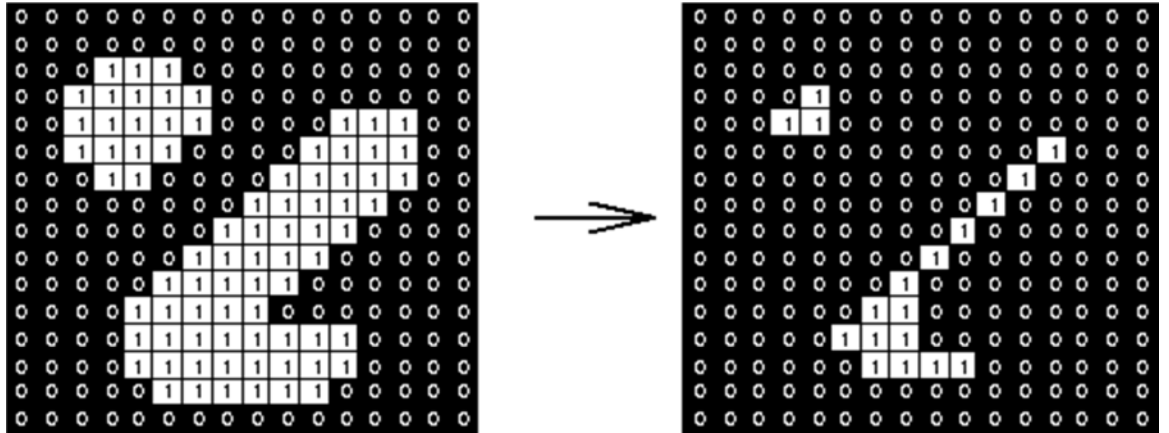


Figure 4: Erosion using 3X3 structuring element

It can be observed from Figure 4 that erosion results in a thinning effect, the area of the foreground pixels decreases. Erosion can be used to eliminate small bright spurious noise spots (salt noise) and it can also be used for edge detection. Edge detection can be obtained by subtracting the original image from the eroded image.

Dilation

The dilation [28] of a binary image A by a structuring element B is defined as:

$$A \oplus B = \{z | (\hat{B})_z \cap A \neq \Phi\} \dots (3)$$

Where A and B are sets in Z^2 . $\hat{B}$ is obtained by reflecting B about the origin, and the reflection is then translated by z. The dilation of the image A by the structuring element B consists of the set of all displacements of $\hat{B}$ such that overlap of $\hat{B}$ and A has at least one element. Dilation can be equivalently defined as:

$$A \oplus B = \{z | [\hat{B}_z \cap A] \subseteq A\} \dots (4)$$

As the structural element is translated in the binary image, the value of the output pixel is the maximum value of all the pixels in the input pixel's neighborhood. This neighborhood is defined by the structuring element.

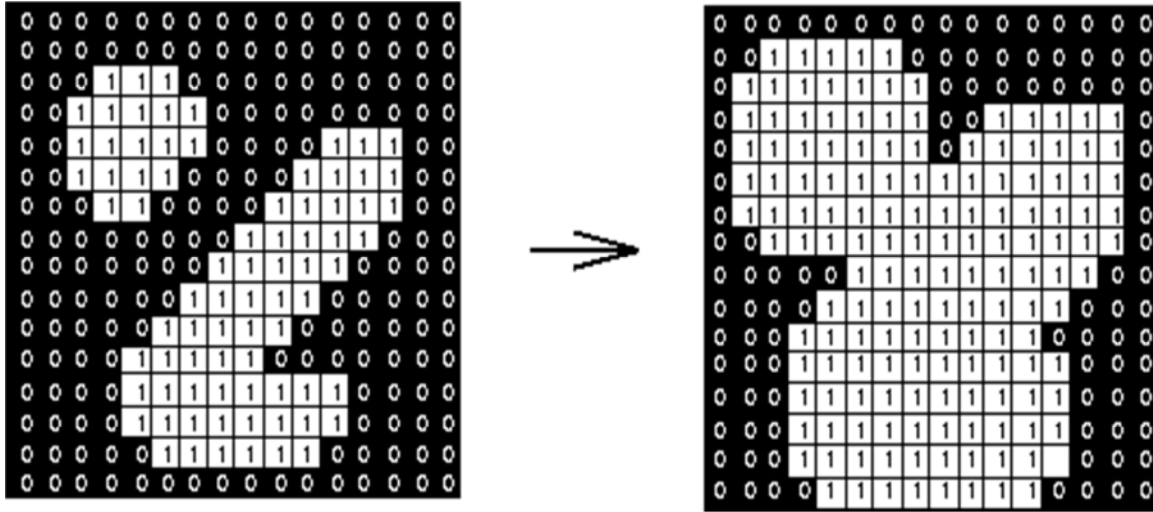


Figure 5: Dilation using 3X3 structuring element

It can be observed from Figure 5 that dilation results in a thickening effect, the area of the foreground pixels increases. Dilation can be used to eliminate small dark spurious noise spots (pepper noise) and it can also be used for edge detection. Edge detection can be obtained by subtracting the dilated image from the original image.

Opening

Dilation expands the components of an image while erosion shrinks the components in an image. Both these operations can have destructive effect on the images based on the nature of the structuring element. Opening[28] is a combination of erosion and dilation and smoothens the contours in an image, breaks narrow isthmuses and eliminates protrusions.

The opening of an image A by structuring element B is denoted as $A \circ B$, it is defined as:

$$A \circ B = (A \ominus B) \oplus B \dots (5)$$

From equation (5) it is clear that the opening of an image A consists of eroding the image by a structuring element B, the eroded image is then dilated with the same structuring element.

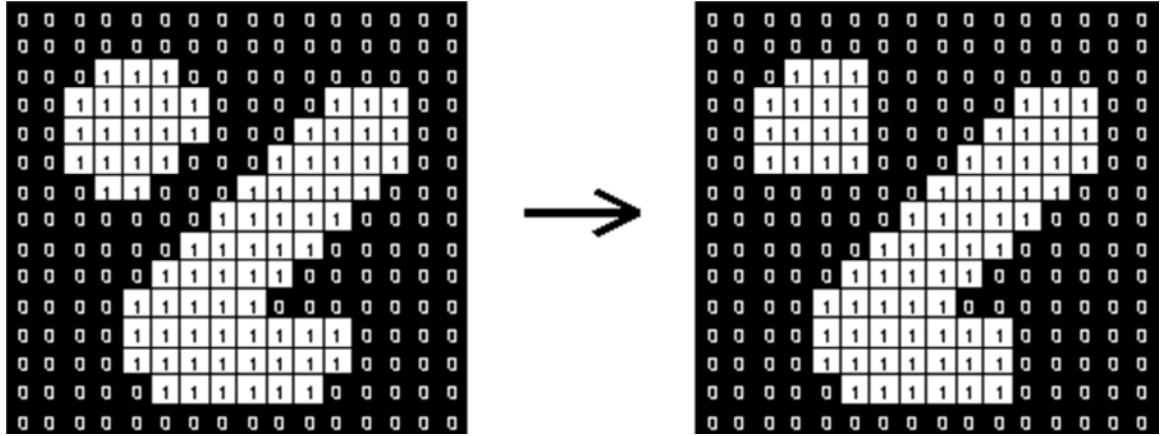


Figure 6: Opening using a 3X3 structural element

Opening of an image results in preserving the foreground pixels that have a similar shape relative to the structuring element, or those pixels that can completely contain the structuring element and erodes away the pixels that do not contain the structuring element.

The binary morphological processes that are defined for binary images can be extended to grey-scale images. When performing dilation the value of the pixels in the output image is set to the maximum value of all the pixels in the input pixel's neighborhood. In case of binary images this value is 1, but in case of grey-scale images the value is the maximum in the neighborhood. Similarly, in case of erosion the value of the pixels in the output image is set to the minimum value of all the pixels in the input pixel's neighborhood. In case of binary images this value is 0, but in case of grey-scale images the value is the minimum in the neighborhood.

4.2. Top hat transform

Top hat transform [28] is a morphological image processing technique that can be used to process images based on shape or morphology of features of an image. Morphological processing typically consists of applying a structural element to an input image which results in an output image of the same size. The value of each pixel in the output image is based on a comparison to the corresponding pixel with its neighborhood in the input image. The right choice of shape and size of the structural element determines the features that will be extracted from the input image.

Top hat transform extracts small elements and features from images. Top hat transform helps us extract light objects from a dark background which fits the nature of our input images. There are two kinds of top hat transform: white top-hat transform and black top-hat transform. White top hat transform is defined as the difference of the input image and its opening by the structural element and black top hat transform is defined as the difference of the closed image with the structural element[6][7][8] from the input image.

White top hat transform has been implemented as a part of the detection technique. The white top-hat transform of the binary image A is given by:

$$T_w(A) = A - (A \circ B) \dots (6)$$

Where ‘o’ denotes the opening operation on an image.

Top hat transform of an image results in a processed image which contains objects that are smaller than the structuring element and brighter than its surroundings. The size and width of the elements extracted from top-hat transform are controlled by the choice of the structuring element.

4.3. Image segmentation

Image segmentation is the process of dividing an image into different components in order to extract relevant information from digital images. This process typically involves clustering pixels into regions which correspond to our objects of interest. Segmentation of nontrivial images is one of the most difficult tasks in image processing and the accuracy of this process usually determines the eventual success or failure of analysis procedure. Successful segmentation of an image results in identification of objects of interest and elimination of the rest of the data in the digital image. When the objects of interest have been segregated, it is an indication that the segmentation should be stopped and further analysis should be performed on the segmented images.

Optimum global thresholding using Otsu’s method[10] is incorporated into the detection technique. Thresholding results in conversion of gray scale images to binary

images with pixels below a certain intensity value being assigned 0 and the pixels above a certain intensity value is assigned 1.

Otsu's global thresholding

Intensity based thresholding is the technique used to perform image segmentation in the detection process that is developed. Thresholding results in conversion of gray scale images to binary images with pixels below a certain threshold value being assigned 0 and the pixels above a certain threshold value is assigned 1. On successful thresholding the objects of interest will be separated from the background. Optimum global thresholding using Otsu's method[10] is incorporated into the detection procedure. Otsu's method selects the threshold by minimizing the within-class variance of the two groups of pixels separated by the thresholding operator. The basic idea is that well-thresholded classes should be distinct in terms of the intensity values of their pixels and conversely a threshold giving the best separation between classes in terms of their intensity values would be the optimal threshold.



Figure 7: Example of thresholding

4.4. Detection technique illustration.

This section explains the detection technique developed for this application in a step by step manner. It also illustrates the effect each of the steps has on the images.

Figure (9) represents a sample frame from the experimental image data. This image is processed to separate the particles of interest from the data. This image is first converted to a grey-scale image.

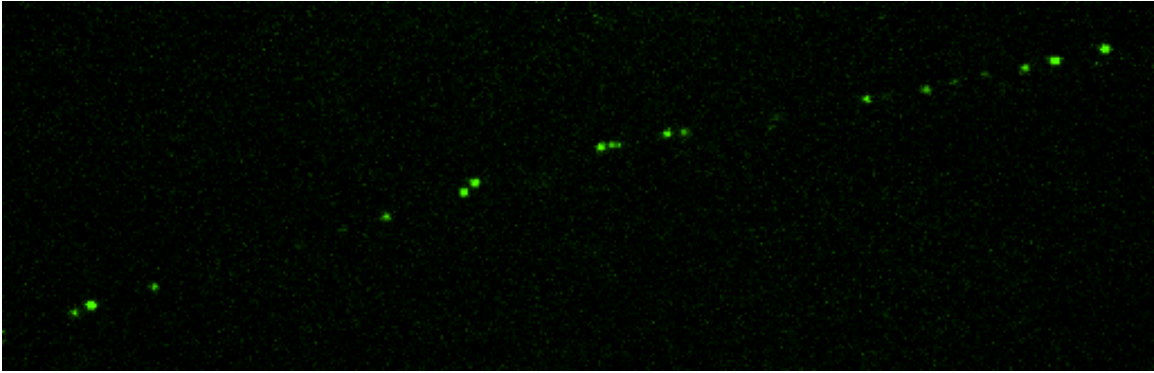


Figure 8: Frame from image data

Top-hat transform is performed on the grey-scale image. The structuring element to perform the top-hat transform is a disk of radius 3 pixels. It comprises of all the pixels within a radius of 3 pixels from the center of the disk. Top-hat transform helps eliminate some of the noise and even out the non-uniform illumination which aids in image segmentation.



Figure 9: Top hat filtered image

Figure (10) depicts the result of top-hat transform. The top-hat transform is followed by image segmentation using Otsu's global thresholding.

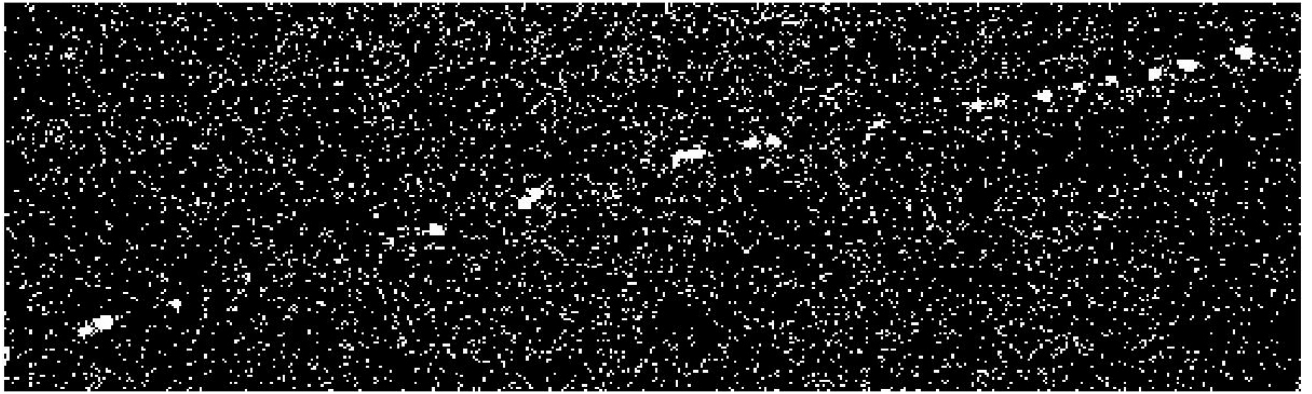


Figure 10: Otsu's optimum thresholded image

On observing the segmented image, it is observed that the noise still persists and the objects have not been separated from the rest of the data. In order to obtain the objects of interest, opening operation is then performed on the thresholded image.

Opening of the image is performed using a disk shaped structural element of radius 1. The shape and radius of the structuring element is a key factor, since the shape and the size of the noise and the particles are comparable. This small radius is chosen in order to ensure that only the noise is eroded and the particles persist.



Figure 11: Result of opening the image

Once the opening of the image is successful, the position of the objects of interest is estimated by taking the centroid of the objects of interest. The X co-ordinate and the Y coordinate of the centroid position is then stored. The intensity value at the estimated centroid coordinate is also stored for further processing.

Chapter 5

TRACKING ALGORITHM

The tracking algorithm studied is the single particle tracking (SPT) described in the reference [1]. The algorithm described addresses the challenges faced in SPT which typically involve a high density of particles, the nature of movement of particles, the various types of particles, temporary disappearance of particles. It also addresses the scenario which involves the merging and splitting of particles during the course of a phenomenon which complicate the tracking process since it is not limited to detection and localization of particles of interest. The association of particles through time and space under the various conditions mentioned above is one of the key factors that govern the success of a tracking algorithm.

The most accurate solution to the SPT is provided by the Multiple Hypothesis Tracking (MHT) [16]. MHT involves constructing all the possible tracks for a single particle in the entire sequence of data assuming the positions of the particles is known. The solution is then chosen as the longest sequence of tracks that do not conflict with any other particle. MHT provides us a solution that is globally optimal both in time and space. This method however is computationally very expensive even in the case of low density of particles as it involves keeping track of every possibility for every particle. The best solution would involve to obtain results similar to the one provided by MHT but in a computationally less expensive and feasible way. There are a number of methods which try to achieve the results obtained by MHT in a computationally efficient way, but most of them however try to obtain this solution in a locally optimum manner. Some methods establish particle correspondence between simultaneous frames (accounting for locally temporal solution) [15] and then combine these in order to achieve globally optimum spatial solution [17-23]. Some methods treat merging and splitting of particles as temporary disappearance of particles [17-19] while others consider them as separate events [24][25]. Most of the existing algorithms can handle one or the other concerns associated with SPT, but none of them can address all the issues involving SPT

successfully. Hence, based on the application under consideration the various concerns regarding SPT must be weighed and the appropriate tradeoffs will have to be decided upon.

A Linear Assignment Problem (LAP) [26] [27] based tracking algorithm is used to track the particles detected in the sequence of images. The details of the algorithm are described in the next section.

5.1. Linear Assignment Problem (LAP) based tracking algorithm.

The tracking algorithm described and studied here is independent of the detection and localization of the particles. Once the particles in the sequence of images have been detected and localized the results are then applied to the tracking algorithm described below.

The tracking algorithm is a two-step process. The first step involves linking of particles in consecutive frames which results in track segments; the second step involves linking the track segments obtained in the first step in order to obtain the complete the trajectories of the particles in the entire sequence of images. Both the steps are formulated as a GLOBAL COMBINATORIAL OPTIMIZATION PROBLEM and result in the most likely set of particle trajectories in the image sequence.

The key factor that determines the success of this algorithm is establishing the correspondence between particles in the consecutive frames which are affected by particle density, nature of particle motion, temporary disappearance and reappearance of particles, merging and splitting of particles, the nature of the particle, fluorescence and the light conditions that prevail during image acquisition.

The initial particle assignment is greedy since it only considers particles in consecutive frames in order to generate track segments; this step looks for a locally optimum solution in time. The second step which involves linking the track segments to generate complete particle trajectories is a globally optimum solution both spatially and temporally.

In the Linear Assignment Problem framework, each assignment is characterized by a cost and the solution to this problem is given by a combination of assignments which minimizes the cost.

$$\hat{A}_{\arg \min} = \sum_{i=1}^{\text{Number of rows}} \sum_{j=1}^{\text{Number of columns}} A_{ij} C_{ij} \dots (7)$$

$$\sum_{i=1}^{\text{Number of rows}} A_{ij} = 1 \quad \sum_{j=1}^{\text{Number of columns}} A_{ij} = 1 \quad \dots (8)$$

Equations (7) and (8) denote the representation of LAP in the mathematical format.

In the above equations A is an assignment matrix where the value 1 indicates that there is a possibility of a link and the value 0 indicates that the link is not possible and the assignment matrix $\hat{A}$ indicates that the assignment minimizes the sum of the costs.

The second equation ensures that every particle/track can link only to one particle/track making them mutually exclusive. As a consequence of equation (8), we can be assured that every particle will have only one assignment. In case of potential multiple assignments the different possibilities compete against each other in order to achieve a minimum.

5.1.1. Frame to frame linking: Cost function parameters.

There are 3 potential types of assignments that can occur in case of frame to frame linking of particles:

- A particle in frame (t) can link to a particle in frame (t+1). (Cost function l)
- A particle in frame t may not link to anything in frame (t+1) indicating the death of a track segment. (Cost function d)
- A particle in frame (t+1) may not have any link in the previous frame indicating the birth or track initiation. (Cost function b).
- x indicates impossible link whose cost exceeded the cut-off.
- Lower right block is an auxiliary block required to satisfy the topological constraints of LAP framework.

This step results in locally optimum solutions since we restrict the linking to consecutive frames.

		Linking						No linking			
		Particle index						Particle index			
Frame $t+1$	Frame t	1	2	3	4	...	n_{t+1}	1	2	...	n_t
Linking	Particle index 1	ℓ_{11}	ℓ_{12}	x	ℓ_{14}	...	x	d	x	...	x
	2	ℓ_{21}	ℓ_{22}	ℓ_{23}	x	...	x	x	d		x
	$\vdots$	$\vdots$						$\vdots$	$\ddots$		
	$\vdots$										
	Particle index n_t	x	x	...	$\ell_{n_t, n_{t+1}-1}$	$\ell_{n_t, n_{t+1}}$		x	x	...	d
No linking	Particle index 1	b	x	...			x				
	2	x	b				x				
	$\vdots$	$\vdots$	$\ddots$								
	$\vdots$										
	Particle index n_{t+1}	x	x	...			b				

Lower right block

Figure 12: Cost matrix for frame-to-frame linking step.

5.1.2. Track segments linking: Cost function parameters

The second step involves linking the incomplete track segments resulting from the first step in order to generate complete particle trajectories in the sequence of images. This involves more assignments to the cost function as they also consider the particle disappearance/ reappearance and also the phenomenon of particle splitting/merging.

There are 6 potential types of assignments that can occur in case of closing gaps between track segments:

- The end of one segment links to the beginning of another segment.(Cost function g)

- End of segment could link to middle of another segment, accounting for merging of particles. (Cost function m)
- Beginning of a segment could link to the middle of another segment, accounting for splitting of particles. (Cost function s)
- End of a segment could link to nothing, leading to the end of a particle trajectory termination. (Cost function d)
- Beginning of a segment could link to nothing, leading to a particle trajectory initiation. (Cost function b)
- A middle segment could link to nothing, this leading to a no merge or split. (Cost function b' and d').

The different segments across the length of the movie compete in both space and time, hence resulting in a globally optimum solution in both time and space.

The cost functions described above have to be tailored in order to suit the application under consideration.

In the application under consideration the cost function is demonstrated considering isotropic random motion like pure or confined Brownian motion of particles due to their abundant presence in biological phenomenon.

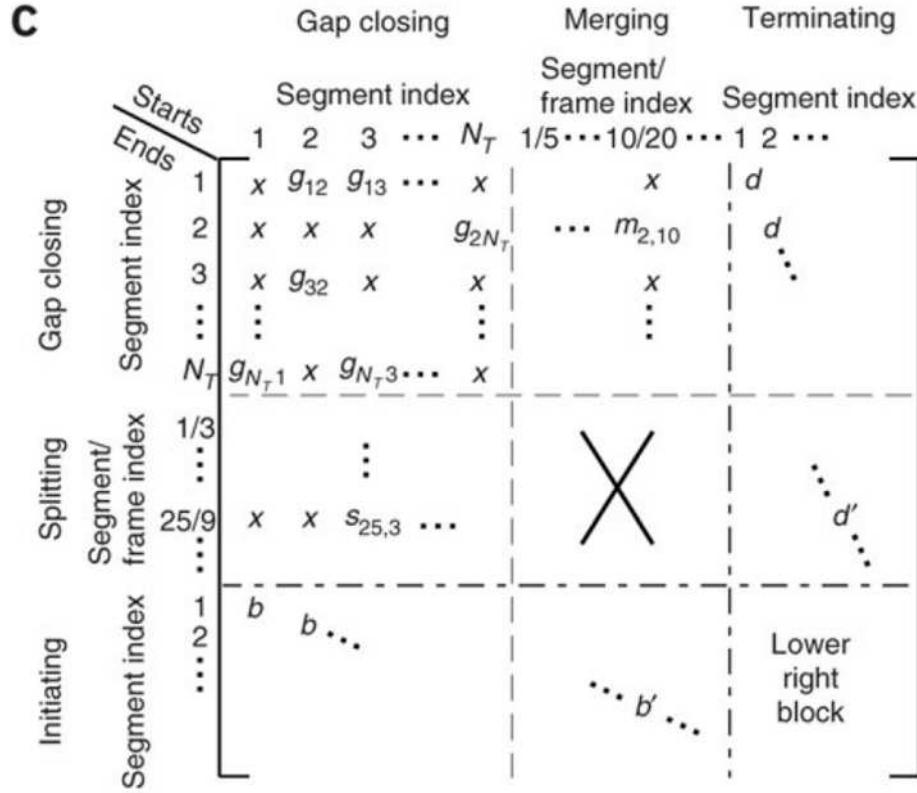


Figure 13: Cost function for track segments linking step.

The cost functions were parameters of distance and intensity:

$$l_{ij} = \delta_{ij}^2, \dots \dots \dots (9)$$

$$g_{IJ} = \delta_{IJ}^2, \dots \dots \dots (10)$$

$$m_{IJ}, s_{IJ} = \begin{cases} \delta_{IJ}^2 * \rho_{IJ}, \rho_{IJ} > 1 \\ \delta_{IJ}^2 * \rho_{IJ}^{-2}, \rho_{IJ} < 1 \dots \dots \dots \end{cases} (11)$$

$$\rho_{IJ}(\text{merge at time } t) = \frac{A_j(t)}{A_I(t-1) + A_j(t-1)}, \rho_{IJ}(\text{split at time } t) = \frac{A_j(t-1)}{A_I(t) + A_j(t)} \dots \dots \dots (12)$$

The intensity factor made sure that the cost function accounted for the difference in intensities before and after merging or splitting occurred. This made sure that the track segments were not linked only based on how close the track segments were located, but also only if the intensities were consistent with the merging or splitting were the track segments linked.

Cut-offs was introduced in order to make sure that physically impossible solutions were not being considered during the computation of trajectories. Every particle or track segment was not allowed to link to any particle or track segment, cut-offs were introduced in the form of apriori to ensure that impossible links were not a part of the solution. These cut-offs were both a function of distance and the intensity of particles.

Cost Matrix Computation:

The steps in order to compute the cost matrix were:

- Cut-offs was introduced in order to eliminate physically impossible solutions.
- The cost function was calculated for all potential assignments.

5.1.3. CUT-OFFS

Frame to frame linking:

1. A maximum search distance was calculated and beyond this distance particle linking was considered to be impossible. This distance was calculated for each particle based on its previous displacements and the particle density is also considered by taking into account the nearest neighbor distance in calculating the search distance. The details for calculating this parameter in supplementary information of [1].

2. In order to reduce initial false assignments, the user also set minimum and maximum limits on the distance. The minimum search radius was set 2 pixels and the maximum search radius was set 10 pixels, and any particle detected beyond this could not link to the detected particle.

3. The flag corresponding to local density was set to 1 to indicate that the local density has to be taken into account while calculating the search radii for linking of detected particles in the consecutive frames.

4. It is a possibility that a particle in one frame doesn't link to anything in the consecutive frame and that a particle in a frame does not link to anything in the previous frame. In order to account for this, the cost of no link should be decided. Any particle with a cost greater than this value will not link to anything in the consecutive frame.

Similarly, if a particle had potential links of lower costs but it lost all the competitions against all other particles, then it does not link to any particle.

Gap closing, merging and splitting parameters:

1. A time window within which track segment ends can be linked to beginning of other track segments.

A very small window results in a number of incomplete trajectories while a big window may result in erroneous linking. A time window of 10 frames was chosen with the assumption that a particle does not disappear for more than 10 frames before it can reappear.

2. A search radius was defined for every track segment start and end in a manner similar to frame to frame linking. Both the displacements of the track segments and the local density of the particles were considered in order to arrive at this constraint.

3. The flag corresponding to local density was set to 1 to indicate that the local density has to be taken into account while calculating the search radii for linking of track segments.

4. We should be able to differentiate between track segment start and end resulting from particle disappearance and reappearance, splitting or merging from true track initiations and terminations. A number of cost function parameters are defined in order to achieve this successfully.

5. Alternative costs b and d corresponding to true initiations and terminations had to be defined. The costs have to be comparable to other potential assignments to make rejection of gap closing, merging and splitting a feasible option at the same time they had to be high enough to ensure that most of the gaps were closed. On empirical testing this was chosen to be 90th percentile since the gap closing did not vary with change in alternative cost in the 80th-100th percentile.

6. The flag corresponding to merge/split was set to 1 to ensure that the algorithm accounted for merging and splitting of particles.

7. Lower and upper limits on the ratio of intensities before and after merging or splitting were defined. Any ratio of intensities beyond the range was not considered as possible events of merging and splitting.

8. Alternative costs in order to refuse a merge or split were defined.

Whenever information was not available, initial guesses were used in order to compute the cost matrix parameters and steps were taken in order minimize the impact of wrong initial guess. A detailed description of the cost parameters are described in the Supplementary information associated with [1]

Chapter 6

RESULTS

The algorithm (both detection and tracking) was implemented in MATLAB. It was tested in Matlab2014b.

A GUI was designed as the front end of the implementation. A video file (AVI) format has to be loaded into the GUI. Once the file is successfully loaded, it is followed by the detection and tracking. The entire process has been automated and when the tracking is successfully completed, the results are displayed on the GUI.

A provision to generate the Kymograph of the tracked results is also available. The kymograph is generated with the assumption that the movement of particles is one dimensional and the particles move only along the X axis while the movement along Y direction is considered to be insignificant and ignored.

This entire process is simple, straight-forward and quick as the user does not have to worry about filling in parameters or deal with the intricacies of the code. The automation of the entire process further reduces the time taken to compute the relevant details.

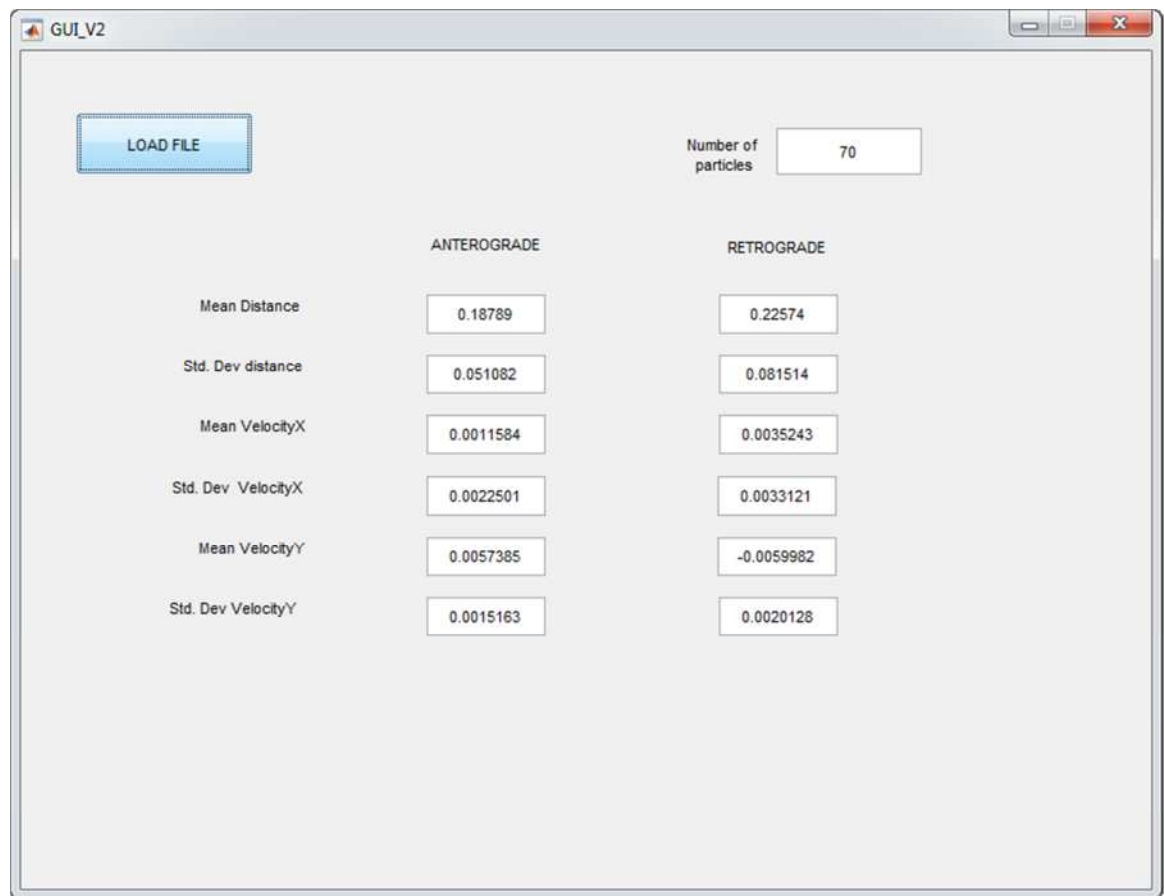


Figure 14:GUI

Anterograde and retrograde denote the direction of movement of the objects of interest. Anterograde means movement of the particles towards the body of the cell and retrograde means movement of the particles away from the body of the cell. The velocity statistics generated regarding the anterograde and retrograde directions play a vital role in drawing conclusions regarding the conditions and abnormalities that may prevail.

The associated parameters and the values used in the implementation are as follows:

Structural element: Shape- Circle, Radius-3

Maximum time difference to consider closing gap: 10 frames

Merge/Split flag: 1.

Minimum track length: 5 frames.

Minimum search radius: 2 pixels

Maximum search radius: 10 pixels

The number of tracks that were successfully tracked in the sequence of images was 63.

The distance is in micrometer. The velocity is in micrometer per frame.

The velocity statistics are as follows:

ANTEROGRADE		RETROGRADE	
Mean distance	0.18	Mean distance	0.225
Distance standard deviation	0.05	Distance standard deviation	0.08
Average velocity X direction	0.001	Average velocity X direction	0.003
Velocity standard deviation X direction	0.002	Velocity standard deviation X direction	0.003

Table 1: Velocity Statistics

Chapter 7

VALIDATION

One of the biggest challenges faced by researchers in particle detection and tracking is the ability to verify and validate the results of the tracking since ground truth is not available in case of real image data, also manual tracking of particles can be subjective and inferior in comparison to automated and algorithmic tracking especially when the number of particles are large.

The detection and tracking algorithm developed as a part of this project is validated independently via 2 different methods. In the first method the algorithm described above is tested on different types on synthetic data and the performance metrics are measured. In the second method the results of some of the existing detection and tracking algorithms is compared with the results of manual tracking and the algorithm developed as a part of this dissertation.

7.1. Method 1

It uses the metrics and the data defined in [2].

The synthetic data is generated for a wide range of features and conditions [2]. The algorithm is tested for synthetic data containing receptors and vesicles. This data is generated for two different SNR values and also for 3 different density ranges of the particles themselves. The various conditions for which the algorithm is validated include:

- Receptors with SNR 4 and particle density low, medium and high.
- Receptors with SNR 7 and particle density low, medium and high.
- Vesicles with SNR 4 and particle density low, medium and high.
- Vesicles with SNR 7 and particle density low, medium and high.

In order to validate the results and quantify how good and accurate the algorithms are, performance metrics have been defined. In this case 4 performance metrics have been defined and the algorithm is validated by calculating these metrics.

If an algorithm is accurate, it essentially means that most of the tracks in the data are not only successfully detected but it is also accurately paired to the track in the sequence of images. Consider the set of tracks in the ground truth (denoted by X) and the set of estimated tracks by the algorithm constitute the set Y . It is likely that Y will be missing tracks; in order to complete the set of estimated tracks the set Y is extended with dummy tracks denoted by Z . Once this is complete, the tracks are assigned based on optimal subpattern assignment based on Munkres algorithm (reference 59). This yields us the best possible global pairing (minimum total distance) for each track in ground truth X (θ_k^X) with either an estimated track Y (θ_k^Y) or a dummy track Z (θ_k^Z). The distance between the tracks is calculated as the sum of the distances between all the points on the track in the sequence of images. Euclidean distance is considered to calculate the distance between the estimated and the ground truth tracks and if at any point in time if a particle is missing from the estimated track a dummy point is considered.

The total distance between the track sets X and Y is the sum over all k of the distances $d(\theta_k^X, \theta_k^Y)$ between the track pairs minimized by Munkres algorithm by optimizing Z . The performance metrics are developed based on this and defined below:

- $\alpha(X,Y)=1-d(X,Y)/d(X,\Phi)$. Φ is a set of dummy tracks and $d(X,\Phi)$ denotes the maximum possible distance from the ground tracks.(error). α lies in the range of (0 1) and denotes the overall degree of matching. 0 indicating the worst, and 1 indicating that everything matched without taking into account the spurious tracks.
- $\beta(X,Y)= (d(X,\Phi)-d(X,Y))/(d(X,\Phi)+ d(Y(\text{bar}),\Phi))$, where the spurious tracks are into account to analyze the overall similarity in the estimated tracks. $Y(\text{bar})$ is the set spurious tracks and $d(Y(\text{bar}),\Phi)$ is the compensation term. The value of β ranges from (0, α), where 0 is the worst and α is the best possible value it can take.
- $JSC= (TP+FP+FN)$, JSC is the Jaccard Similarity Coefficient for track points. It ranges from (0,1) where 0 is the worst, and 1 is the best. TP is True Positives and denotes the optimally matched track points, FP is False positives which constitute the non-matching points including spurious detections and FN is False negatives which denote the dummy points in the optimally paired tracks.

- $JSC_0 = (TP_0 + FP_0 + FN_0)$, JSC_0 is the Jaccard Similarity Coefficient for the entire tracks. It ranges from (0,1) where 0 is the worst and 1 is the best. TP_0 is True Positives and denotes the optimally matched complete tacks, FP_0 is False positives which constitute the non-matching tracks including spurious tracks and FN_0 is False negatives which denote the dummy points in the optimally paired tracks.

Case 1: The maximum limit on the distance between the reference and tracked particles is considered to be 5 pixels.

Synthetic data parameters	Performance metric(alpha)	Performance metric(beta)	Similarity between tracks(Jaccard)	Paired track statistics	Similarity between detections(Jaccard)
Receptor SNR: 4, density:high	0.2079	0.1471	0.4569	813 of 1307	0.3321
Receptor SNR:4 Density:low	0.2421	0.1881	0.5320	191 of 274	0.4333
Receptor SNR:4 Density:mid	0.2308	0.1781	0.5817	388 of 561	0.4029
Receptor SNR:7 Density:high	0.2168	0.1608	0.5188	814 of 1302	0.3647
Receptor SNR:7 Density:low	0.2618	0.2075	0.6449	207 of 282	0.4633
Vesicle SNR: 4, Density:high	0.1930	0.1501	0.5628	927 of 1325	0.3634
Vesicle SNR: 4, Density:low	0.2477	0.2	0.4822	203 of 251	0.4716
Vesicle SNR: 4, Density:mid	0.218	0.1704	0.5791	344 of 493	0.4056
Vesicle SNR: 7, Density:high	0.1867	0.1407	0.5340	847 of 1304	0.3449

Vesicle SNR: 7, Density:low	0.2649	0.2302	0.7422	190 of 230	0.5283
Vesicle SNR: 7, Density:mid	0.2342	0.1956	0.6434	406 of 539	0.4669

Table 2: Performance metrics for Case 1

Case 2: The maximum limit on the distance between the reference and tracked particles is considered to be 10 pixels.

Synthetic data parameters	Performance metric(alpha)	Performance metric(beta)	Similarity between tracks(Jaccard)	Paired track statistics	Similarity between detections(Jaccard)
Receptor SNR: 4, Density:high	0.3523	0.2635	0.5274	895 of 1307	0.4364
Receptor SNR:4 Density:low	0.4321	0.3572	0.6320	213 of 274	0.5927
Receptor SNR:4 Density:mid	0.4127	0.3461	0.6961	433 of 561	0.5779
Receptor SNR:7 Density:high	0.3791	0.2982	0.6112	904 of 1302	0.4934
Receptor SNR:7 Density:low	0.4589	0.4015	0.7483	226 of 282	0.6491
Vesicle SNR: 4, Density:high	0.3757	0.3121	0.6660	1029 of 1325	0.5224

Vesicle SNR: 4, Density:low	0.4721	0.3981	0.5407	219 of 251	0.6359
Vesicle SNR: 4, Density:mid	0.4231	0.3542	0.6840	381 of 493	0.5799
Vesicle SNR: 7, Density:high	0.3688	0.2989	0.6351	945 of 1304	0.5046
Vesicle SNR: 7, Density:low	0.5016	0.4550	0.8130	200 of 230	0.7059
Vesicle SNR: 7, Density:mid	0.4540	0.4017	0.7197	434 of 539	0.6586

Table 3: Performance metrics for Case 2

On analyzing the results in the above table, it can be observed that depending on the performance metric being considered, the performance of the algorithm varies drastically. Another important feature that determines the performance of the algorithm is the maximum boundary threshold distance that is allowed for a detection to qualify as a true detection. The performance can considerably improve or deteriorate depending on the metric under consideration.

When the maximum boundary threshold distance is considered to be 5 pixels, the metrics JSC and the JSC_0 metrics which denote the overall particle and track detection performance fair better than α and β which are a measure of overall similarity in the tracks. On increasing the maximum threshold distance to 10 pixels the performance metrics α and β improve as a consequence of relaxing the upper bound on the maximum search radius for particle detection and association. From the following observations it

can be concluded that on relaxing the upper bound on the maximum search radius, the performance of the algorithm improves.

It can also be observed that the algorithm performs best when the density of particles is low, as expected. This arises due to the fact that it is easier to link particles along frames when the density is low. The results are similar in case of both receptors and vesicles as both particles possess similar features, both of them have similar size and shape which gives us similar results in either of the case. The best results are obtained then the maximum threshold distance is set to 10 pixels, density of particles is low and SNR of the images is 7.

7.2. Method 2

The method developed and tested as a part of this study was designed to track the movement of motor proteins. As a consequence of the fact that the nature of the experimental images acquired was different from the synthetic data that was used to validate the algorithm in the previous section an alternative method to quantify the performance measure of the developed algorithm on experimental data is used. In order to validate the performance of the algorithm, the results of the algorithm on the experimental data was compared against the results of manual tracking on the same set of experimental data. Furthermore, some of the algorithms that were developed as a part of the study “Objective tracking of particle tracking methods” [2] was also used on the experimental data and the results obtained were compared against the results obtained by manual tracking. Some of the key features of interest that need to be extracted from the experimental data include the number of particles in the sequence of images, to be able to successfully track the different particles, and to successfully generate velocity statistic of the particles along different directions(mean and standard deviation).

7.2.1. Manual Tracking

This method is currently the most reliable but also the most time consuming method for the experimental data under consideration. In case of manual tracking, once

the experimental data is obtained, kymograph of the data is obtained. All the necessary observations and conclusions are made by observing the generated kymograph.

In order to generate the kymograph, once the experimental data is obtained an axis which contains the movement of maximum number of particles is chosen. A kymograph is then generated considering 1 dimensional movement of particles along this axis. On obtaining the kymograph, conclusions regarding the number of particles, number of tracks are obtained.

The total number of tracks in the entire sequence of images was found to be 58.

Calculating the slope of the trajectories, statistics regarding the mean of velocities and standard deviation of the velocity of particles is also calculated.

The alternative methods are first described briefly and their results are then compared with the results of the manual tracking.

7.2.2. Method developed

This method was specifically targeted to calculate the required parameters from the experimental data. All the algorithm associated parameters were fixed since the nature of images and the particles that were being imaged remained constant.

The associated parameters and the respective values were:

Structural element: Shape- Circle, Radius-3

Median Filter size: [2 2]

Maximum time difference to consider closing gap: 10 frames

Merge/Split flag: 1.

Minimum track length: 5 frames.

Minimum search radius: 2 pixels

Maximum search radius: 10 pixels



Figure 15: Detected objects in the first frame

The number of tracks that were successfully tracked were 70.

7.2.3. Alternative Method 1

This method was developed by Yannis Kalaidzidis [11] [12].

The algorithm consists of a pre-filtering stage which involves background subtraction, followed by detection by fitting a Lorentzian function to structures above noise level. Once the detection is complete, the linking of the particles in time is based on dynamic programming using a weighted sum of different features.

The parameters for the method were the same as the parameter values for the condition Vesicles with SNR 7 and for mid density of particles.

Associated Parameters and their respective Values:

- **Window size** for estimating the background (60 x 60 pixels).
- **Merging ratio** of intensities for overlapping particles : 0.90
- **Position weight** in the cost function for track assignment: 0.7.
- **Speed weight** in the cost function for track assignment: 0.01.
- **Area weight** in the cost functions for track assignment: 0.3.
- **Peak intensity weight** in the cost function for track assignment: 0.1.
- **Sum intensity weight** in the cost function for track assignment: 0.01.
- **Deviation weight** in the cost functions for track assignment: 0.01.

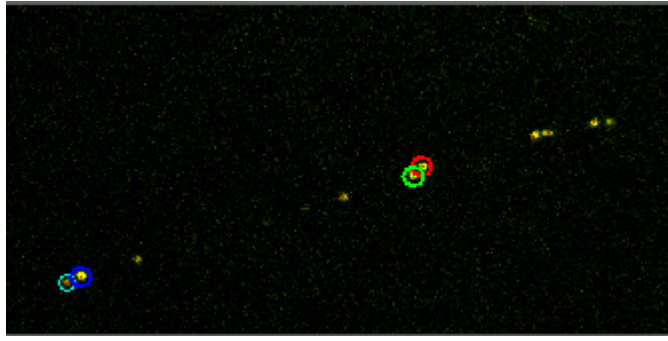


Figure 16: Image data overlapped with detected objects

It is observed from this image that some of the particles of interest are not detected for the data under consideration.

18 tracks were detected and tracked in this case.

7.2.4. Alternative Method 2

This method was developed by Ivo F. Sbalzarini, Yuanhao Gong, Janick Cardinale [13].

This algorithm consisted of detection of particles of interest by iterative intensity weighted centroid calculation followed by combinatorial optimization which involves greedy hill optimization with topological constraints for linking of particles in time.

The parameters for the method were the same as the parameter values for the condition Vesicles with SNR 7 and for mid density of particles.

Associated Parameters and their respective Values:

- **Radius** for local maxima detection and centroid computation: 2.
- **Cutoff** for discarding detections based on intensity moments: 3.
- **Percentile** of image intensities that detected maxim: 0.1%.
- **Link-range** used by the combinatorial optimizer for linking detections: 1 frame.
- **Link-length** cutoff for linking detections in successive frames: 10 pixels.

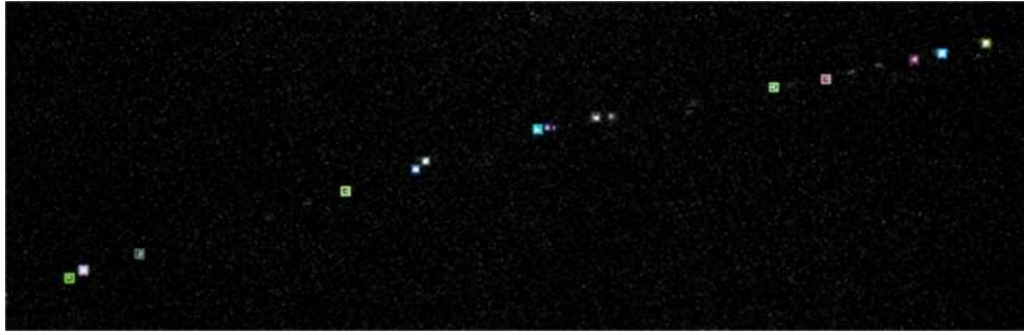


Figure 17: Detected particles in frame 1 of experimental data

A total of 158 tracks were detected and tracked in the sequence of images.

7.2.5. Alternative Method 3

This method was developed by Perrine Paul-Gilloteaux [14].

The pre-filtering is performed by Laplacian of Gaussian or Gaussian filtering and the detection is done with either maxima detection with pixel precision or a combination of thresholding and Gaussian fitting. The linking of particles is done by global optimization of associations using simulated annealing.

The parameters for the method were the same as the parameter values for the condition Vesicles with SNR 7 and for mid density of particles.

Associated Parameters and their respective Values:

- **Standard deviation** (sigma) of the Laplacian-of-Gaussian filter: 1.5pixels.
- **Tolerance level** used in the flood-fill algorithm: 20.
- **Search radius** used in the nearest-neighbor algorithm: 10.
- **Disappearance time** allowed in the linking algorithm: 2.
- **Number of iterations** in the simulated annealing algorithm: 100.

Based on the different levels of tolerance selected the number of particles detected and tracked vary.

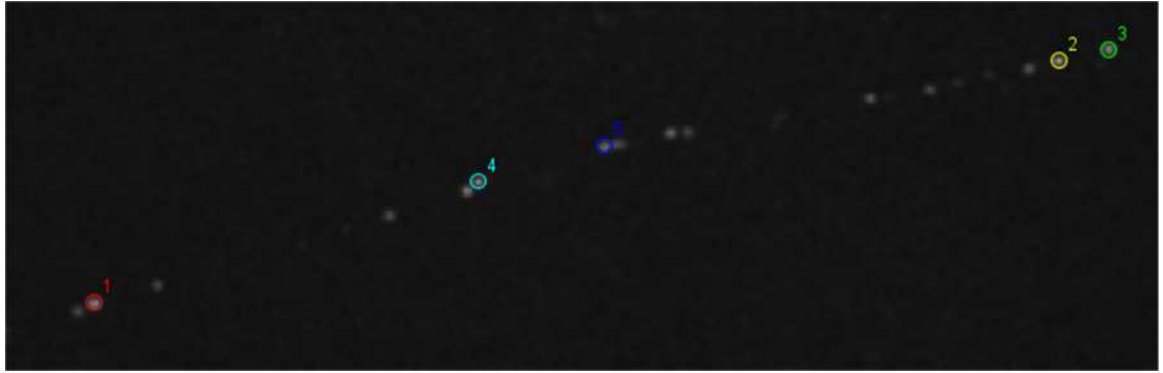


Figure 18: Tolerance 80-Particles detected

It can be observed that some particles have not been detected in this case. In case of the tolerance being set to 80, 12 tracks are tracked by the algorithm in the experimental data.

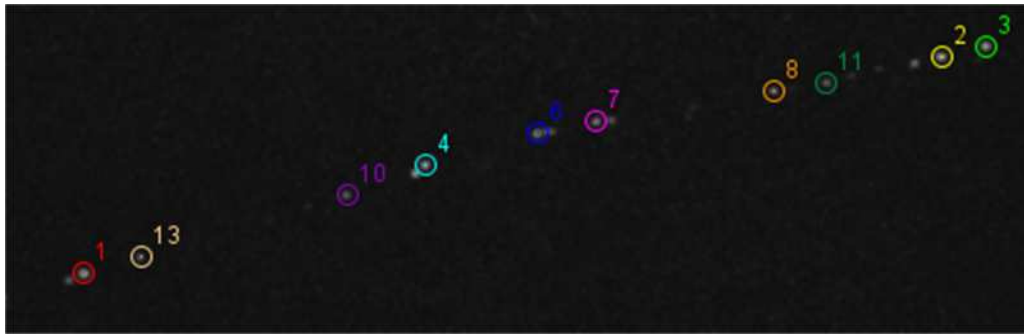


Figure 19: Tolerance 40-Particles detected

In case of the tolerance being set to 40, 25 tracks are tracked by the algorithm in the experimental data. However, some particles have still not been detected in the data.

More number of particles was detected when the tolerance was set to 40 in comparison to the number of the particles tracked when the tolerance was set to 80.

7.2.6. Alternative Method 4

This method was developed by Joost Willemsse, Katherine Celler and Gilles P. van Wezel [15].

Pre-filtering is first performed in order to increase the contrast between the particles of interest and the other particles. This is performed by Gaussian filtering or top

hat filtering. The detection is then performed by watershed-based clump splitting. The linking of the detected particles is based on the nearest neighbor approach which is capable of handling particle disappearance and reappearance. It also uses a linear motion model for the particles.

The parameters for the method were the same as the parameter values for the condition Vesicles with SNR 7 and for mid density of particles.

Associated Parameters and their respective Values:

- **Standard deviation** (sigma) of the Gaussian filters for noise reduction (1 pixel).
- **Intensity threshold:** 30 units
- **Minimum size** of the thresholded objects: 10 pixels.
- **Maximum range** within which to find nearest neighbors: 10 pixels.
- **Backward tracking range** within which to close gaps: 3 frames.
- **Minimum track length** in numbers of frames: 3 frames.

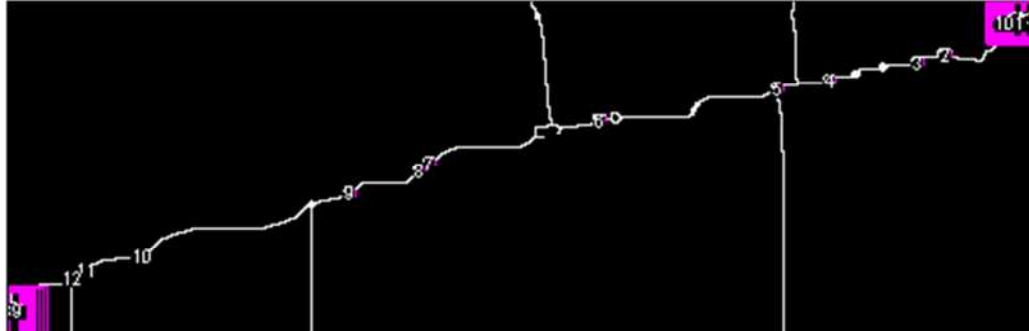


Figure 20: Number of particles detected in frame 1 of the experimental data

It was observed that 6 tracks were tracked in the sequence of images.

Method	Number of tracks detected
Manual tracking	58 tracks
Developed method	63 tracks
Alternative method 1(Yannis Kalaidzidis)	18 tracks

Alternative method 2(Ivo F. Sbalzarini, Yuanhao Gong, Janick Cardinale)	158 tracks
Alternative Method 3(Perrine Paul-Gilloteaux)	With tolerance set to 80: 12 tracks. With tolerance set to 40:25 tracks.
Alternative Method 4(Joost Willemse, Katherine Celler and Gilles P. van Wezel)	6 tracks

Table 4: Comparison of the different techniques studied for particle detection and tracking

Since the ground truth is not known in case of experimental results, the results of manual tracking are considered to be a yard stick to measure the performance of the other methods. On comparing the number of tracks that were tracked successfully, it can be observed that for the images under consideration the method developed as a part of this study is most effective and closest to the results of manual tracking.

The next step is to validate the accuracy of the algorithm with the results of the manual tracking as a reference. Kymograph is generated for the tracks detected and tracked by the algorithm described in this study. This generated kymograph is then compared against the kymograph which formed the basis for the manual tracking.

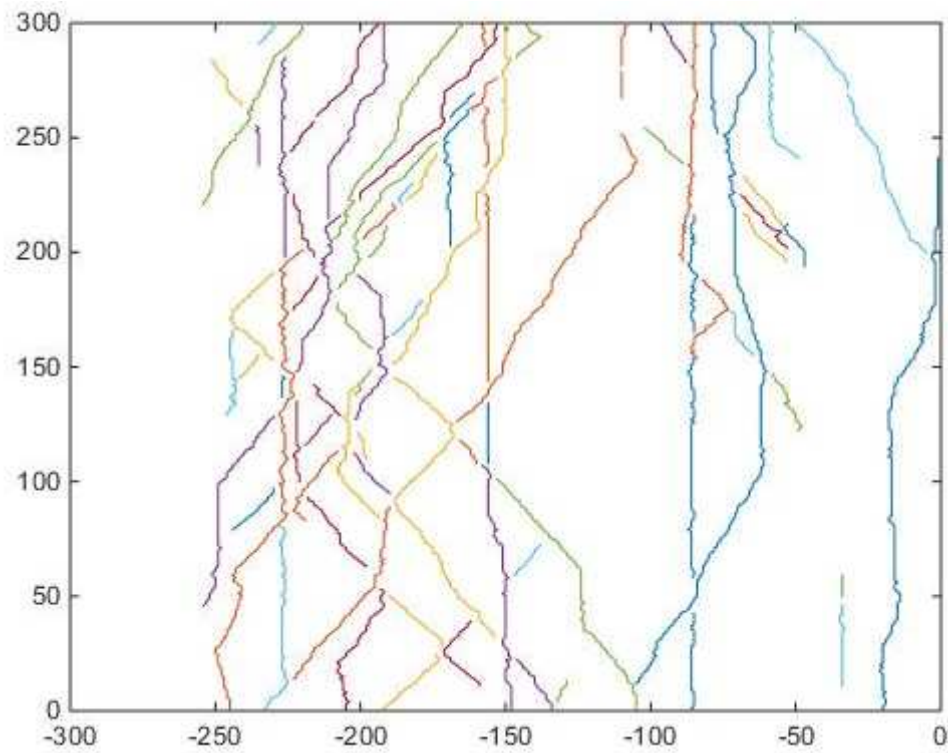


Figure 21: Kymograph generated from the tracking results

On comparing the kymographs it can be all the tracks that are tracked by manual tracking are also successfully tracked by the algorithm developed.

Based on the details described above it becomes clear that the method developed as a part of this is study is the most effective one in comparison to the other methods for the application under consideration.

Chapter 8

CONCLUSION

The technique developed as a part of this dissertation is used to study motor protein motion. The objects of interest are labelled using a fluorescent tag which aids in distinguishing the motor proteins in the presence of other objects. The algorithm is robust to the density of objects, variation of SNR in the sequence of images and also handles temporary disappearance and reappearance of objects. The objective of this study is to gather statistics pertaining to the motor proteins.

The results that are required to be generated include the number of particles that present in the sequence of images, verifying the tracking of the detected objects, the velocity mean and standard deviation of the objects along both anterograde and retrograde directions. The velocity statistics generated help in drawing valuable conclusions regarding the behavior of the motor proteins. Their behavior is directly linked to multiple human diseases which can be concluded from velocity statistics.

This algorithm was tested on the experimental data and the results were validated against the results obtained by manual tracking since there was no access to ground truth. The number of particles was within a close range of the number of particles found with manual tracking. Further, kymograph of the tracked particles was generated and compared with the kymograph of the manual tracking procedure. It was observed that both the kymographs had close resemblance leading to the conclusion that most of the detected particles were tracked successfully.

The velocity statistics in case of manual tracking was generated by calculating slopes of the individual track segments from the kymograph and then calculating the mean and the standard deviation for both anterograde and retrograde direction. Kymograph is a graph of 1Dimensional displacement on Y axis vs time on the X axis and there is not enough data to get the units for velocity since the scale is unknown. However, in case of the technique developed the velocity statistics was calculated by taking into account the displacement in each consecutive frame. This data was then used to generate

to the mean and statistics of the particles; the unit being $\mu\text{m}/\text{second}$ or $\text{pixels}/\text{frame}$. Due to this reason, it becomes difficult to compare the velocity statistics generated by both the techniques. In the future, a reasonable technique to compare and validate the results of the velocity statistics generated by both the above mentioned methods will prove to be a vital tool in the study of motor protein motion.

In conclusion, this study has provided the framework for future work to be built on it. It has also provided success in achieving some of the objectives which were intended to be accomplished. These results have been successfully validated which lets us conclude that the technique was successful.

Chapter 9

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