

DEVELOPMENT OF AUTOMATIC TRACKING METHODS FOR THE ANALYSIS OF ANIMAL BEHAVIORS

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Engineering

University of Hyogo, JAPAN

September 2017

Acknowledgments

I would like to thank Associate Professor Teijiro Isokawa at University of Hyogo, first and foremost. I would also like to appreciate Professor Naotake Kamiura, Associate Professor Masakazu Morimoto and Professor Hidetoshi Ikeno. Their supports and encouragements have been of much help to me. This dissertation would not have been completed without their contributions.

I would also like to appreciate Professor Hidetoshi Ikeno, Professor Mizue Ohashi and Dr. Ryuichi Okada at University of Hyogo, and Etsuro Ito at Waseda University for giving me the opportunity of this research under them and for providing experimental videos. They devoted their precious time for supporting my research and for giving me invaluable advice and their knowledge in the ethology. This dissertation also would not have been completed without their supports.

I am grateful to Professor Karl Crailsheim, Dr. Thomas Schmickl, Dr. Ronald Thenius, Ms. Martina Szopek and Mr. Gerald Radspieler and the laboratory members of Artificial Life Laboratory at Karl-Frazens-University Graz for allowing me to stay their laboratory for one year and helpful discussion in the filed of both engineering and zoology and providing experimental videos.

I would like to thank Dr. Naomi Kodama, Dr. Seiichiro Yonemura and Dr. Satoshi Kaneda at National Institute for Agro-Environmental Sciences, and Dr. Hitoshi Aonuma at Hokkaido University for their useful comments and suggestions.

A special thank is for the friendships and interactions from all people that are involved in this research.

Finally, I am extremely grateful to my wife for patient support.

Preface

Clarifying the mechanisms of animal behavior is an important issue in ethological research. Many previous studies have observed and analyzed target animals to elucidate their behavioral mechanisms. Animal behavioral analyses are important for basic research, informing not only ecology, but also engineering, medical science, and economics.

Recent developments in digital video camera technology have increased the performance and reduced the cost of recording devices, enabling researchers to easily record long durations of video in experiments. Researchers can then confirm the recorded video, and analyze the behaviors of interest. After the videos are recorded, data can be manually obtained from them for objective analysis of an animal's behavior. However, this approach requires substantial time and effort. Furthermore, it is difficult to obtain multiple data sets using the same criteria, increasing the likelihood of human error. The development of software for automatically tracking multiple animals or insects could have widespread applications in animal behavioral research. Image processing is a method of data acquisition from images using a computer. Image processing can obtain data using consistent criteria and reduce the work of manual data acquisition traditionally carried out by researchers.

The dissertation examines three computer-aided tracking methods: 1) a method for tracking multiple bees in an observation hive under natural light; 2) a method for tracking multiple bees on a flat area under controlled lighting in laboratory experiments; and 3) the tracking method for multiple points of an earthworm on a Petri dish under controlled lighting. All of these methods comprise three main processes: detection, identification and tracking. Appropriate methods were adopted for each of these processes, in each proposed method. In addition, this dissertation describes the development of specialized software based on the proposed methods. Honeybees, a well-known social insect, and earthworms, which play an important role as a soil organism, served as target animals. These methods do not require identification marking, such as tags or codes, which can affect behavior.

This research involves the application of image processing in ethological research, utilizing the techniques of computer science. In most related studies, engineering researchers have developed tracking software from an engineering perspective. However, it can be difficult for ethological researchers to use engineering-oriented software. To produce software that meets the needs of ethological researchers, the author developed and tested the tracking software with colleagues involved in ethological research. Thus, this software was developed from both engineering and ethological perspectives. For this reason, the current research provides new insight into an emerging field of engineering research that integrates engineering and ethological research. The author hopes that this research will contribute to advancing research in both fields.

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Chapter 1

Introduction

Behavioral analyses of humans and animals can provide important information. For example, the development of collision avoidance technology was based on observation of the collision avoidance behavior of bees during flight [3] [57] [82]. Furthermore, new methods for cancer cell detection are based on processes observed in roundworms [51]. To examine the mechanisms underlying the behaviors of humans and animals, it is important to observe targets in detail. In behavioral experiments, researchers can record the behaviors of the target animals using digital video cameras or web cameras, and systematically analyze the video. However, acquiring the data required to analyze behavioral mechanisms in recorded videos is challenging.

To solve this problem, many researchers have developed tracking methods and software for studying humans and animals [18] [19] [80] [91]. The effectiveness of recently developed systems for tracking humans and methods for automatically identifying and tracking animals and insects in ethological research has been demonstrated. However, applying human tracking methods for tracking animals and insects is challenging, because most methods use color and figure information, and many animals do not exhibit characteristic of them.

Because the cost and performance of digital video cameras has greatly improved in recent years, video recording of targets for long durations in the laboratory has become easier [74] [84]. The development of software for automatically tracking various animals, including insects, could have useful applications for animal behavioral research. Several software packages for supporting the tracking and analysis of animal behavior have been developed, such as Ctrax [15] and idTracker [36] software for tracking drosophilae, zebra fish and mice. These software packages are effective for performing tracking and analysis of target behaviors. However, the behavior of targets often involves monotonous and continuous movement. Furthermore, because idTracker requires researchers to use high-resolution videos (e.g., more than 4K images), computers with high specifications and substantial storage capacity are required. Thus, using this software is difficult for many researchers.

Many previous studies of social insects have examined honeybees as model animals, investigating a range of phenomena, from nervous system activity to behavior. However, tracking honeybees automatically using current methods is challenging, because the bees have limited characteristic movement, as well as having large populations and complex behaviors. For this reason, researchers have developed tracking software using PFID, QR codes or customized identification codes [22] [24] [49] [68] [71] [115]. Using these methods, codes are typically attached to the backs of targets. These approaches have two main issues that should be solved: 1) behavioral changes under the influence of codes; 2) separation of codes. To reveal the social mechanisms of honeybees, it is important to identify and track each individual without any markings, and to analyze how the animals move as a swarm. In this research, new automatic tracking methods were developed for multiple individuals, to support analysis of complex behaviors, such as interactions between individuals. This dissertation examines a proposed method for identifying each individual, and detecting their location.

The dissertation consists of six chapters. Chapter 1 presents the Introduction, describing the background, significance and purpose of this research in the context of related work. In Chapter 2, a new automatic tracking method for multiple honeybees

in an observation hive is proposed. Separating regions of images containing honeybees from original images is challenging, because the targets are individuals moving in an observation hive are not under controlled environmental conditions. For this purpose, the proposed method adopted regional segmentation using learning vector quantization as a detection method for honeybee regions. The identification of individuals was performed by the relation of their location information, frame by frame. The trajectory line was drawn by connecting the central points of each individual, frame by frame. Chapter 3 describes a new proposed tracking method for multiple honeybees on a flat surface with controlled environmental conditions, and the development of a prototype software package called K-Track, based on the proposed method. Under controlled conditions, the method used background subtraction for the detection of honeybee regions. The identification of individuals was performed using the relation of their location information frame by frame, as in Chapter 2, and the linear moving prediction was based on the bees' movement. Chapter 4 describes the improvement of the K-Track algorithm using both tracking results with videos containing forward and backward play. A weakness was detected in the previous algorithm (K-Track), and tracking was found to fail in situations where bees' movement was limited when they were touched, or when they interacted with other individuals. In such cases, it was sometimes easier to track a target behavior using backward-playing video rather than forward-playing video. Based on this notion, a decision method for trajectories was proposed, by comparing both forward and backward tracking results to improve error rates. Chapter 5 describes the development of a tracking method for a single earthworm, called "Mimizu" in Japanese, in a glass container, applying the proposed honeybee tracking method. For behavioral analysis of an earthworm, the method obtained top, end and central points using temporal relations. Based on the proposed method, prototype software called MimizuTrack was developed and evaluated. Chapter 6 is finished this dissertation with conclusions.

Chapter 2

Tracking method of multiple individuals using vector quantization for analysis of bee's hive behavior

Abstract

Social activities are among the most striking of animal behaviors, and the clarification of their mechanisms is a major subject in ethology. Honeybees are a good model for revealing these mechanisms because they display various social behaviors, such as division of labor, in their colonies. Image processing is a precise and convenient tool for obtaining animal behavior data, but even recent methods are inadequate for the identification or description of honeybee behavior. This is because of the difficulty distinguishing between the large number of individuals in a small hive and their multiple movements. The present study developed a new computer-aided system, using a vector quantization method, for the identification and behavioral tracking of individual honeybees. The vector quantization method enabled separation of honeybee bodies in photographs recorded as a movie. This system succeeded in analyzing a huge number of frames quickly and can thus save both time and labor. Moreover, the system identified more than 72% of the bees in a hive and found and determined the active areas in the hive by extracting the trajectories of walking bees. In addition, useful behavioral data on the honeybee waggle dance were obtained using the present system.

2.1 Introduction

Social activities are among the most striking of animal behaviors, and the clarification of their mechanisms is a major subject in ethology [72]. Honey bees are a good model for revealing these mechanisms because they display various social behaviors such as division of labor [45] [110]. A honey bee colony consists of tens of thousands of worker bees, one queen and some hundreds of drones. While the queen expends her energy laying eggs, worker bees, depending on their age, clean and build the hive, take care of the brood and queen, defend the hive from enemies, and leave the nest to search around the hive and collect food [41] [45] [110]. Thus, individual bees exhibit different behaviors within the hive.

A unique communication behavior in honey bees, to share information regarding profitable food sources, was discovered by von Frisch [41]. After a forager bee has returned to the hive from a successful foraging flight, it often performs a waggle dance to transfer information regarding the direction and distance of the discovered food source to other worker bees. Some workers (followers) surround the dancing bee (dancer), receive the information and then visit the food source that the dancer has indicated [33] [35] [41] [45] [106] [103].

To understand the mechanisms involved in such social behaviors, we must be able to precisely identify individual bees and provide a detailed description of their behaviors. These tasks have generally been achieved by manual analysis of film or video footage. For example, thousands of bees would be marked for an observation of a hive by means of numbers and/or color combinations [1] [45] [64] [106] [103] [109] [114]. Some of the dancers and followers might then be tracked [55] [105] and various dance positions plotted [104] [111]. Thus the dance precision was calculated in many behavioral experiments [7] [29] [86] [87] [112] [116]. However, such manual analyses require large amounts of time and labor.

Image processing is a useful method for identification and tracking of multiple moving elements [13] [14] [122]. Some methods have been developed for studying sequential photographs of these movements, for example tracking ants by body color and movement information [4]; automatic identification of bees using a combination of k-nearest neighbor classification and a hidden Markov model [38]; and tracking of unmarked bees using a Rao-Blackwellized particle filter and several principal images of bees (eigen-bees) [58]. However, it is difficult to directly apply these methods for analyzing social behavior of animal group because they are developed for processing independent behaviors of a few individuals. One problem is that a background image with no tracking objects is required for the extraction of tracking objects [4] [13] [14] [38] [58] [122]. Another is that marks on and / or numbers of tracking objects to be analyzed may also be required [4] [13] [58]. Methods developed for tracking a few individuals are generally inadequate for simultaneously tracking large numbers of individuals [13] [14] [38]. There are no methods that can simultaneously track hundreds of unmarked targets in a changing background. Thus, the development of suitable methods for analyzing animal behaviors is still worthy of considerable attention.

In the present study, a computer-aided system was developed for identification and behavioral tracking of individual honey bees within a hive. The system was based

on vector quantization and temporal contextual information. The vector quantization method (VQ) allowed honey bee bodies in time series photographs to be separated from one another without any preconditions. Individual bees were extracted from images using their body size and locomotion. This new system successfully tracked hundreds of bees simultaneously without the need for marking. The system identified more than 72% of the bees in the hive and successfully traced the trajectory of the waggle dance.

2.2 Tracking method for multiple honeybees under natural light

2.2.1 Procedure

Despite widespread interest in the social behavior of honey bees, a number of factors, such as their small body size, the large population within a hive and the lack of pronounced external features on individual bees, make behavioral analyses problematic. To overcome these problems the following three steps were taken in the development of the analysis system: a) extraction of a honey bee-code image (see below) from the whole hive image using the VQ; b) separation of single and plural regions of honey bees from the honey bee-code image using an average honey bee body size and shape; c) tracking of honey bee movement from temporal contextual information in sequential image data or movies.

2.2.2 Extraction of honey bee body regions using VQ

VQ is used to separate many elements into several groups with approximately the same characteristics. Each group is represented by a centroid vector, which is called the “code vector” (CV) [21]. This method has been applied to various fields, such as data compression, signal processing and data clustering.

Normally, a color image consists of three color components: red (R), green (G) and blue (B). From quantization of the R, G and B components of images, R was found to be the best. It seems likely that this reflects the warm colors such as yellow and orange which predominate in the honey bee body. Thus, the highest contrast image was obtained from the R image (Figure 2.1 A). This image was split into small regions of 2 x 2 pixels and each region was categorized according to spread and the sharpness of the image. Based on the 256 levels of each pixel each 2 x 2 pixel region mapped a point (training vector (TV)) in 4-dimensional space. Analyses were investigated using regions of different pixel numbers (3 x 3, 4 x 4 and more). Increasing the pixel number within each region increases the space mapped but also increases distance between TVs and thus reduces the resolution of the image. 2 x 2 pixel regions were found to be the best for separation of honey bee individuals from their background.

In VQ applications the generation of good CV values is critical. Previously, it was extremely difficult to calculate CV values from image data. However, the Linde-Buzo-Gray (LBG) algorithm ([46] [69]) used in the current study automatically generated CV values from TV values using the following iterative process.

1. An Initial CV (e.g. CV_0) value was calculated using the average of all TV values.

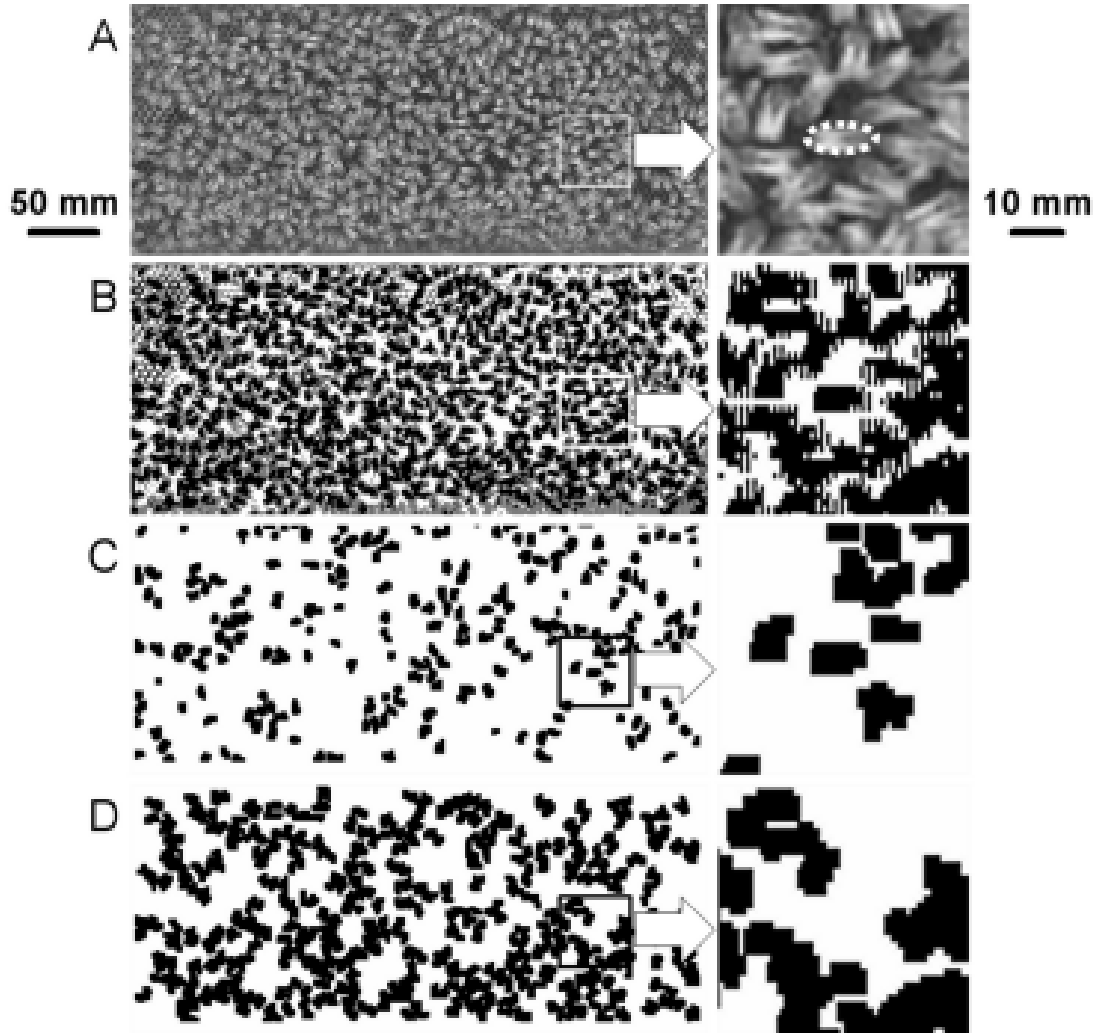


Figure 2.1: The honey bee code image and honeybee region. A: A whole hive image. B: A honey bee code image obtained using VQ and the whole hive image (A). The detected honey bee regions are indicated in black in B. C and D: After repeating our procedure, a single honey bee region image (C) was obtained, which consisted of single honey bee regions alone. By subtraction of the single honey bee region image from the honey bee code image, the remaining image was defined as a plural honey bee region image (D). The boxed areas to the right of each main image (A-D) are enlarged for comparison with the original images. The dotted oval in the enlarged area of A shows the approximation of the honey bee body to an ellipse shape.

2. A first CVs (e.g. CV_1 and CV_2) value was generated from the initial CV (CV_0) value by addition and subtraction of a small value to and from the initial CV value, respectively.
3. The TV values were separated into two groups (TV_1 and TV_2) based upon the new CV values (CV_1 and CV_2).
4. The two CV values (CV_1 and CV_2) were then each recalculated using the average of the relevant reclassified TV values (i.e. from each TV_1 and TV_2).
5. The process was repeated, adding further CV values and TV groups until a minimum Euclidean distance between the CV values and TV values was reached.
6. Each CV was split into two vectors again. The same operation was carried out in target vector space.

In a preliminary experiment, the number of CV values for classification was varied. It was found that eight CV values produced the best result for the separation of objects within the observation hive. These vectors correspond to the honey bee body, the dark and light of the honey bee wing, the hive, the hive frame and the background and noise. Thus, eight CVs were needed to represent all of the objects. The vector distribution and quantization results for each region are shown in Figure 2.2 . Each vector consists of four dimensions ($v1, v2, v3, v4$). The distribution of CV values was examined in two, two dimensional graphs ($v1-v2$ plane, Figure 2.2 A and $v3-v4$, Figure 2.2 B). The CV values, $cv3$ and $cv5$ representing the hive and honey bee body regions, respectively, are approximately at the centre of those regions. In most cases, TV values could classify the central CV values for each region. The system employed in this study generated eight CV values from all of the TV values and extracted an image for honey bee body regions, called a “honey bee-code image”, from each frame.

2.2.3 Separation of a honey bee-code image into single honeybee regions and plural honeybee regions

Two different types of regions in the honey bee-code image were extracted from the hive image. The first type was a single honey bee region (SHR) which consisted of a single honey bee, and the second was a plural honey bee region (PHR) which consisted of a group of two or more honey bees. First all of the SHRs were extracted from the hive image based upon the morphological characteristics of honey bees (Figure 2.1 C). Subsequently the PHRs were processed to separate individuals (Figure 2.1 D). Morphological information, including the body size and shape of single honey bees from the images, was used for detection of the SHR. The shape of a honey bee was approximated visually as an ellipse in Figure 2.1 A and the size of the SHR estimated as 100 ± 18 pixels from 20 manually selected ellipses. Similar sized regions (from 80 to 120 pixels) were extracted as the other SHRs from Figure 2.1 B.

After SHRs were processed, the individual honey bees in these regions were identified. Overlapping areas between the honey bee regions were noted. There were two possible overlapping areas in the SHR. The first type consisted of a temporal overlap between two SHRs that were in fact the same bee (Figure 2.3 A). That is where the SHR for a

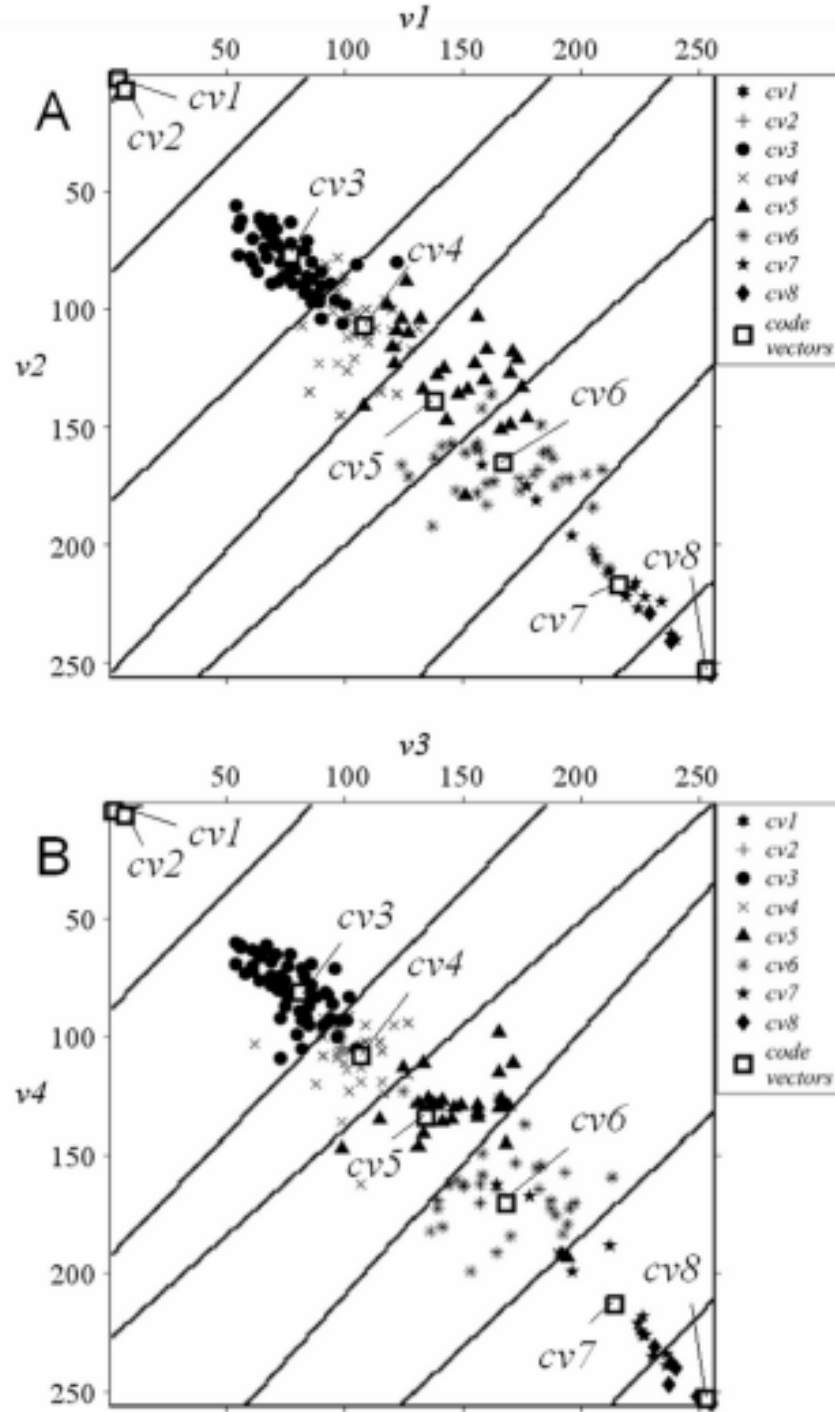


Figure 2.2: The vector distribution and quantization results for each region. The position of each CV is shown as a box and is labeled. Other symbols represent vectors generated using VQ. The solid lines show the borders of each region. Four dimensions were used to generate these two dimensional graphs. In most cases, the training vectors could determine the central CVs for each region.

bee #1 at time t is S_t^1 and at time $t + 1$ the SHR is S_{t+1}^1 and the regions S_t^1 and S_{t+1}^1 overlap. The two images needed to be superimposed to identify the two honey bees as identical. The second type of SHR consisted of an overlap among two or more SHRs (Figure 2.3 B). When SHR (S_{t+1}) overlapped with two or more SHRs at time t , the sizes of the overlapping regions were compared. For example, the size of the overlap between S_{t+1} and S_t^2 were compared that between S_{t+1} and S_t^3 . It was assumed that the largest overlap occurred between the same bee at different times. That is, the overlapping area between S_{t+1} and S_t^2 was larger than that between S_{t+1} and S_t^3 thus S_{t+1} and S_t^2 represent the same bee and S_{t+1} can be renamed S_{t+1}^2 .

When PHRs were divided into SHRs using before and after frames, the identification of honey bees was more complicated. When a PHR was separated into two or more SHRs, the honey bees were identified as following (Figure 2.3 C). A PHR at time t was named P_t . If this PHR overlaps with two SHRs at time $t - 1$ i.e., S_{t-1}^4 and S_{t-1}^5 then it is likely that the PHR P_t actually consisted of two SHRs S_t^4 and S_t^5 . Thus the PHR is separated and identified and from then on is processed as an SHR. This process was repeated until no more PHRs existed which could be processed. Individual movements were tracked by calculating central points by averaging the coordinates of each extracted area. Central points calculated for the same honey bee for different times were connected sequentially. The honey bee trajectory was thus extracted (Figure 2.2 D).

2.3 Experiments and Results

2.3.1 Experimental setup

The behavior of honey bees, *Apis mellifera*, in an observation hive was recorded as a movie using a digital video camera (GR-HD1; JVC, Yokohama, Japan). The resolution was 720 pixels x 480 pixels; the video frame rate was 29.97 fps; and the movie was recorded for a bee hive frame (width 44.0 cm x height 19.6 cm) (Figure 2.4 A). In the movie, there were more than 700 honey bees. They had not been marked and could walk freely in the hive.

2.3.2 Identification of honey bees

A ten-second movie (300 frames) was processed. The system was able to automatically number more than 500 honey bees in each frame (Figure 2.4 B). Three frames were selected at random and we calculated the percentage of correct identification by our software to the total number of individuals in each frame. The total number was a number of honey bees extracted and identified manually. More than 72% of the bees manually identified were also identified using the analysis system (510 of 704, 522 of 718, 516 of 700). This result indicates that most bees in a hive can be identified. The only limitation was that bees which only walk only a short distance overlap with other bees and these individuals were difficult to identify.

2.3.3 Tracking of honey bee movement

Individual trajectories were plotted from sequential temporal identification of individuals. The system succeeded in simultaneously tracking hundreds of honey bees in a single

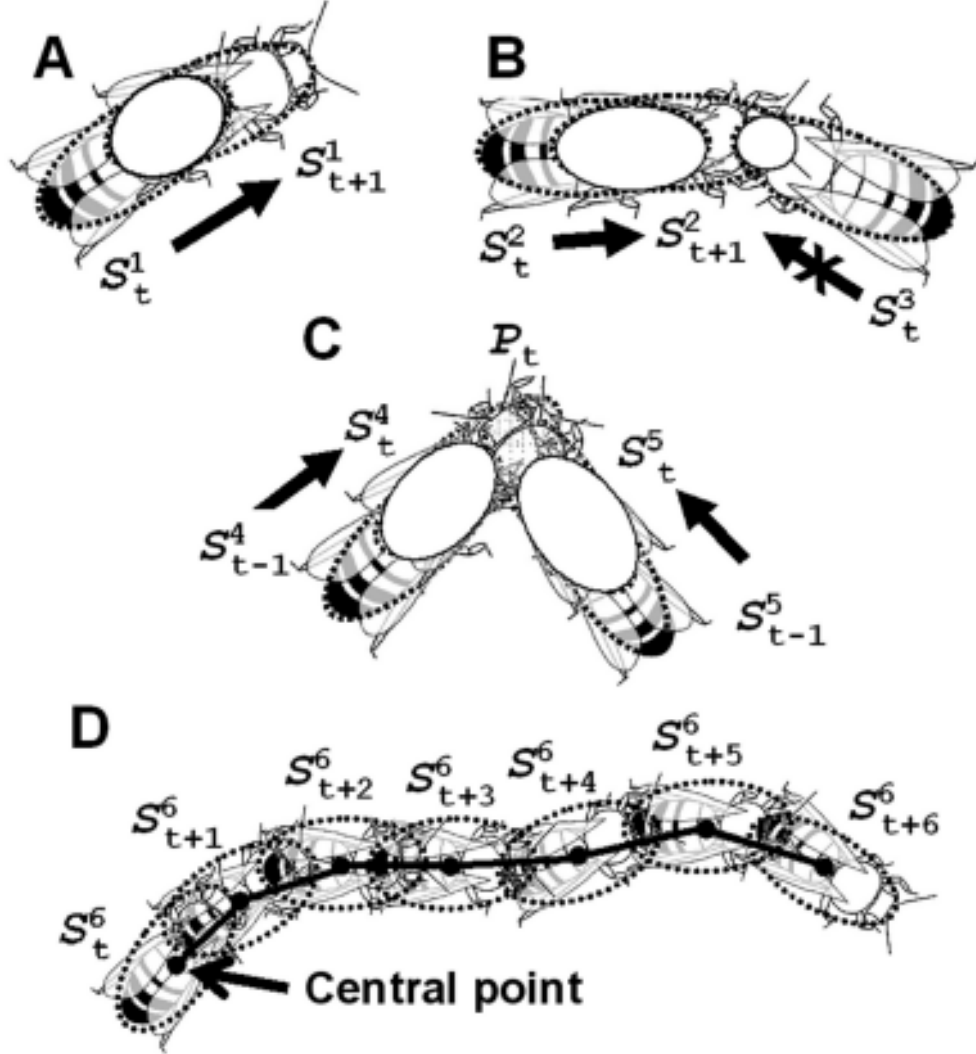


Figure 2.3: Numbering and tracking of individual honey bees. Open circles show the temporary areas of overlap between bees or bee images. The dotted ovals around the bees show the approximation of the honey bee body as an ellipse. A: When a single honey bee region S_{t+1}^1 at a given time $t + 1$ was overlapped with one in the preceding image (S_t^1), we assigned the same individual number to this region, i.e., bee number #1 in this case. B: When a single honey bee region was temporally overlapped with more than one single honey bee region, the individual number belonging to the largest overlapped region was assigned, i.e., bee number #2 in this case. C: When two honey bee regions were merged into one region, the region was separated into two single honey bee regions by examining movement in previous and subsequent frames in this region. D: The trajectory of a honey bee. The central points of an individual that was assigned the same number were connected along a temporal sequence.

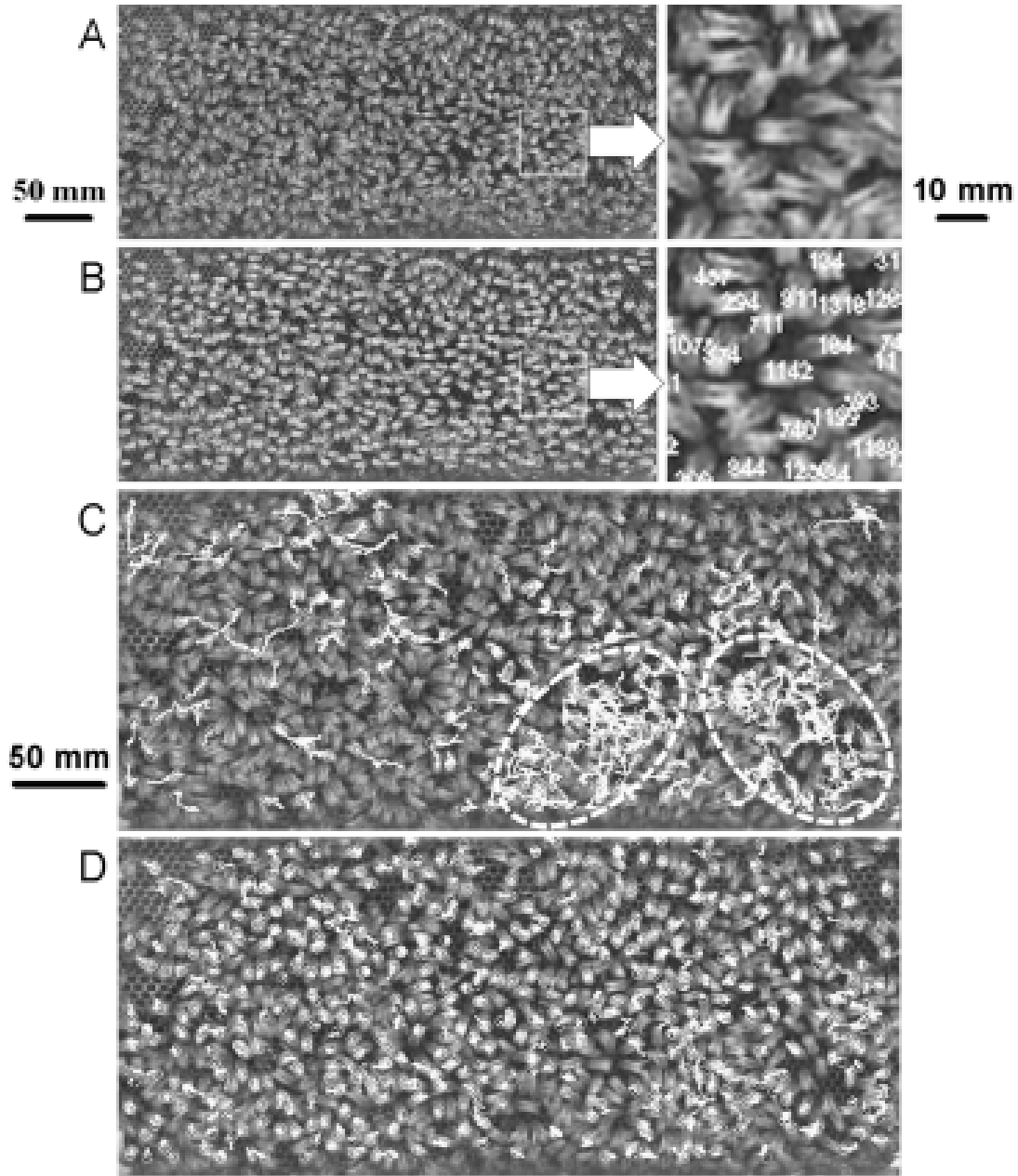


Figure 2.4: The identification and trajectories of bees. A: A whole hive image. Only the red component of a color movie was used to create the hive image. B: Identification of honey bees. The number on each honey bee indicates the individual number assigned using the study method. Enlarged images for (A) and (B) are shown on the right hand side. Most bees were identified and the system only failed to detect a small number. C and D: Trajectories of bees walking a long-distance (C) or short-distance (D). Open circles superimposed on the relevant individuals indicate starting positions. The method identified two areas of high activity in C. These are indicated using a dotted line on the image. The area to the bottom right was particularly active; this area was near the entrance of the hive and is well known as the site of the waggle dances performed by bees returning from foraging. Indeed, dancing behavior was found in this area at this time.

hive, resulting in the production of trajectories for more than 50% of all of the bees. As expected from the analysis of overlapping areas between single and plural honey bee regions, the tracking was easy for honey bees displaying characteristic movements, such as long-distance walks and the waggle dance, but the tracking was difficult for bees that did not walk over a long distance. Thus long-distance walkers (Figure 2.4 C) and short-distance walkers (Figure 2.4 D) were defined according to whether or not a honey bee moved more than 30 mm in 10 sec.

The trajectories of long-distance walkers determined in this study revealed the presence of active areas and static areas within the hive. Figure 2.4 C shows the location of two active areas in the lower right part of the hive frame. As these areas were near the entrance of the hive, they corresponded to the site of dances performed by workers returned from foraging [104] [105].

2.3.4 Extraction of waggle dance

In a hive, some bees have specific behaviors. The system employed in this study succeeded in extracting the trajectories of some of these, such as the waggle dance. The waggle dance consists of a combination of straight wagging walking and right- or left-turn walking to generate a “dance” in a figure-of-eight. The white lines of Figure 2.5 show an example of the waggle dance in one of the active areas extracted using the system. Several parameters, such as the timing of, the duration (coding the distance of the food source from the hive) and the direction (indicating the direction of the food source from the hive) of the straight wagging walk can be calculated from this extraction. These data could be very valuable for ethological studies of honey bees. In Figure 2.5, the honey bee performed two waggle walks within 10 sec. The analysis system developed and employed in this study obtained the behavioral parameters of the waggle dance as obtained by manual tracking [89]. From the system the durations of these walks were 1.10 sec and 0.80 sec (1.10 sec, 0.87 sec from manual). The directions were 138.3° and 143.1° (131.6° and 145.0° in manual).

2.4 Discussion

A new tracking system for honey bee behavior was developed utilizing a VQ. This system had two distinct advantages for identification and tracking. First, the system successfully identified about 500 of more of the 700 bees crowded into a small hive, including overlapping bees. This number is substantially greater than numbers extracted in previous studies where only several tens of the target insects were separated in the image [58]. Second, this system simultaneously tracked about 350 bees, 50% of the entire hive. This is much more than the numbers of individuals tracked in previous studies, e.g. 1 bee [59], 7 ants [4] or 20 ants [58]. Nonetheless, although this new method can track the behavior of most honey bees either extracted as single or plural regions, there are some circumstances where this method does not work. Such cases are when one bee stands directly on top of another bee or when bees hide in holes in the hive structure. Thus there are additional improvements that can be made for tracking such complex behaviors.

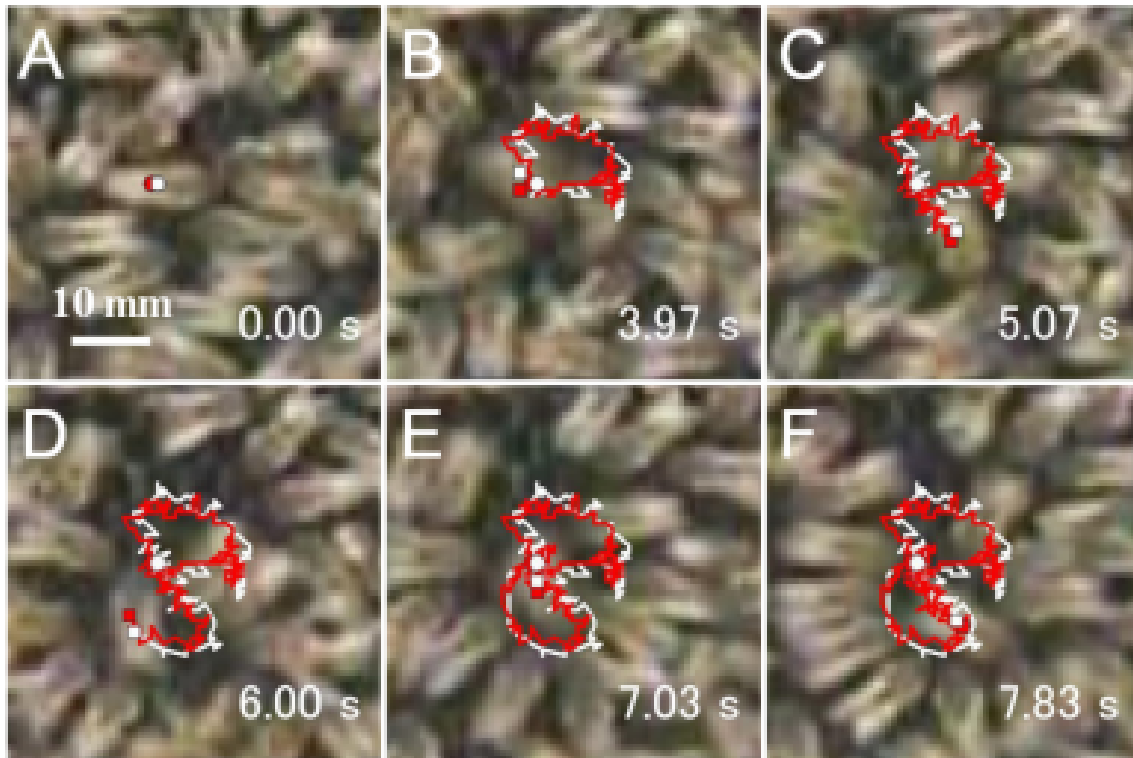


Figure 2.5: The trajectory of a dancer during a waggle dance. The white and red lines indicate the trajectories described by automatic and manual tracking methods, respectively. The dancer indicated by the open circle (A) was tracked during one cycle of a waggle dance. The number in the lower right of each image shows the time elapsed from the start of tracking. First the bee moved in a wide circle from and back to the initial position (B). Then the bee made a first waggles walk (C). Next, the bee turned right and walked in a straight line (D), then returned to her initial position (E). Lastly the bee made a second waggles walk (F). Overall the path taken follows a figure of 8.

Further evidence of the power of this new system was its ability to track hundreds of objects under the relatively poor conditions. For example, resolution of the images was 720 pixels x 480 pixels for 44 cm x 20 cm, which is close to those used by Balch et al. [4] (640 x 480 pixels, 40 x 20 cm), but lower than that used by Khan et al. [58] (720 x 480 pixels, 15 x 10 cm). Our analyses were performed using natural sunlight, which is much poorer and less controlled than the light sources generally used in these experiments. Furthermore, the complexity caused by dynamic change in images is compounded as the number of targets is increased. Thus tracking hundreds or thousands of objects also is much more difficult than tracking small numbers.

Our system could process a movie up to three seconds due to memory limitation. For a longer-time movie, it is necessary to split it into the series of processable time movies. Utilization of a computer with high-capacity memory will solve this problem. We can also process the longer-time movie by reducing the frame rate, but it is not recommended because some important information of movements, such as overlapping, might be lost by the deduction, which will decrease the accuracy of tracking.

It is expected that the accuracy of tracking would decrease with time due to the complex movements of individuals. However, this problem will be avoided by modifying the trajectories of bees manually and visually as necessary. Additionally, the following attentions are recommended during image recording; 1) the focus of the digital video-camera is accurate on the whole hive during the record, 2) the lighting is uniform on the whole hive, 3) a homogenous background and new combs are used for the record.

This computer-aided system using VQ was found to be highly applicable for bee experiments. A single colony of social insects like honey bees contains a large number of individuals. Conventional computational methods are limited by CPU, memory, and other hardware ceilings and cannot meet the requirements of complex processing to deal with data sets for such a large number of targets simultaneously. However, VQ can select the essential information and drop the unessential data, thereby reducing the workload for the computer [44] and enabling the analysis of hundreds of bees simultaneously. To identify and track 700 individual bees manually would require more than 50 days for 11 frames of a 10 s movie. Using the present system this required only 1 hour for 300 frames of the same movie.

These results indicate that the developed system is an effective tool for behavioral analyses in honey bee experiments. In addition, the system was able to at least partly resolve the problem of overlapping honey bees. Making improvements in the accuracy of individual identification by using a high resolution digital video camera and a motion estimation algorithm could resolve this problem. This system should ultimately not only be applicable to honey bees but also to the social behavior of human beings.

Chapter 3

Development of tracking software of multiple individuals, honeybees, in a flat area using background subtraction and spatio-temporal locational information

Abstract

A computer program that tracks animal behavior, thereby revealing various features and mechanisms of social animals, is a powerful tool in ethological research. Because honeybee colonies are populated by thousands of bees, individuals co-exist in high physical densities and are difficult to track unless specifically tagged, which can affect behavior. In addition, honeybees react to light and recordings must be made under special red-light conditions, which the eyes of bees perceive as darkness. The resulting video images are scarcely distinguishable. We have developed a new algorithm, K-Track, for tracking numerous bees in a flat laboratory arena. Our program implements three main processes: (A) The object (bee's) region is detected by simple threshold processing on gray scale images, (B) Individuals are identified by size, shape and spatiotemporal positional changes, and (C) Centers of mass of identified individuals are connected through all movie frames to yield individual behavioral trajectories. The tracking performance of our software was evaluated on movies of mobile multi-artificial agents and of 16 bees walking around a circular arena. K-Track accurately traced the trajectories of both artificial agents and bees. In the latter case, K-track outperformed Ctrax, well-known software for tracking multiple animals. To investigate interaction events in detail, we manually identified five interaction categories; 'crossing', 'touching', 'passing', 'overlapping' and 'waiting', and examined the extent to which the models accurately identified these categories from bee's interactions. All 7 identified failures occurred near a wall at the outer edge of the arena. Finally, K-Track and Ctrax successfully tracked 77 and 60 of 84 recorded interactive events, respectively. K-Track identified multiple bees on a flat surface and tracked their speed changes and encounters with other bees, with good performance.

3.1 Introduction

The majority of ethological studies rely on accurate observation of animal behavior. Animal behaviors have been studied by monitoring the movement of target animals in both field and laboratory environments. In such experiments, the model animals are contained in circular or rectangular arenas. Behavioral information is gathered by recording the trajectories and variation of animal movements within the arena. To date, small animals such as flies [15], mice [9] [16] [32], spiders [90], and cockroaches [11] [42] have been used as model animals. Social insects such as bees [119] and ants [53] are also popular for studying animal social mechanisms. In these ethological studies, necessary data on animal sociality are collected by means of video recordings and computer analysis. Recent developments in recording equipment, such as digital video cameras and webcams, provide high functionality at reasonable cost, enabling long-term movements of target animals to be captured rapidly and easily. However, although human observers can easily monitor the target animals by these recordings, extracting behavioral data from the movie images remains a laborious and time-consuming manual task. In addition, manual analyses of sequential images may yield insufficient quantitative and objective ethological data.

Recent automatic tracking programs for collecting ethological data from video images have enabled us to analyze various animal behaviors in the laboratory quickly and precisely (e.g. [4] [38] [58]). For example, the program developed by Delcourt et al. has successfully tracked juvenile Nile tilapias (*O. niloticus*), which often swim in schools, and has identified three different crossing patterns [30]. The open-source program Ctrax, published by Branson et al. [15], was developed for tracking multiple flies walking in an arena. Ctrax has been widely used for tracking not only flies [15] [75] [92] [101] [118] but also ants [39] [94], cockroaches [8] [26] and fish [5], [93]. These programs detect individuals by subtracting background images. The location of target animals at time t is estimated from a constant-velocity model, based on positional change from time zero to $t - 1$. Therefore, these models are of limited applicability: target animals must move on a constant background such as an arena, and their movements are assumed continuous, streamlined and monotonous. Because of these limitations, automatic tracking remains an important challenge in the behavioral analysis of animals with diverse movements, such as ants and honeybees.

Various equipments and methods have been developed and applied for tracking animal behaviors such as flies, mice and ants. For example, the video-based systems tracked targets based on shape and/or color [20] [27] [85]. These characteristics are usually used to identify and track them. For more accurate identification, the barcodes were used for identification by Mersch et al. [76].

The RFID chip was also used for tracking animal behavior [40]. As same as the video-based tracking using a barcode, the animals can be identified by attached unique RFID chip. Furthermore, the fusion video-RFID tracking method was already applied by Weissbrod et al. [117]. Even the methods with physical attachments are helpful for identification; many researchers want to use the video-based tracking because of its convenience and little influence on the animals.

The waggle dance of honeybees, discovered by Karl von Frisch in 1967 [41], is one

of the most famous social behaviors. By conducting this dance, the honey bee shares information of profitable food sources with her nest mates. The waggle dance has roused much interest among ethologists, rendering the honey bee a popular model animal for studying social behaviors. Other social behaviors displayed by honeybees include division of laborious tasks such as cleaning and building of combs, caring for the queen and brood, defending the hive from potential predators and controlling the moisture and temperature in the hive [31]. Adult honeybees also transfer food to other adults in their colony (trophallaxis), which serves a communicational, nutritional and transport function [17] [37] [65] [79]. From the type, quality and willingness of the donation, the recipient obtains information about the food condition in the colony or within a smaller subgroup of bees. Trophallaxis is typically conducted with the bees facing each other in a line. The trophallactic strategy has been adopted in robots [98]. Although the capabilities of an individual are limited and few, the bees collectively achieve high performance. Cooperation enables the colony to survive cold winters, which individual bees could not survive, and rapidly boosts the foraging workforce in spring, when other social insects (such as bumble-bees and wasps) remain in the colony-founding phase. Monitoring and analyzing individual behaviors together with social interactions is expected to reveal the social structure and performance of a honeybee colony.

The social behaviors of bees are generally investigated on flat surfaces. Some researchers have analyzed the waggle dances on a flat vertical observation hive [88], while others have monitored the response of young bees to temperature in a flat circular horizontal arena [100]. Such flat-surface experiments are important for observing and analyzing the social behaviors of honeybees. However, automatic tracking of honeybee behavior is not readily achieved using existing computer software, because honeybees display complex and unique behaviors such as contact with other individuals and accidental movement, i.e. resting or stopping within the hive. Such behaviors require analysis by a new tracking method.

Previously, we developed a method that tracks hundreds of unmarked honey bees walking in an observation hive [61]. In this method, individuals are distinguished and tracked based on body size and shape, and the spatiotemporal overlapping of bee regions. Within a few minutes, the algorithm tracked more than 350 in a colony of about 700 bees in an observation hive. However, precise, longer-term tracking of several bees in an arena could not be undertaken without losing bees from time to time. Number retention is an important prerequisite for studying the social behavior of honeybees.

In the present article, we describe a new and improved method for tracking unmarked multiple honeybees. Our method implements three main processes: (A) Regions occupied by bees are detected, (B) Individuals are identified, and (C) The behavioral trajectory of an individual is constructed, based on the known complex behaviors of bees. Overlapping individuals are identified in our program by one of two processes; predicting their linear movement or regional matching. Our new software, named “K-Track”, is validated by tracking 16 honey bees moving on a circular arena, and comparing the results with those obtained from Ctrax (0.3.9) [15], a well-known free software package for animal tracking. We demonstrate the superior tracking performance of our program, compared to “Ctrax”.

3.2 Tracking method for multiple honeybees under controlled light

To precisely assess the movements of an animal species, the behavioral properties of the animal must be ascertained. The main problem in simultaneously tracking multiple bees is the difficulty of identifying and separating overlapped or contacting individuals. Complex patterns arise from the combination of individual’ movements, especially when three or more bees interact. We assume that, in the absence of interaction (a reasonable proposition on frame-rate time scales) a single bee moves linearly forward. However, in practice, the movement of a bee is often influenced by interactions with other bees, and the linear prediction is incorrect even on short time scales. In this case, our tracking algorithm would lose contact with the bee. As a contingency strategy for such frequent events, the neighboring regions were searched for the target bee and the target position updated by matching and detecting all local individuals.

The new method is adopted for tracking multiple bees in a movie depicting spatiotemporal changes of bee body sizes, shapes and locations. Individual bees were treated as rigid objects, distinguished and separated by “size” and “shape”. Our algorithm can assign each tracked object a unique identification number (ID) by analyzing temporal changes of two key aspects. The trajectory of a bee is obtained by connecting the centers of mass of individuals assigned specific ID numbers in sequential frames. The workflow of our proposed method proceeds as shown in Figure 3.1: (A) The region occupied by an object (bee) is delineated by simple threshold processing of gray scale images, (B) Individuals are identified from spatiotemporal contextual information of size, shape and location, and (C) Behavioral trajectories are drawn by connecting the centers of mass of individuals sharing the same ID number through all movie frames.

In process A, an image is split into two images; ‘foreground’ and ‘background’. Ideally, all tracked objects should exist in the foreground image. If the background image is obtained first, the source image (Figure 3.2A) is readily divided into the two categories. If no background image is recorded before the bees enter the arena, the background must be deduced from the movie data. This is achieved as follows: the gray scale levels of both bees and background are constant under stable light conditions. In our honeybee arena, the background is brighter than the animals, so the gray scale values of bee-associated pixels are lower than those of the background pixels. The background image (Figure 3.2B) is obtained by allocating the maximum gray scale value to each pixel within all movie frames. A series of foreground images (Figure 3.2C) is then obtained by subtracting the generated background image from the source images. To identify the sizes and shapes of bees, all foreground images are converted to binary images (Figure 3.2D) based on a predetermined threshold.

Process B aims to detect and identify all bees from the previously fabricated binary images. Honey bees possess almost no distinguishing features that allow individual identification on the recorded video images. They are very similar in size, shape and color, and individual differences are smaller than the discretization variability and the noise introduced by the video recording technique and poor light conditions (red light). Regardless of such difficulties, our algorithm assigns every tracked bee a unique number that holds over the entire video period. To achieve this, we assume that the size of

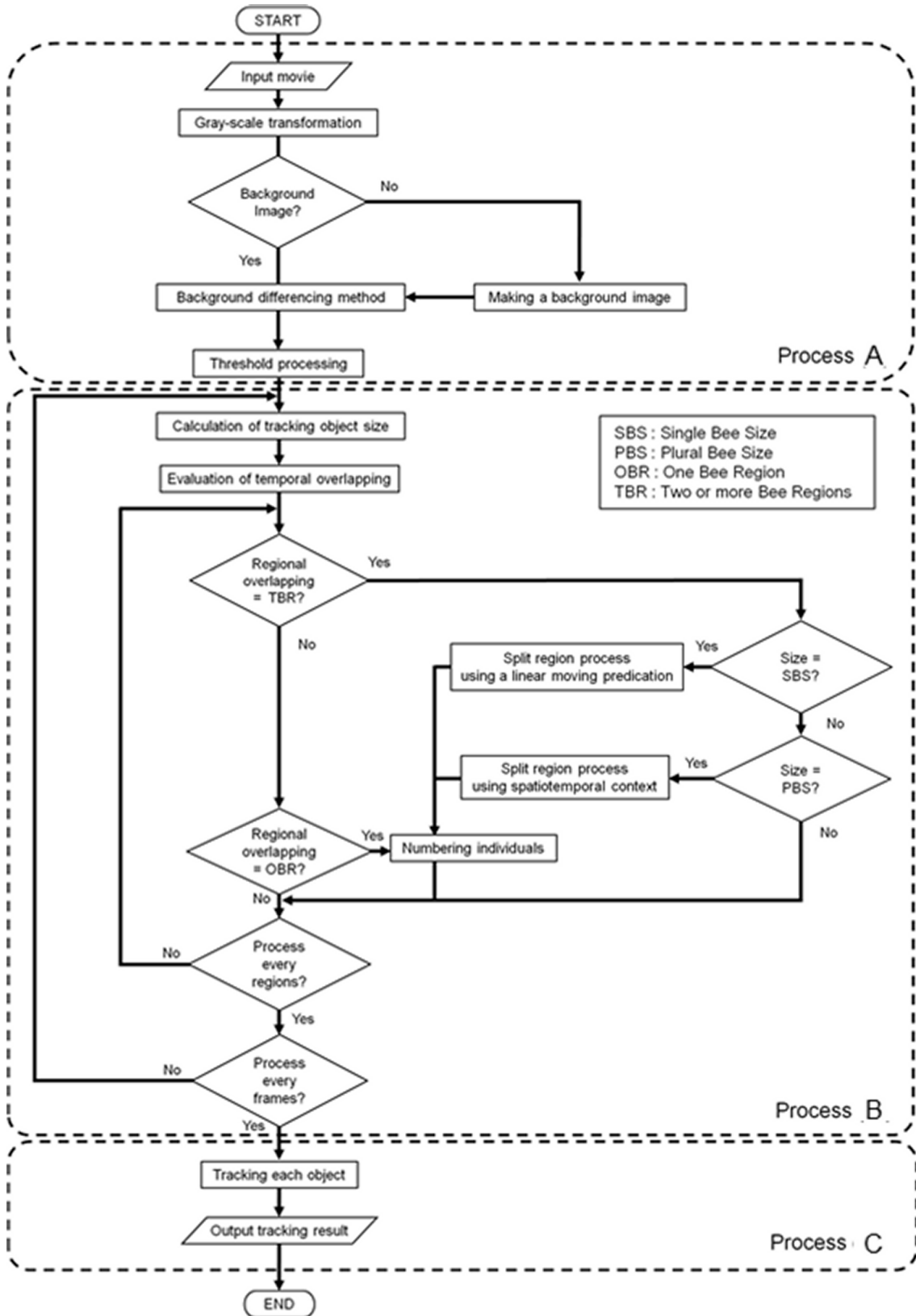


Figure 3.1: Workflow of our proposed method. The method consists of three main processes; (A) Detecting bee candidate regions using gray-scale transmission and threshold processing; (B) Identification and numbering of individuals, achieved by extracting individuals from regions containing two or more bees; and (C) Assigning x and y position coordinates to each bee, outputting the results and connecting them into trajectories.

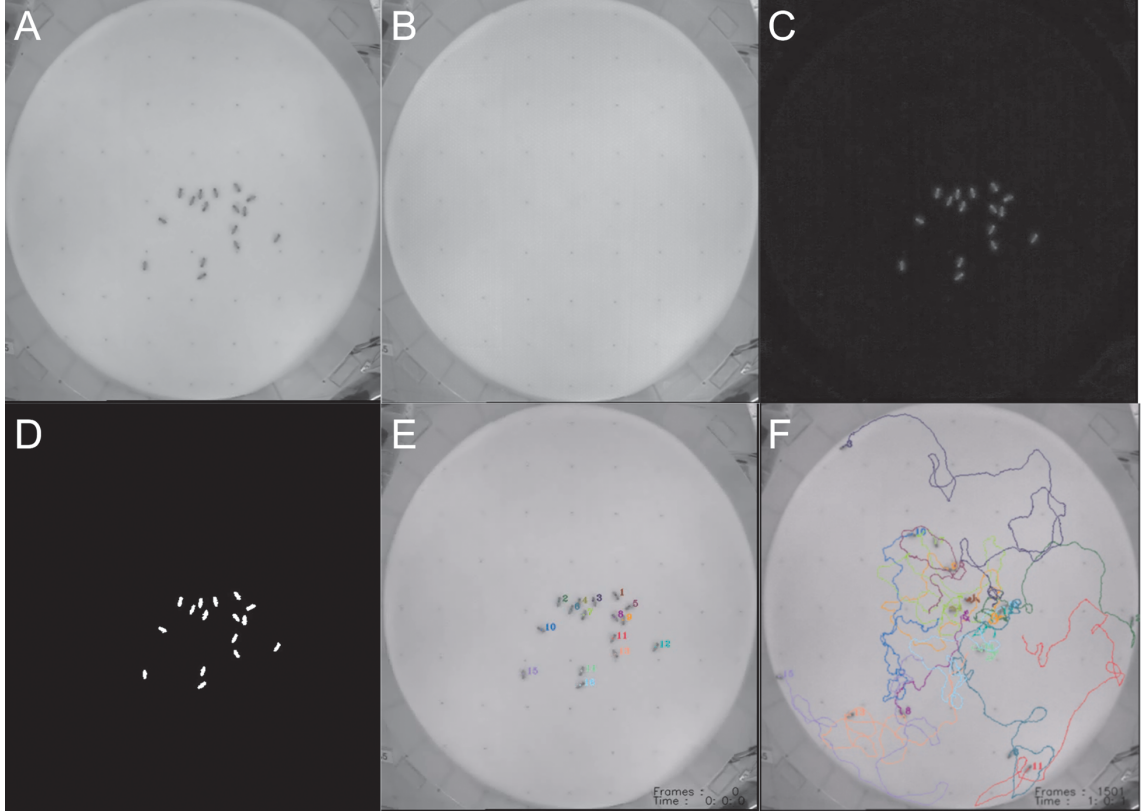


Figure 3.2: Snap shots to illustrate each step of the data analysis. (A) original image, (B) background image to make from original images, (C) the image to process background subtraction, (D) the image to process the binarization, (E) the result image of identification of bees and (F) the trajectories of every individuals.

one bee varies slightly during each run. As a pre-processing step, our method detects the bee regions from the body size of a single bee, without individually identifying the bees. Identified numbers are then assigned to the bee regions to produce a prediction and identification model for bees, parameterized from each focal movie. The number of pixels in a single bee region is calculated from the initial movie frames (20 seconds; 500 frames), which also reveal the valid size range of single honey bees (RSS: Range of Single-bee Size). The RSS is an effective measure for detecting individuals in all subsequent video frames. To allow the tracking of multiple bees, our method imposes an important restriction: The regions of an individual bee at time t and time $t-1$ must be overlapped (Figure 3.3). In other words, a bee cannot move further than its own body length in any two consecutive frames. The algorithm then categorizes all bee-associated regions by size and by spatiotemporal overlapping in the movie frame sequence.

Our program defines the size and shape of one bee. Moreover, we assume that the bee's shape remains unchanged in an overlapping situation of two bees. Therefore, the program can calculate the number of individuals and the location of each bee at current time using the current overlapping area and the location of each bee at previous time [61]. In the next step of the algorithm, bee regions are first classified into three categories based on region size; 1) SBS: the region fits within the RSS, 2) PBS: the region is bigger than the maximum RSS and 3) NBS: the region is smaller than the minimum RSS. Next, the number of regions in the previous frame overlapped on each current region is determined. This quantity is also divided into three classes; 1) NBR: No overlapping bee regions, 2) OBR: One overlapping bee region, and 3) TBR: Two or more overlapping bee regions. From these two characteristics (size and overlap), individual bees can be distinguished in each movie frame. In the first frame, unique ID numbers are assigned to SBS regions (Figure 3.2E). In subsequent frames, ID numbers are recovered as follows:

1. SBS and NBR: This region contains a single new bee. A new unique number is assigned to this region.
2. SBS and OBR (Figure 3.3 A): The current bee $SBS(t)$ is assigned the ID number of the previous overlapping bee $SBS(t-1)$.
3. SBS and TBR (Figure 3.3 B): The current region $SBS(t)$ contains two individuals. The correct $SBS(t)$ s are delineated by the linear motion assumption. The current $SBS(t)$ s are assigned the ID numbers of the overlapped $SBS(t-1)$ bees.
4. PBS and TBR (Figure 3.3 C): The current region $PBS(t)$ is divided into two $SBS(t)$ s by regional matching, using spatiotemporal contextual information between the current region and $SBS(t-1)$ s. The divided $SBS(t)$ region is assigned the ID numbers of the $SBS(t-1)$ bees [61].
5. Other cases: No processing is executed.

In process C, the location data and the behavioral trajectory of individual bees are logged during the image processing (Figure 3.2F). Bee location is the position of the center of mass of each identified bee's region. These coordinates are finally exported into a CSV-format file. From the bee location data, the velocity, acceleration and direction

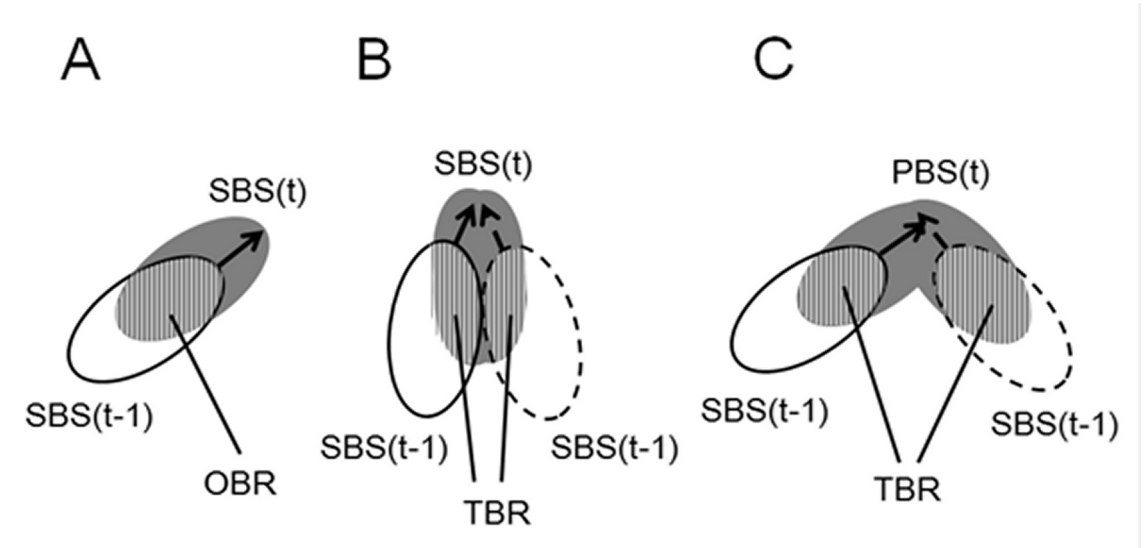


Figure 3.3: Spatial overlapping patterns are classified by size changes between current and former regions. (A) Single bee moves straight ahead. Region size is within range of single-bee size (SBS) and overlap is one-bee region (OBR). (B) Two bees appear as a merged entity in the source image. Region size is SBS and overlap is (two-or-more bee region) TBR. (C) Two bees bump into each other. Region size exceeds range of single-bee size (PBS) and overlap is (TBR).

of the bee’ movements are simultaneously determined, as well as the distance moved by individuals (estimated from temporal changes in the bees’ locations). Furthermore, our method can generate visual trajectories of bee behavior by interpolating between all locations of all individuals frame by frame [61]. The tracking results are also exported as an image file, with the overlapping locus of each bee on the original frames. These quantitative values will assist the further analysis of individual and collective honeybee behaviors. The method is also applicable to many other animals that move and interact in comparable arena setups.

3.3 Experimental Results

Our tracking software, called “K-Track”, is developed in Microsoft Visual Studio 2010 (Visual C++) with Computer Vision Library: OpenCV 2.31 on a laptop computer with an Intel Core i5 – 2.50GHz (CPU), 8GB (Memory), 256GB (HDD) and Microsoft Windows 7 Professional 64bit (OS). Because a large RAM (more than 4GB) is required to store the individual positions over all time frames, our software runs as a console application in a Windows 64bit environment. Larger memory space would allow researchers to realize more efficient image processing and longer-term tracking of multiple individuals.

We prepared four sets of 1-minute movies (1,500 frames), named ‘movie-A’, ‘movie-1’, ‘movie-2’ and ‘movie-3’, to evaluate our software. In ‘movie-A’ the movements of software-simulated and multi-artificial agents are recorded. These agents are driven by the honeybee-inspired BEECLUST algorithm [6][34][36]. This movie contains relatively simple movements and was used to evaluate the basic performance of our software. The

movie was played at 25 frames per second, the rate of PAL format, and the frame size of each image was 600 x 600 pixels. The sizes of individual honeybees and the arena were extracted from the experimental movies, which incorporated two additional behavioral components; a bee could vary its direction by rotating its body axis, or it could suddenly stop. These behaviors were added to the original BEECLUST algorithm in order to mimic real bee’s behavior.

Movies 1-3 are experimental benchmark-movies of sixteen young honeybees (*Apis mellifera* L.) walking in a circular arena (radius 30 cm). These movies were recorded by an infrared camera fixed 175 cm above the arena in the Artificial Life Lab. at the Karl-Franzens-University Graz, Austria [62]. The behaviors displayed in the movies differ widely in terms of (1) average speed of movement, (2) number of interactions among two or more individuals, and (3) long-term resting behavior. More specifically:

Movie-1 characterized by slow movement, few interactions, and periods of long-term resting.

Movie-2 characterized by moderate movement, some interactions, and no long-term resting.

Movie-3 characterized by rapid movement, many interactions and no long-term resting.

As described below, the frequency and quality of movie images (25 frames per second (PAL format) and 532 x 576 pixel size for the circular arena) was sufficient for tracking the behaviors of young bees in the arena. Furthermore, when evaluating our software, we paid attention to overlapping patterns, including the interactions among bees, which embody the most important social behaviors. Prior to each experiment we investigated the overlapping patterns in the arena, obtained from the experimental movies, and manually classified them into five categories; 1) crossing, in which the paths of two bees cross without interaction (Figure 3.4 (a)), 2) touching, in which two bees contact during walking (Figure 3.4 (b)), 3) passing, in which one bee contacts another bee while walking (Figure 3.4 (c)), 4) overlapping, in which two bees overlap as they cross (Figure 3.4 (d)), and 5) waiting, in which one bee contacts another bee and stops (Figure 3.4 (e)). Three of these classifications (Figure 3.4 (a), (b) and (d)) have been previously identified by Delcourt et al. [30]; the passing and waiting categories were deduced from our behavioral analysis of bees.

3.3.1 Experiment 1 (movies of multi-artificial agents)

In the first experiment, K-Track tracked multi-artificial agents that mimic honey bee movements (movie-A). The aim of this experiment was to evaluate the basic performance of our tracking system. The algorithm performance was compared to that of the current state-of-the-art algorithm Ctrax [15]. Results were obtained as a new movie containing the dynamics of the trajectories of all objects (Figure 3.5). As shown in Figure 3.5 A, K-Track offered excellent tracking results with no misidentification, whereas Ctrax mis-tracked twice (Figure 3.5 B) on the same movie. We also evaluated the accuracy of position estimation by measuring maximum, minimum and average Euclidean distances between the assigned and calculated values (Figure 3.5 C). The average errors in object

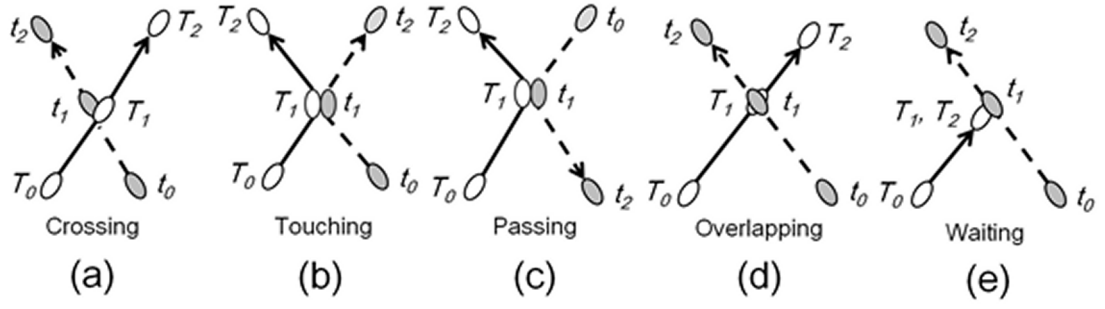


Figure 3.4: Five types of interaction patterns between two bees were extracted from the movies and classified as follows; (a) crossing: two bees cross without overlapping; (b) touching: two bees touch, then separate; (c) passing: one bee walks past another with touching (d) overlapping: two bees cross and overlap; (e) waiting: after touching, one bee waits until another bee has moved forward.

centers of mass were less than 1.2 pixels in K-Track (less than 1.0 mm in real space). In Ctrax, the average errors were below 1.7 pixels for correctly identified individuals, but the distance errors were very large because individuals were exchanged in frames 580 and 1366. Clearly, K-Track can track multiple bees on a flat surface more accurately than Ctrax (Mann-Whitney U-test, $P = 3.443 \times 10^{-7}$).

3.3.2 Experiment 2 (movies recorded from honey bee experiments)

Next, the performance of the K-Track and Ctrax algorithms was tested on three honeybee movies (honeybees being the target animals of our algorithm). Interactive behaviors were the focus of this investigation. We also checked the trajectories of each individual bee. In each movie, five interaction patterns (see Figure 3.4) were manually counted (Table 3.1). Each tracking result was evaluated by the success rate of identifications and the trajectory of each bee. In each movie, five interaction patterns (see Figure 3.4) were manually counted (Table 3.1). Each tracking result was evaluated by using a standard measure called the Trajectory Fragmentation Factor (TFF) and the Trajectory Completeness Factor (TCF) to measure the performance of multi-target tracking software [43,46]. The TFF value is the necessary number of calculated trajectories to draw a correct trajectory of the target and the TCF value is the accuracy of tracking trajectory. If the calculated trajectory of tracking software fits in the correct trajectory, both TFF and TCF values are 1. If the software fails to track a target, the TFF value is more than 1 and the TCF value is less than 1. The K-Track's TFF average of all three movies is 1.29 (movie-1: 1.00, movie-2: 1.13, movie-3: 1.75). The K-Track's TCF average of the movies is 0.84 (movie1: 1.00, movie2: 0.89, movie3: 0.63). Both numbers suggest K-Track is highly accurate for tracking multiple honey bees.

First, K-Track and Ctrax were tested on relatively simple bee movements (Movie-1). The bees in this movie moved slowly and interacted less than in other movies. The tracking results of the two programs are shown in Table 3.1 A and Figure 3.6. While K-Track captured all bee interaction events, Ctrax failed in 7 instances (passing: 1, waiting: 6). Thus, Ctrax did not always identify the behavioral states 'waiting' and

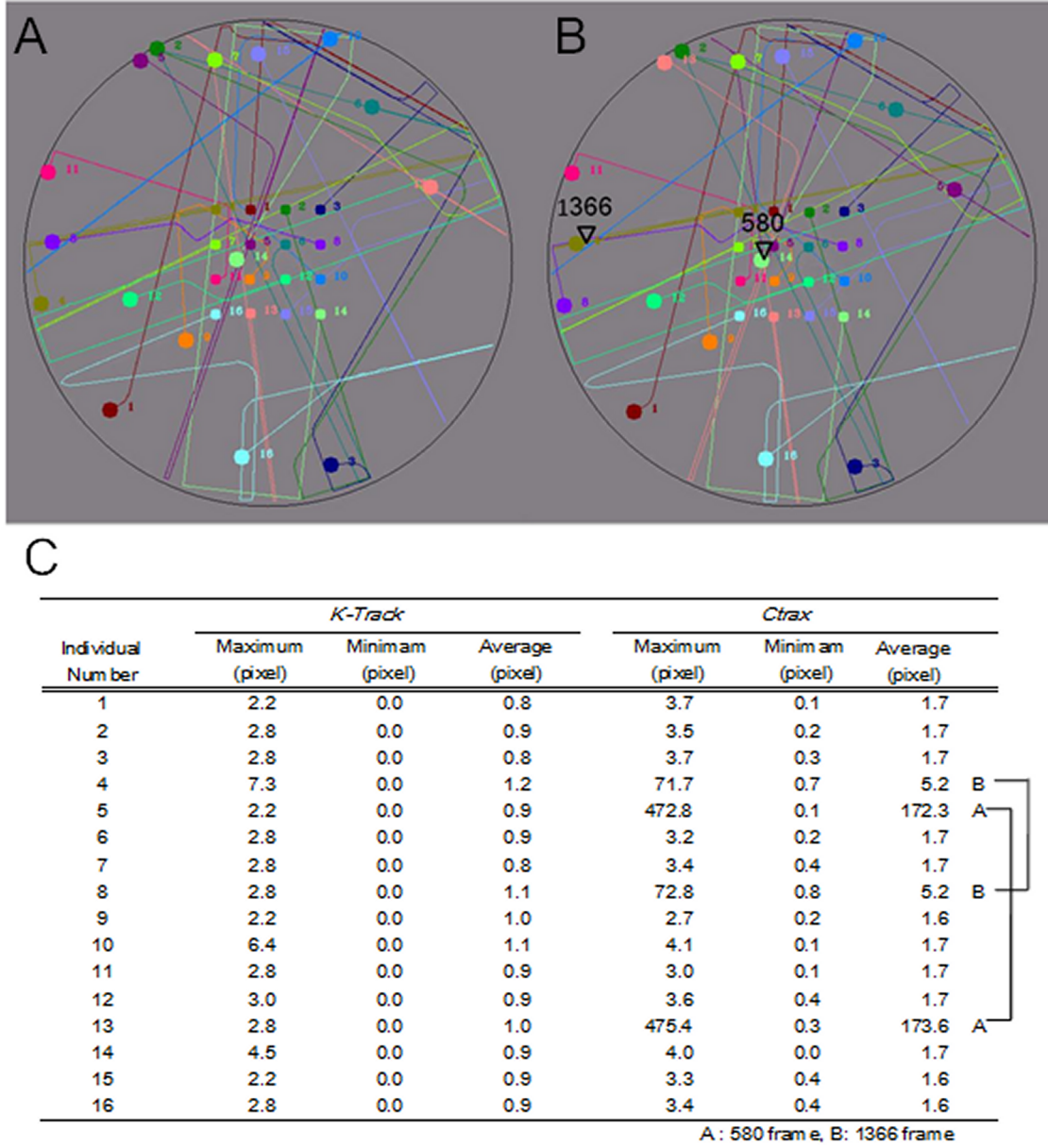


Figure 3.5: Tracking movements of bee-inspired agents in K-Track and Ctrax. (A) K-Track's trajectories. K-Track correctly identifies the positions of all agents. (B) Ctrax's trajectories. Several mistakes occur in two frames (t=580 and t=1366). (C) Comparison table of the average errors made by K-Track and Ctrax.

Table 3.1: The numbers and errors of interaction patterns detected from experimental movies. The movements from crossing to waiting are represented in Figure 3.4. The “multiple” indicates that three or more bees interact. Failure occurs when two or more bees interact near the edge of the arena. The high false rate of the “Overlapping” state may be caused by motion rather than by the interaction pattern. Our program K-Track outperforms “Ctrax” in terms of tracking accuracy.

				K-Track		Ctrax	
				False	False	False	Flase
				number	rate(%)	number	rate(%)
				Occurence			
A	Movie-1	Touching	2	0	0.0	0	0.0
		Passing	3	0	0.0	1	33.3
		Waiting	10	0	0.0	6	60.0
B	Movie-2	Crossing	1	0	0.0	0	0.0
		Passing	5	0	0.0	2	40.0
		Overlapping	1	0	0.0	0	0.0
		Waiting	15	1	6.7	6	40.0
		Multiple	1	0	0.0	1	100.0
C	Movie-3	Crossing	3	0	0.0	1	33.3
		Passing	18	1	5.6	0	0.0
		Overlapping	2	2	100.0	1	50.0
		Waiting	14	2	14.3	3	21.4
		Multiple	9	1	11.1	3	33.3
	Sum		84	7	8.3	24	28.6

‘passing’, which are frequently exhibited by bees. Regarding trajectory tracking, K-Track completely tracked all movements with no duplicate ID assignments (see Figure 3.6 A). By contrast, Ctrax lost the movements of some individuals and could not thereafter identify them (see Figure 3.6 B). In this movie, some of the bees remained stationary over significant periods of time. Ctrax regarded these bees as part of the background and permanently lost their locations.

We then tested both algorithms on movie-2, which contains more complex honeybee movement patterns than movie-1. The comparison results are summarized in Table 3.1 B. We note that K-Track made one mistake while Ctrax missed nine interaction events. As before, Ctrax tended to misinterpret ‘waiting’ and ‘passing’ states. Finally, both algorithms were tested on the third movie (movie-3) in which complex bee behavior is displayed. In this movie, K-Track and Ctrax made 6 and 8 tracking errors, respectively (see Table 3.1 C). The positions at which K-Track fails, and the switched identification numbers and their timing, are shown in Figure 3.7 A and Figure 3.7 B. All errors occur near the edges of the arena. Our behavioral tracking algorithm assumes linear forward motion of the bees in a short period. However the circular wall forces the bees to turn and move along the bended edge of the arena. Under such conditions, K-Track cannot always correctly separate the individuals.

In summary, K-Track and Ctrax failed to separate and identify overlapped individ-

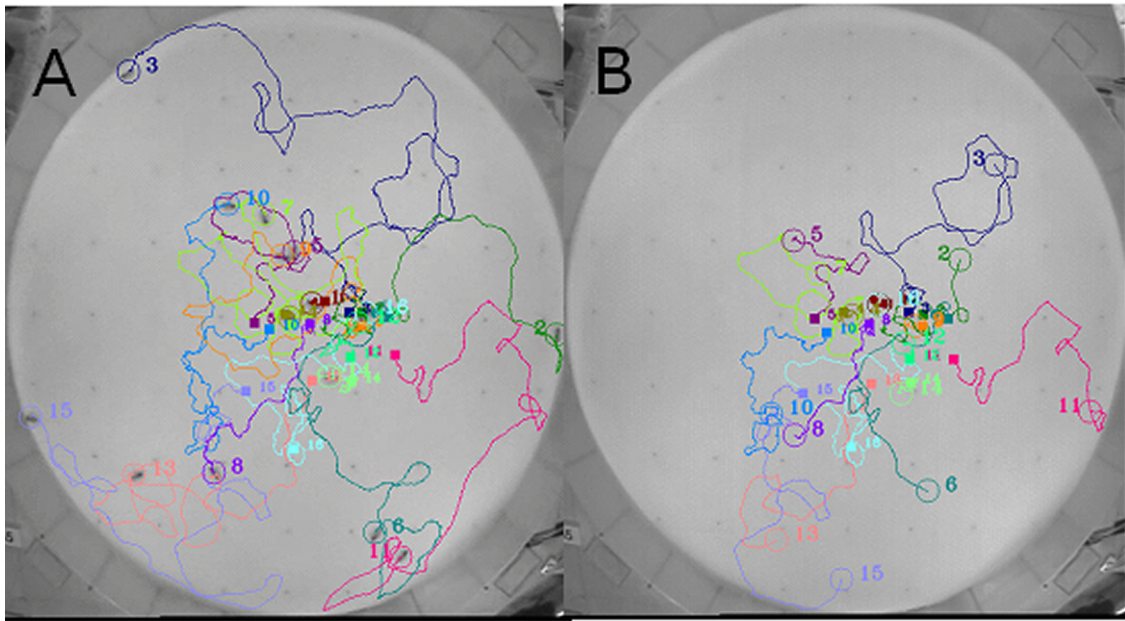


Figure 3.6: Tracking results of movie-1 by K-Track and Ctrax. The numbers shown on individuals are the identified IDs. Squares and big circles represent the start and the end points of tracking without losing the bee and re-identifying it with a new ID after the tracking. (A) Tracking results achieved by K-Track, (B) Tracking results achieved by Ctrax. The trajectories achieved by Ctrax are shorter than those by K-Track, indicating that Ctrax more frequently loses track of the bees.

uals in seven (8.3%) and twenty four (28.6%) interactions, respectively. Even in the middle of the arena, Ctrax failed to capture ‘waiting’ and ‘passing’ interactions, while K-Track could adequately process these data. Both algorithms failed around the arena edge, where linear movements are curtailed by the rounded boundary. The superior performance of K-Track for tracking multiple interacting bees was confirmed.

Automatic image processing and tracking has several advantages over manual image processing. For example, K-Track automatically detects the position and timing of contacts between two or more bees from the distances between individuals (Figure 3.8). The interactions among multiple individuals, such as approach, contact and separation of one bee from another, are crucial for analyzing group behavior of animals. We classified such events by calculating the Euclidean distance between two bees. A bee-to-bee encounter was defined as one bee facing another at a distance of less than one body length. As an example, the Euclidean distances between target bee #9 and another colony member (#2 or #6) were calculated at different times. The temporal changes in these distances are plotted in Figure 3.8 A. K-Track also calculated the velocity of bee #9 and assessed five candidates for interaction by whether the distance between individuals reduced below a specified threshold during 30 seconds (Figure 3.8 B). In this experiment, the threshold value was 15 pixels (the length of the major axis of the honeybee body). The walking speed of bee #9 was suddenly slowed by all five encounters, but was recovered in three cases. Thus, we confirmed that K-Track can observe detailed interactions and movements among multiple agents, and can evaluate

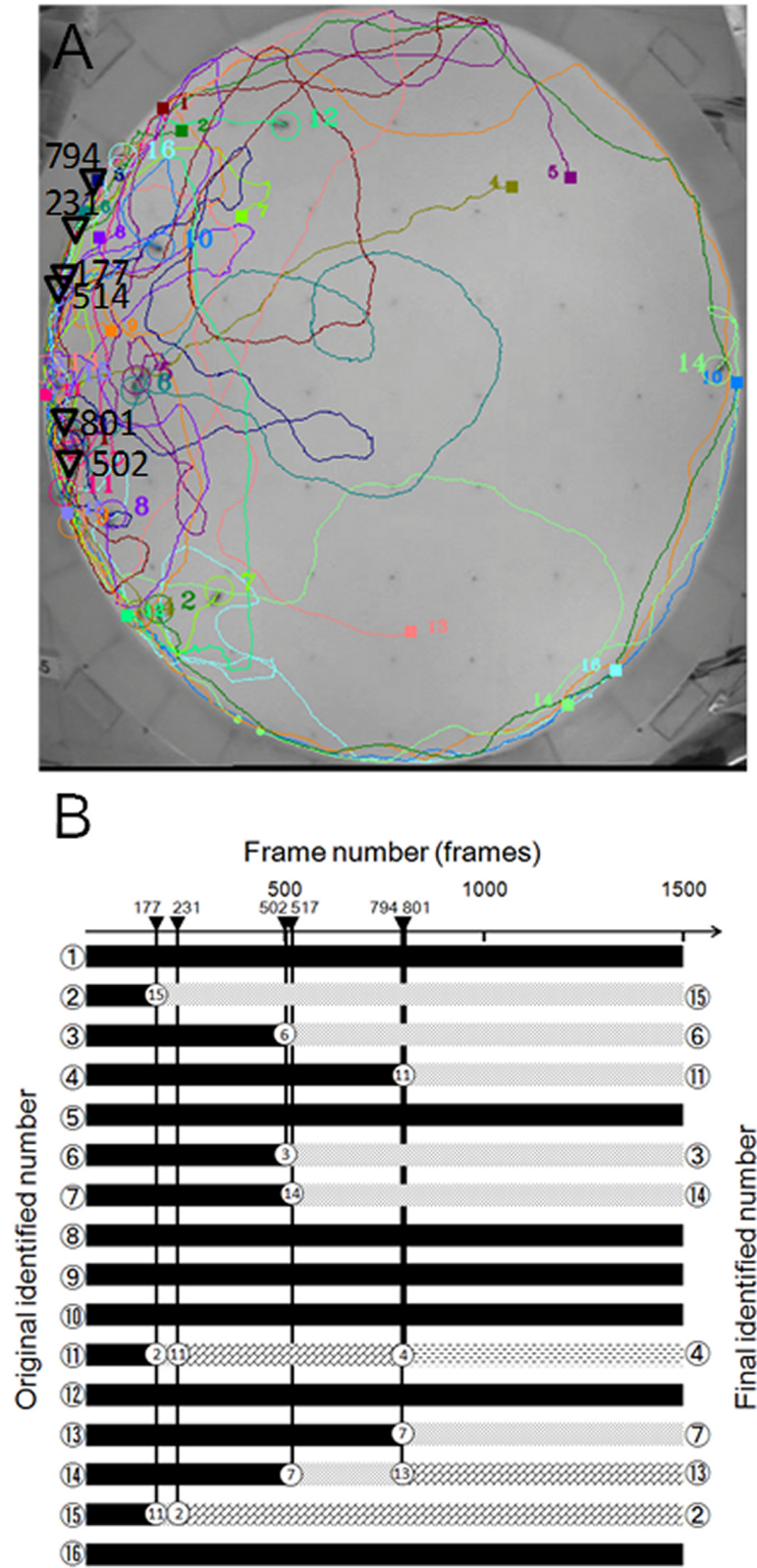


Figure 3.7: Processing of movie-1 by K-Track. (A) Trajectories produced by K-Track. Squares and circles represent start and end points of individual trajectories of each bee. Triangles show the interaction points between two or three bees. Triangles tend to aggregate around the edge of the arena, indicating that interactions frequently occur there. (B) The temporal state transition of each bee. The numbers on the inverted triangles are the frame numbers in which bee IDs were exchanged. The numbers in the right-hand circles are the exchanged IDs. Six exchanges occurred in Movie-3.

them quantitatively.

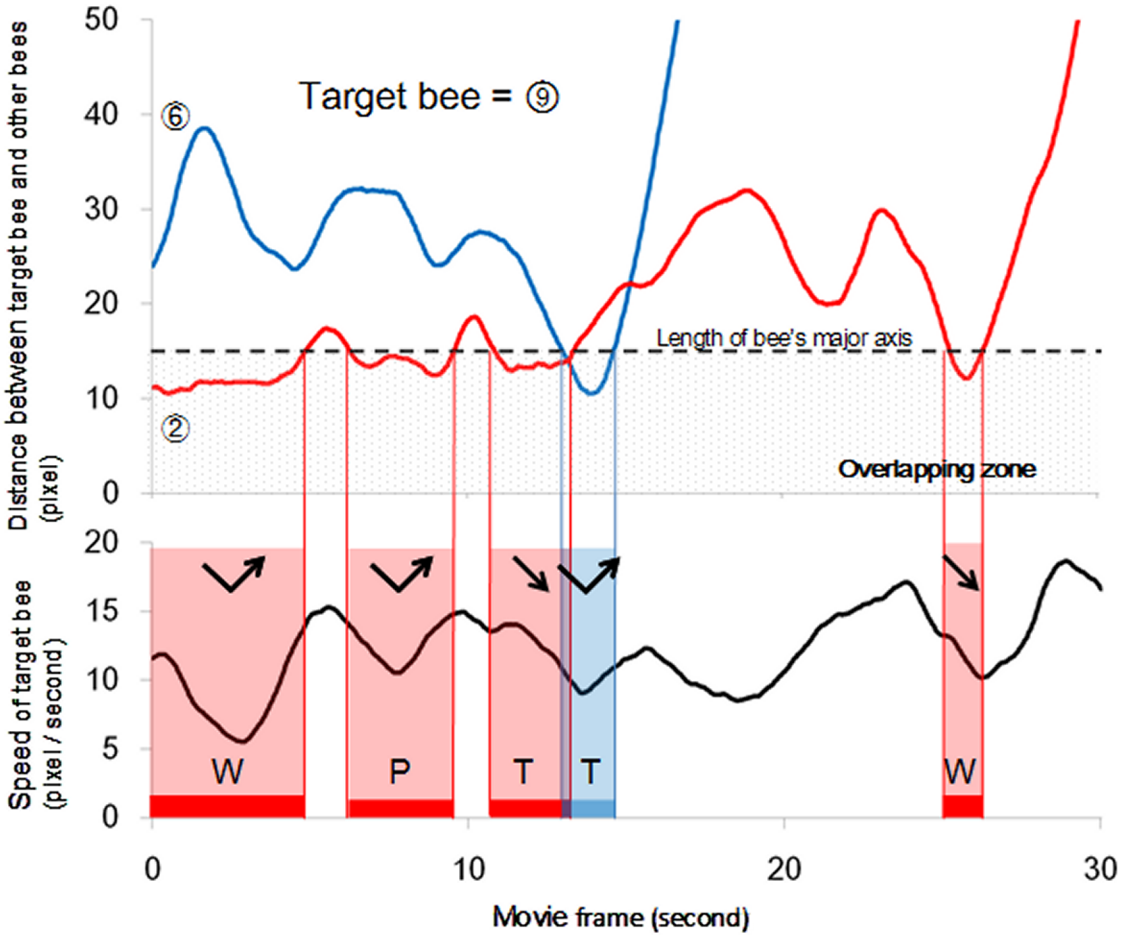


Figure 3.8: Temporal changes of distance and speed between two bees. (A) The distance between bee #9 and bees #2 and #6, which contact bee #9. (B) The area spans less than 20 pixels. The dotted-line shows the length of the bee’s major axis. Below the dotted line, the two bees are assumed touching. In the lower part of the figure, “W”, “P” and “T” represent the identified interaction patterns “waiting”, “passing” and “touching”, respectively (see Figure 3.1).

3.4 Discussion

K-Track demonstrates superior performance in tracking multiple honeybees compared to the current state-of-the-art algorithm, Ctrax. Among 84 crossing events observed in three experimental movies, K-Track and Ctrax successfully tracked 77 and 60 honeybee interactions, respectively. Of the two algorithms, K-Track provided higher accuracy for tracking multiple honey bees with complex crossing and contacting. We note that K-Track was specifically designed for complex honeybee interactions. Analyzing their movies of interacting juvenile Nile tilapias, Delcourt et al. [30] classified crossing events as “crossing”, “touching” or “overlapping”. In addition to these categories, we observed “passing” and “waiting” in honey bees’ interaction behaviors. Of the five main honeybee interactions, “crossing”, “touching” and “overlapping” occurred only 12 times in 84 crossing

events. The majority of events (72) comprised ‘passing’ and ‘waiting’, which was not reported in Delcourt et al. [30]. To compare the results of the two algorithms in detail, we reclassified the five crossing patterns into two groups, one comprising the behavioral states ‘crossing’, ‘touching’ and ‘overlapping’, the other holding the states ”passing” and ”waiting”. In the first group, both algorithms successfully tracked individuals throughout 10 out of 12 events (83.3% tracking accuracy in both algorithms). In the second group, however, K-Track achieved 93.1% tracking accuracy (67 out of 72 events), while that of Ctrax was 69.4% (50 out of 72). Such a difference in tracking accuracies indicates that conventional software is less suitable than K-track for tracking the behaviors most commonly observed in honey bee collectives. In a broader sense, this implies that K-track can more accurately track animals displaying variable crossing events.

All of the 7 tracking failures observed in K-Track occurred around the edges of the circular arena, indicating that future improvements to the algorithm should especially focus on these regions. K-Track assumes that a bee travels ahead without changing the direction of her body axis during a crossing event. However, at the arena wall, the focal honeybee is prevented from linear movement and often bends her body near the circular edge, thereby following the curvature of the arena wall. Currently, our program does not reproduce this behavior. In future work, the movements of bees near the walls will be studied in detail to analyze the interaction patterns and the variation of moving directions in those regions. These new dynamics should be adopted into a new rule set which accounts for the special conditions at the wall. Furthermore, we plan to apply K-Track to a statistical estimation model of behavioral attributes, based on the bees’ individual motion histories.

Potentially, K-Track may collect and present target interaction images for ethological studies. For example, the interaction patterns of honeybees tend to scatter throughout the movie. To extract interaction information, researchers must therefore check all frames in the movie. This manual checking is time consuming, labor consuming and error-prone, and constitutes a large problem for researchers. Our system specifies that interactions occur only when the distance between approaching bees becomes less than the bee’s body size. Consequently, K-Track can easily extract only those scenes involving bee interactions, and specify the exact locations of interactions in successive frames. While animal interaction is generally regarded as a tracking problem, K-track is especially designed for such interactions and considers them in predicting the future movements of individuals. The algorithm retains individual IDs after interaction events. Because our algorithm exploits interaction data and identifies and classifies interaction events, it may greatly assist ethological honeybee research. Determining the localized interactions among clearly identified bees provides valuable information for models of pheromone exchange among bee groups. It is also useful for investigating trophallactic interactions and for analyzing the inhomogeneous distribution of social interactions in subgroups of the honeybee collectives. From the movie scenes, our software extracts the particular area in which two bees interact and identifies the bees by their ID numbers. In this way, the bees’ historical behavior before the interaction event is available for automated or computer-aided analysis. Additionally, we developed an automatic program for editing specific areas in images, which can efficiently present the target area in the frames using various functions such as zooming [60]. By combining this automatic

editing program with K-Track, we can extract specific areas in specific scenes containing interactive behaviors of target animals. K-Track can be automatically upgraded to collect such target images and display them effectively and emphatically. This new software should greatly benefit ethological researchers in analyzing the interactive behaviors of their target animals.

The performance of the tracking software depends on the target animals. K-Track succeeded to track the walking movements of the Argentine ants (*Linepithema humile*) [78]. It can be applied for small insects, but it still has some problems, such as animal size and frame rate of movie, in general use. We also applied K-Track for tracking grovelling behaviors of earthworms, but we failed it because of the big changes of body size during their movements. Other tracking methods were already developed by many researchers. De Chaumont et al. used a body model of a mouse through a set of geometrical primitives linked by physical constraints to track individuals [20]. However, this software can track only two mice. Ohayon et al. used a unique back pattern of a mouse [85]. The mice have unique patterns of their backs, but the bees would not have identifiable features as mice. Freund et al. used mice with PFID transponders to detect their locations [40] and Weissbrod et al. used a method in mixing a video-based tracking and a PFID-based tracking [117]. It is easy to tag them with PFID, but is difficult to apply bees with these devices.

Branson et al. used the method that each detected fly in frame t is associated with a fly tracked in the previous frame $t - 1$ [15]. Kabra et al. applied the Ctrax for classification of fly behaviors [56]. These methods are good performance for the flies that always move linearly, but would be not adapt for the complex behaviors of bees with long-time resting or waiting. Dankert et al. used the localized body model by fitting a Gaussian mixture model (GMM) with three Gaussians (background, other parts and body) to the histogram of the values using the Expectation Maximization (EM) algorithm [27]. This method, however, track only two flies, simultaneously.

Moreover, Mersch et al. [76] developed the tracking software for multiple ants. They used the ants with ARTags to identify individuals. Similar to PFID, it is impossible to set a ARTag to each bee without a stress. Therefore, our software is quite effective to track multiple bees easily.

3.5 Conclusion

In this paper, we proposed a novel method for tracking unmarked multiple honey bees in a flat laboratory arena, which focuses on identifying interaction events among honeybees. Based on this method, we developed a prototype software named “K-Track”. The performance of “K-Track” was compared with that of the open-source tracking software “Ctrax”. The test subjects were one movie of sixteen agents and three movies of experiments involving sixteen young bees moving in a circular arena. The proposed algorithm showed significantly superior performance in tracking multiple bees compared to Ctrax, in terms of both robustness (fewer tracking errors and losses in movies showing complex motion patterns), and richness (number of identified behavioral states) of the behavioral classifier.

Chapter 4

Improvement of the software using tracking results of both forward and backward play

Abstract

In recent ethological studies, the behaviors and interactions of animals have been recorded by digital video cameras and webcams, which provide high functionality at reasonable cost. However, extracting the behavioral data from these videos is a laborious and time-consuming manual task. We recently proposed a novel method for tracking unmarked multiple honeybees in a flat arena, and developed a prototype software named “K-Track”. The K-Track algorithm successfully resolved nearly 90% of cases involving overlapped or interacting insects, but failed when such events happened near the edge of a circular arena, which is commonly employed in experiments. In the present study, we improved our K-Track algorithm by comparing the interaction trajectories obtained from forward and backward playing of video episodes. If the tracking results differed between the forward and backward episodes, we chose the trajectory with the smaller maximum moving distance per frame. Based on this concept, we developed a new software, “K-Track-kai”, and compared the performances of K-Track and K-Track-kai in honeybee tracking experiments. In the cases of 6 and 16 honeybees, K-Track-kai improved the tracking accuracy from 91.7% to 96.4% and from 94.4% to 96.7%, respectively.

4.1 Introduction

Most ethological studies rely on accurate observations of animal behavior. Social mechanisms in animals are popularly observed in insects such as bees [53] and ants. The social behaviors of honeybees, such as the waggle dance discovered by Karl von Frisch in 1967 [41], are of great interest to ethologists. Consequently, the honeybee has become a popular model animal for studying social and swarm behaviors. By monitoring and analyzing the individual honeybee behaviors alongside their social interactions, we could better understand the social structure and performance of a honeybee colony. In recent experiments, behavioral information was gathered by recording the trajectories and variation of animal movements within a circular arena [102] [108]. Recording equipment such as digital video cameras and webcams, which provide high functionality at reasonable cost, acquire important ethological data on animal sociality. Video recordings capture the long-term movements of target animals rapidly and easily, but extracting the behavioral data from video images is a laborious and time-consuming manual task.

Ethological data in video images can now be acquired by automatic tracking programs, enabling quick, precise analyses of various animal behaviors (e.g. [4] [38] [58]). However, these methods are of limited applicability because the target animals are assumed to move against a constant background with continuous, streamlined, unvarying movements. Such software is unsuitable for automatic tracking of honeybee behavior, which is both complex and unique. Bees frequently contact each other and display many irregular movements, such as resting or stop-start walking within their hive. These behaviors require analysis by a new tracking method such as video-RFID (Radio Frequency Identification) tracking and barcode labeling, in which individuals are identified by part of an RFID chip or by a barcode paper attached to their bodies [40] [76]. However, a purely video-based tracking method that imposes no artifacts or colors on the animals is highly desired. Such a method would minimize the experimental interference with the focal animals. The automatic tracking of unmarked honeybees remains an important challenge in animal behavior analysis.

Recently, we proposed a novel method that tracks unmarked multiple honeybees in a flat laboratory arena, focusing on their interaction events [62]. Based on this method, we developed a prototype software named “K-Track” [63]. The K-Track program implements three main processes; (A) detecting the object (bee) region by a simple threshold processing on gray scale images, (B) identifying individuals by their size, shape and spatio-temporal position changes, and (C) connecting the centers of mass of the identified individuals through all video frames to obtain the individual motion trajectories. The tracking performance of K-Track was evaluated on videos of 16 bees walking around a circular arena. The algorithm successfully tracked nearly 90% of all recorded interaction events, but failed when interactions occurred near the wall (edge) of the circular arena. Failure is probably caused by the honeybees’ nonlinear movement and the sudden directional changes of their bent bodies as they follow the curvature of the arena wall.

In the present study, we improve the existing K-Track algorithm by acquiring additional information on the motion properties of the tracking target. To this end, interaction events are extracted from both normal forward tracking and by time-inverted picture streaming. Based on this idea, we developed a new software named “K-Track-

kai”, and validated its performance in tracking 6 and 16 honeybees on a circular arena. The novel results were compared with those obtained from the existing original K-Track algorithm as a reference.

4.2 Materials and Methods

4.2.1 Tracking algorithm

To improve the existing K-Track algorithm [63], we played the video recordings both forward and backward, and compared the trajectories obtained from the initial tracking results during interaction events (Figure 4.1). If the forward and backward trajectories were very similar, both tracking results were regarded as successful. However, if the two trajectories were different, one of them had probably resulted from incorrect tracking, characterized by sudden changes in the moving distance and direction of the target between two continuous frames. Therefore, when comparing the forward and backward trajectories of the same interaction, we assumed that the maximum moving distance per frame is larger in the incorrect tracking than in the correct tracking. This concept was adopted in our new program “K-Track-kai”, which improves out original K-Track algorithm. Our new program generates the sequential image data in the forward-time and back-ward-time directions from one original video. In a window of time around each overlap or merger event of two individuals, the K-Track-kai algorithm extracts the both individuals’ trajectories from both streams of sequential images. Depending on the interaction situation, the overlap or merging duration extended from a few seconds to tens of seconds. In each case, whether the correct trajectory was the forward or backward tracking result (see Figure 4.2) was decided by the following steps:

1. Generate sequential images in the forward- and backward-time directions.
2. Apply K-Track’s tracking process on the forward and backward image sequences (Figure 4.3).
3. Detect the time and position of two overlapped animals (Figure 4.3).
4. Calculate the trajectories of the two animals by tracing their positions in the forward- and backward-time directions (Figure 4.3).
5. If the two tracking results differ, calculate all moving distances of both objects from the forward sequence $Fs(t)$, and from the backward image sequence $Bs(t)$, in a frame by frame manner. Here, $Fs(t)$ and $Bs(t)$ are functions of the forward time t . The moving distance was measured as the Euclidian distance. If the maximum value $maxBs$ of $Bs(t)$ is lower than the maximum value $maxFs$ of $Fs(t)$, adopt the tracking results from the backward sequence. Otherwise, adopt the tracking results from the forward sequence (Figure 4.4).

4.2.2 Image data and computer

The videos were recorded at the laboratory of Karl-Franzens University Graz, Austria, and were also analyzed in our previous study [63]. Under the same conditions, we ac-

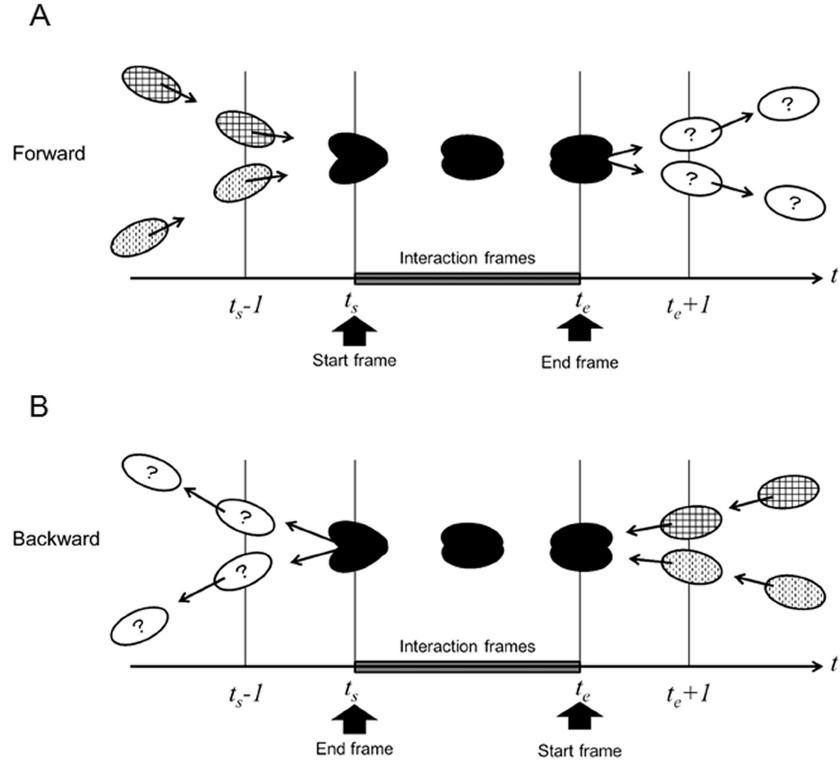


Figure 4.1: Tracking trajectories in a two-bee interaction. Bold line indicates the range of the interaction frames. (A) trajectory of forward-running image sequences and (B) trajectory of backward-running image sequences.

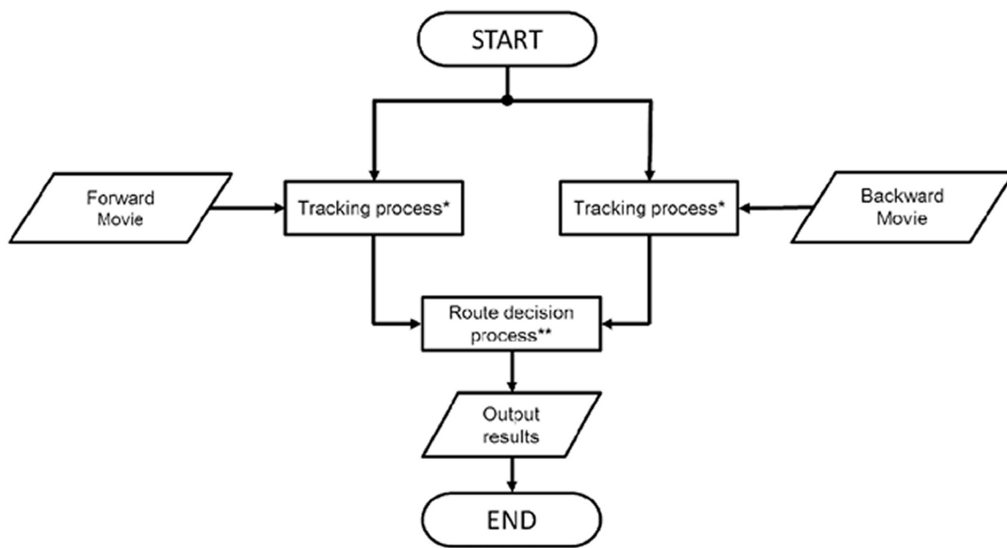


Figure 4.2: Flowcharts of the proposed method

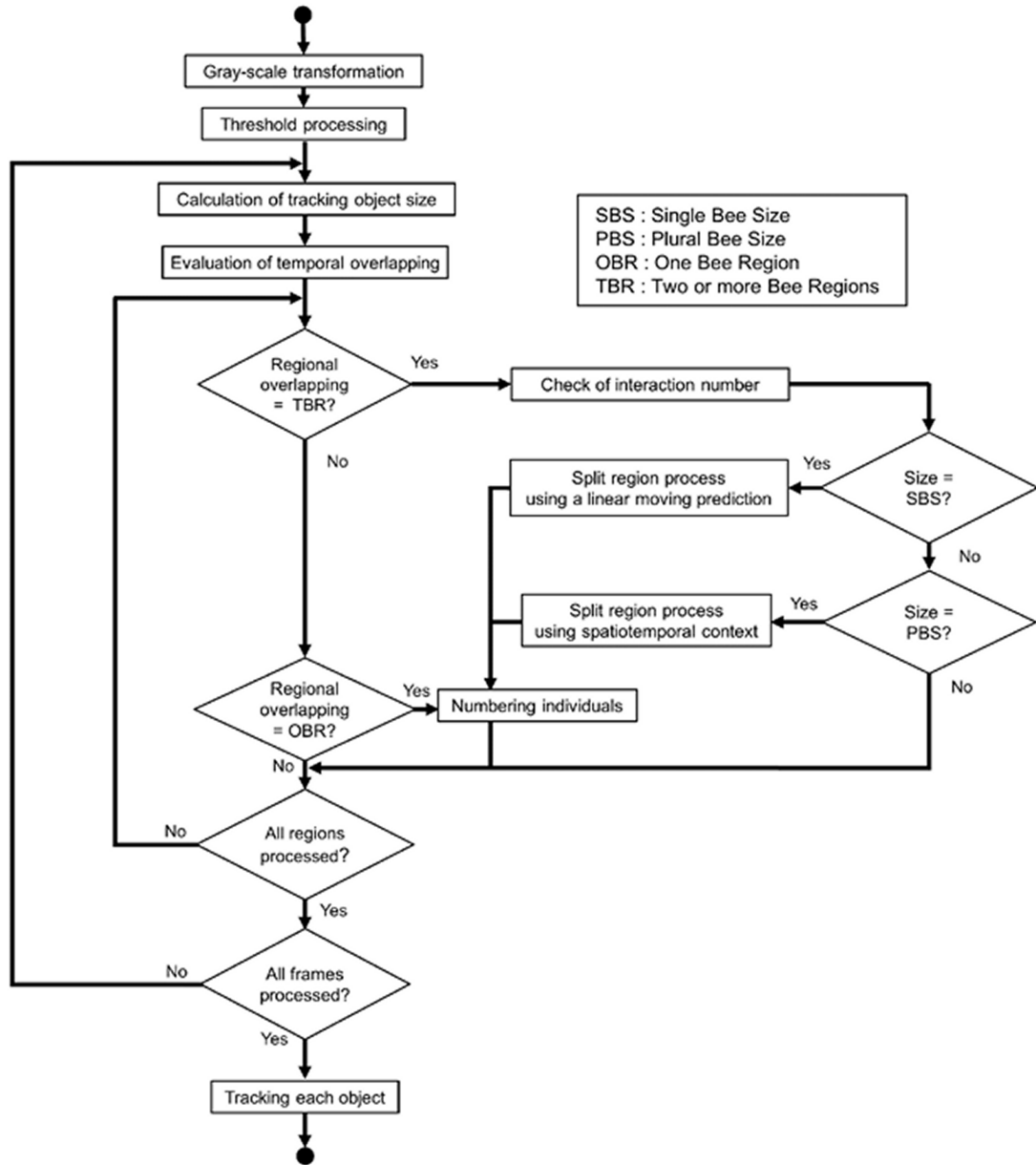


Figure 4.3: Flowcharts of the proposed method, flowchart of “Tracking Process” that gives the detailed processing steps in Figure 4.2.

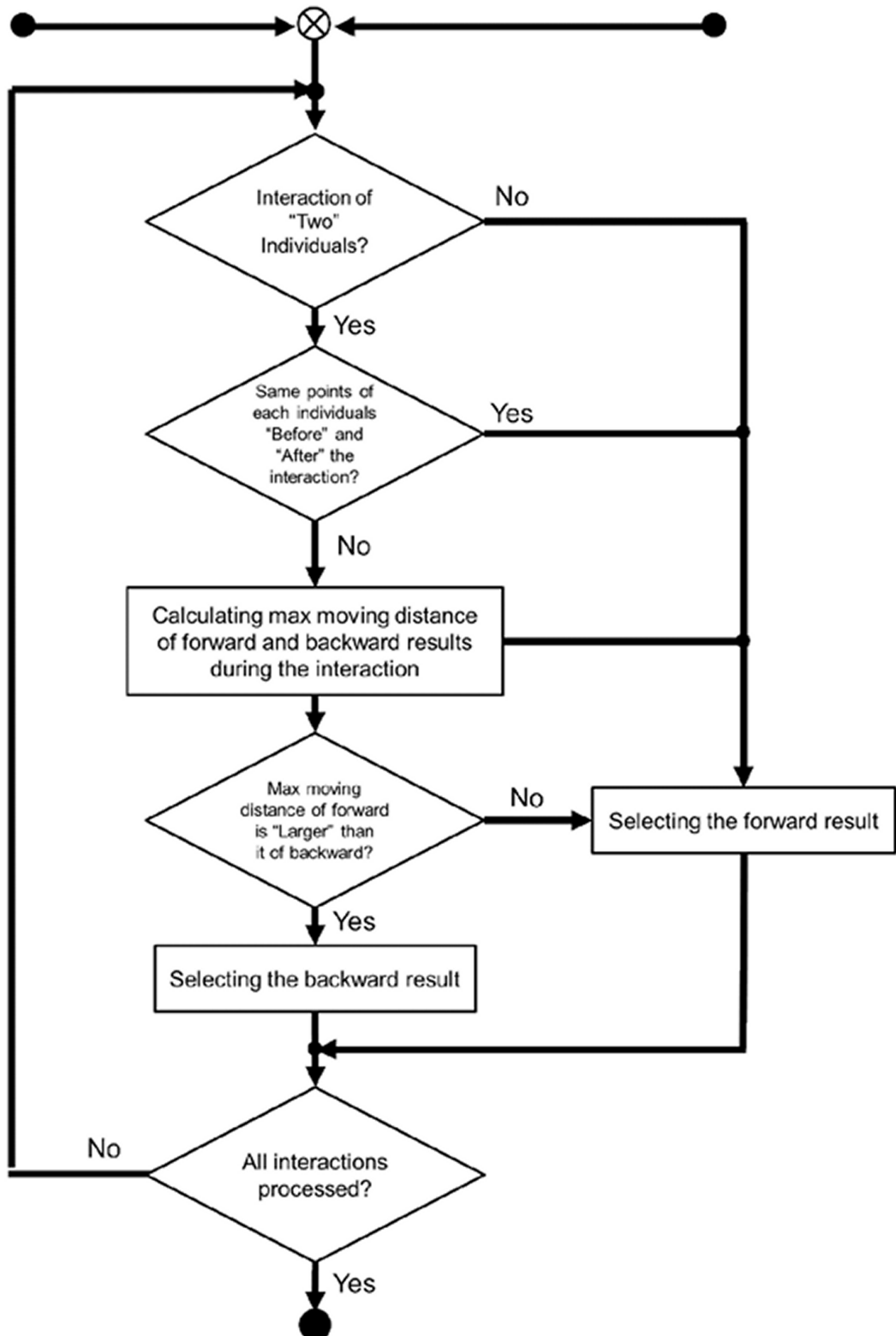


Figure 4.4: Flowcharts of the proposed method, flowchart of “Route Decision Process” that gives the detailed processing steps in Figure 4.2.

quired several new videos for evaluating our new algorithm. In those videos, 6 or 16 juvenile female worker honeybees were entered into the flat circular arena (60 cm diameter) in a darkroom. A temperature gradient from 32°C on the right to 36°C on the left was imposed on the arena floor. The animal behaviors under infrared overhead lighting were recorded by an infrared-sensitive CCTV (Closed-Circuit TeleVision) camera. The videos were recorded in PAL format, generating (720 x 576)-pixel digital image sequences.

Our software was developed and evaluated on a personal computer (Intel Core i7-3970X, 3.50 GHz, 64 GB memory, 1TB SSD). The software was developed on a Windows 8 Enterprise 64-bit operating system, Visual Studio 2013 Ultimate (C++ 2013). We used OpenCV 2.4.10 as the image processing library.

4.3 Results

4.3.1 Tracking of 16 honeybees

Applying the above methods, we evolved our original algorithm K-track into K-Track-kai. To confirm the performance of our improved method, we applied K-Track-kai to three videos (Movie16-1, Movie16-2 and Movie16-3) of 16 moving honeybees. The same videos had been previously applied in the evaluation of K-Track (Table 4.1). By analyzing the backward results, K-Track-kai successfully tracked the honeybees in four out of seven trackings that had failed in K-Track. As shown in Figure 4.5, the honeybees were back-tracked without sudden changes in distance and direction, and the *maxBs* of the backward trajectory was lower than the *maxFs* of the forward trajectory in the successful cases. The total success rate was improved from 91.7% in K-Track to 96.4% in K-Track-kai (Table 4.1). The success rate of ‘waiting’, a situation in which one bee remains still until passed by another bee, increased from 93.3% to 100.0% in ‘Movie16-2’ and from 85.7% to 92.9% in ‘Movie 16-3’. In both videos, K-Track-kai correctly tracked the two interaction events that were erroneously treated by K-Track. The success rate of the ‘passing’ and ‘overlapping’ situations also increased from 94.4% to 100.0% and from 0.0% to 50.0% respectively, because K-Track-kai successfully tracked the interactions that failed in the original K-Track.

However, K-Track-kai failed to track the honeybees in three interaction events (‘overlapping’, ‘waiting’ and ‘multiple’) in ‘Movie16-3’ (Table 4.1). In the ‘overlapping’ event, K-Track-kai incorrectly selected the forward trajectory rather than the backward trajectory (Figure 4.6). In the ‘waiting’ event, both the forward and backward trajectories were incorrect; consequently, K-Track-kai yielded the wrong results irrespective of the selection. In this study, K-Track-kai selected the forward trajectory because the maximum moving distance was identical in the forward and backward videos (Figure 4.7). K-Track-kai did not improve the tracking results of ‘multiple’ events because the decision process operates only when two honeybees interact. Honeybee tracking by the original K-Track failed in one of 9 ‘multiple’ event cases. Because this failure was not corrected by K-Track-kai’s decision process, it reappeared when the same video was analyzed by K-Track-kai.

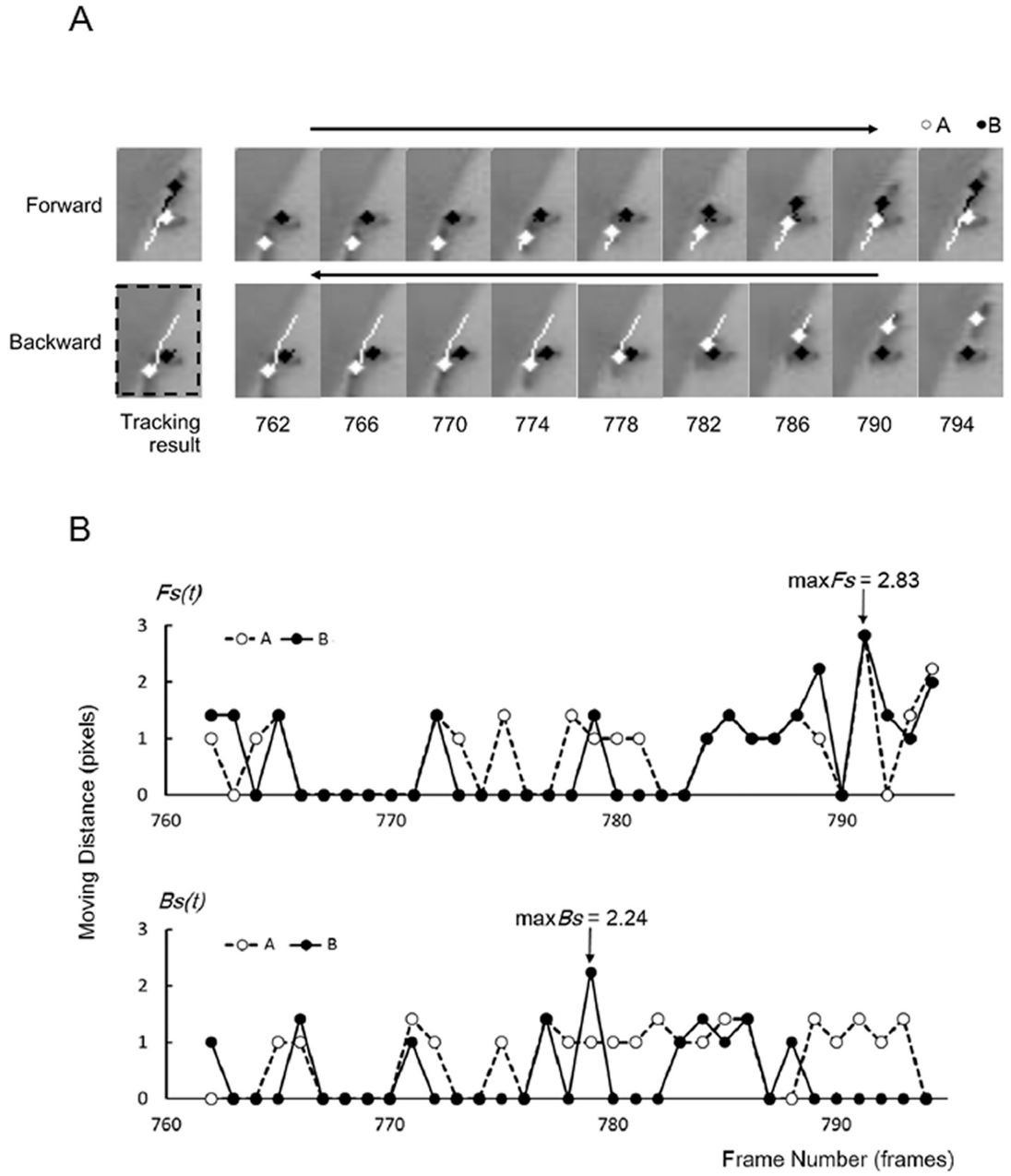


Figure 4.5: Route decision process. (A) Comparison of two tracking results and (B) moving distances of each individual in the forward and backward videos. In the tracking results of the forward and backward sequences, the maximum moving distances are 2.83 and 2.24, respectively. The backward-running sequence, with smaller maximum moving distance than the forward sequence, is selected by K-Track-kai. The square with dashed line indicate the selected trajectory.

Table 4.1: Success rates of tracking 16 honeybees by K-Track and K-Track-kai software. The asterisk (*) marks indicate the improved items.

			K-Track	K-Track-kai	
		Occurrence	Success rate(%)	Success rate(%)	
Movie16-1	Touching	2	100.0	100.0	
	Passing	3	100.0	100.0	
	Waiting	10	100.0	100.0	
Movie16-2	Crossing	1	100.0	100.0	
	Passing	5	100.0	100.0	
	Overlapping	1	100.0	100.0	
	Waiting	15	93.3	*	100.0
	Multiple	1	100.0	100.0	
Movie16-3	Crossing	3	100.0	100.0	
	Passing	18	94.4	*	100.0
	Overlapping	2	0.0	*	50.0
	Waiting	14	85.7	*	92.9
	Multiple	9	88.9	88.9	
Total		84	91.7	*	96.4

4.3.2 Tracking of 6 honeybees

Both variants of our tracking software (K-Track and K-Track-kai) were applied to five videos (Movie6-1, Movie6-2, Movie6-3, Movie6-4 and Movie6-5) recording the motions of six honeybees. In interaction cases, the total success rate of K-Track-kai was 96.7%, versus 94.4% for K-Track. K-Track failed to track two out of eight ‘waiting’ cases in ‘Movie6-2’ and two out of 13 ‘waiting’ cases in ‘Movie6-4’. K-Track-kai provided the correct results in the one ‘waiting’ case that were failed by K-Track in ‘Movie6-2’ and the one in ‘Movie6-4’ (Table 4.2).

4.4 Discussion

K-Track-kai improved the tracking accuracy of our previous tracking software K-Track. In our previous study, K-Track failed a total of seven interaction events. All of those events occurred near the wall of the circular arena in the video tagged ‘Movie 16’ [63]. In contrast, our present K-Track-kai algorithm successfully tracked four out of six cases, and two out of five cases in ‘Movie 6’ that were failed by K-Track. This means that K-Track-kai corrected 40–70 % of the interacting events that were failed by K-Track. The K-Track-kai algorithm compares the two trajectories obtained from the forward- and backward- played image sequences of a video in a window of time around an interaction event, then selects the trajectory with the lower maximum moving distance of the honeybees in each frame. The maximum distance was superior to other parameters (such as the average or sum of the moving distances) in the trajectory-selection process.

The improved tracking accuracy of K-Track-kai over K-Track means that when track-

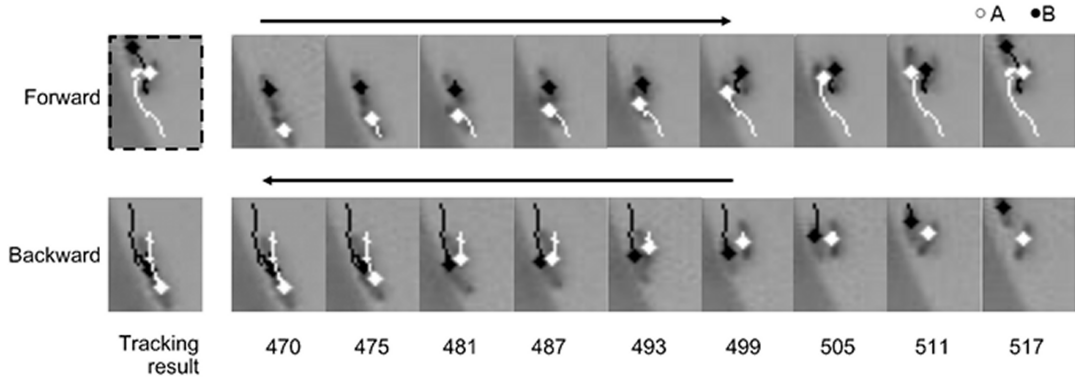


Figure 4.6: Erroneous K-Track-kai results when the trajectories extracted from both forward and backward videos are incorrect. The square with dashed line indicate the selected trajectory.

ing interacting honeybees, a backward-running image sequence sometimes yields a more accurate trajectory than a forward-running sequence. The forward and backward trajectories may differ if the extracted shapes of the honeybees before their interaction differ in the forward and backward sequences. In this study, the shape of an interacting honeybee was estimated from its shape just before the interaction. Thereafter, the estimated shapes were used for estimating the honeybee locations. The accumulated locations produced the whole trajectories of the bees over time. Therefore, if a bee's shape is distorted during the interaction and the altered shape remains in a few successive frames until the undistorted shape is recovered, the extracted shape immediately after interaction will differ from that before the interaction. In this scenario, the extracted shapes of the honeybees (used for estimating the shapes during the interaction) will differ in the forward- and backward-running sequences, generating different trajectories in the image sequences. In some cases, the honeybees' shapes and positions during the interaction are more accurately estimated from the backward videos, which trace the distorted animal images after an interaction.

The failure results of tracking two-bee interactions by K-Track-kai were categorized into two types: (1) incorrect tracking in both forward and backward directions and (2) incorrect selection of the trajectory. The first type of failure occurs when the bee's shape distorts only during the interaction, and recovers after the interaction. In this case, the trajectories from both the forward and backward image sequences can lead to incorrect tracking. In our present study, the second type of failure occurs only in overlapping interactions. If one bee moves over another bee to avoid being pushed aside, no unnatural changes in moving distance will appear. Therefore, the maximum moving distance informs an incorrect trajectory decision.

Our new software K-Track-kai can obtain the movement trajectories of plural animals from video data, enabling new ethological analysis and quantitative evaluation of animal behavior. Such studies will improve our understanding of the complex interaction-based self-regulation of social insect colonies, which have led to significant bio-inspired algorithms and robotic applications in past studies [89] [97] [99] .

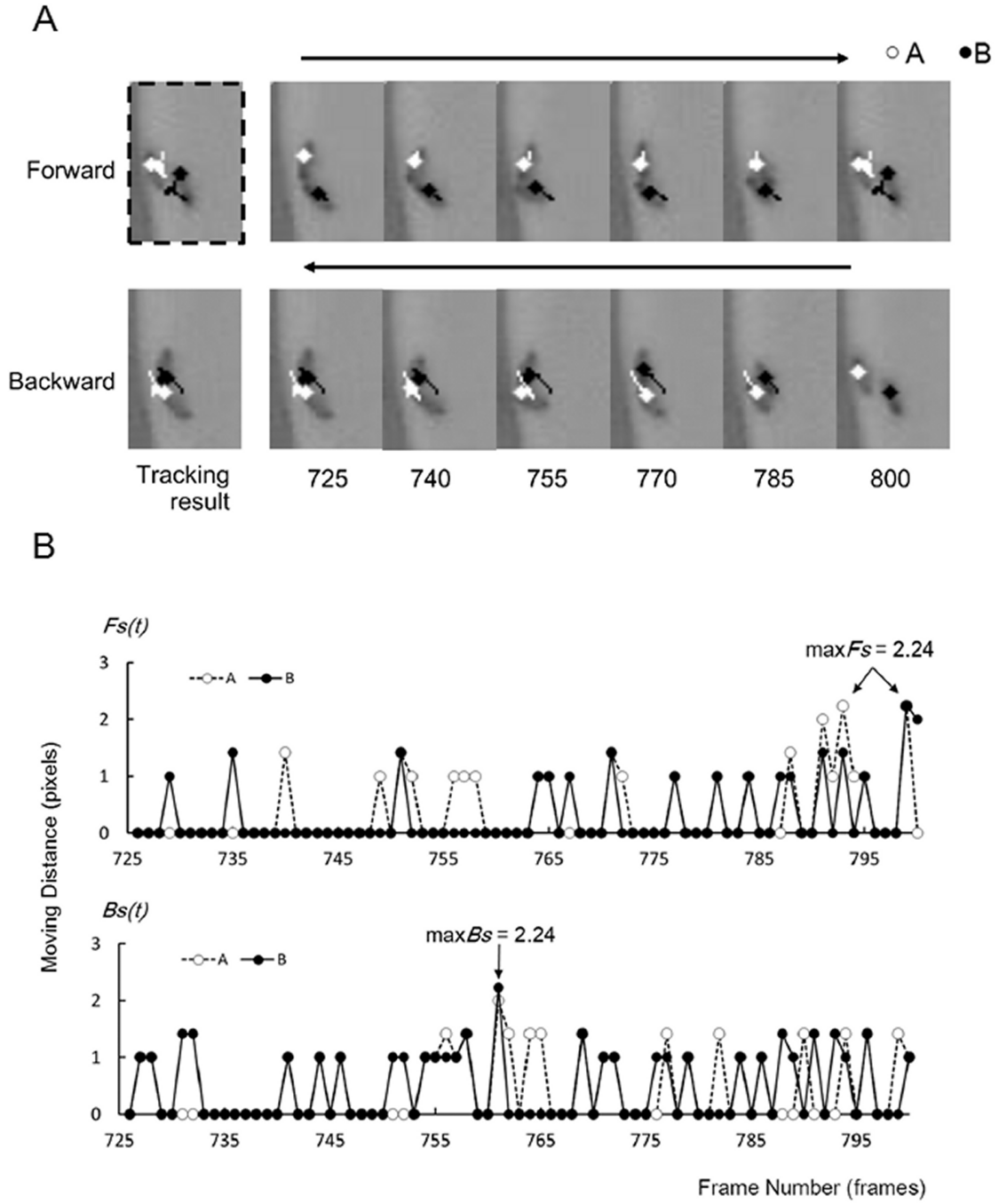


Figure 4.7: Erroneous K-Track-kai results when the wrong trajectory is selected from the trajectories yielded by the backward and forward analyses. The square with dashed line indicate the selected trajectory.

Table 4.2: Success rates of tracking 6 honeybees by K-Track and K-Track-kai software. The asterisk (*) marks indicate improved items.

		K-Track		K-Track-kai	
		Occurrence	Success rate(%)		Success rate(%)
Movie6-1	Touching	4	100.0		100.0
	Waiting	1	100.0		100.0
Movie6-2	Passing	3	100.0		100.0
	Waiting	8	75.0	*	87.5
Movie6-3	Touching	9	100.0		100.0
	Passing	1	100.0		100.0
	Waiting	13	100.0		100.0
	Multiple	2	100.0		100.0
Movie6-4	Touching	8	100.0		100.0
	Passing	3	66.7		66.7
	Waiting	13	84.6	*	92.3
	Multiple	1	100.0		100.0
Movie6-5	Touching	18	100.0		100.0
	Waiting	6	100.0		100.0
Total		90	94.4		96.7

Animal tracking can reveal various behavioral properties, such as aggregation, scattering and avoiding. Such behaviors can be further quantified by considering factors such as the number of animals in an aggregation or the moving directions preferred by animals. Moreover, the energy consumptions of such behaviors could be estimated from the measured moving distance, speed and observed acceleration. Our software was developed for analyzing honeybee videos, but can be adapted to the analysis of various other animals by changing the possible target object shapes.

In K-Track-kai, the target is assumed as a solid object with a linear movement. Our experiments were performed on videos with a standard frame rate, but higher frame rates should improve the tracking accuracy by providing a smaller, more linear movement in each time step. As infrared light is a necessary requisite of biological honeybee experiments, we recorded the bees' motions by an infrared-sensitive camera. However, infrared light degrades the image quality, rendering the tracking effort significantly more difficult. For example, if animals are recordable under normal light conditions, the animals in the images can easily be separated by considering the subtle differences in their color-shades. The resolution of the used infrared camera is not high and the contrast in the produced images is poor. Improving the contrast and resolution of the images would enable more precise segmentation of the target individuals. More advanced image acquisition equipment would yield finer images for extracting precise data on animal behavior. However, many video data have been already acquired under poor conditions. Quantitative measurements from these image data are important for utilizing the existing scientific resources.

Even in our current situation, our tracking software could be improved in several ways. We assumed that individuals are solid objects; in reality, an object’s shape can change during and after collisions. An elastic object model would further improve the estimations of shape and center of gravity of each object [96]. To improve the tracking accuracy, we could consider the irregularity of movement near the edge of a flat arena. However, these improvements would not entirely remove tracking errors in complex interactions and collision cases. Thus, in practical applications of our software, an additional manual tracking function, in which automatic tracking results can be edited and corrected by human operators, could be developed in future. To achieve this functionality, questionable tracking estimates must be automatically identified in the videos, then presented to the human operator. By admitting a human into the loop, K-Track-kai might achieve perfect tracking results (100% tracking accuracy) with minimum involvement of human handwork.

4.5 Conclusions

In this study, we improved our K-Track algorithm by comparing the interaction trajectories obtained from forward and backward playing of video episodes. In the case of tracking results are different between the forward and backward, we chose the trajectory with the smaller maximum moving distance per frame in our new software, K-Track-kai. In the cases of 6 and 16 honeybees, K-Track-kai improved the tracking accuracy from 91.7% to 96.4% and from 94.4% to 96.7%, respectively. An automatic tracking is useful for various behavioral experiments, but the development of the method and technique depending on image quality is not easy. So, it is important that we use and improve software in the experiments.

Chapter 5

Tracking method of single individual, an earthworm, in a laboratory experiment

Abstract

Earthworms are important soil macrofauna inhabiting almost all ecosystems. Their biomass is large and their burrowing and ingestion of soils alters soil physicochemical properties. Because of their large biomass, earthworms are regarded as an indicator of “soil health”. However, primarily because the difficulties in quantifying their behavior, the extent of their impact on soil material flow dynamics and soil health is poorly understood. Image data, with the aid of image processing tools, are a powerful tool in quantifying the movements of objects. Image data sets are often very large and time-consuming to analyze, especially when continuously recorded and manually processed. We aimed to develop a system to quantify earthworm movement from video recordings. Our newly developed program successfully tracked the two-dimensional positions of three separate parts of the earthworm and simultaneously output the change in its body length. From the output data, we calculated the velocity of the earthworm’s movement. Our program processed the image data three times faster than the manual tracking system. To date, there are no existing systems to quantify earthworm activity from continuously recorded image data. The system developed in this study will reduce input time by a factor of three compared with manual data entry and will reduce errors involved in quantifying large data sets. Furthermore, it will provide more reliable measured values, although the program is still a prototype that needs further testing and improvement. Combined with other techniques, such as measuring metabolic gas emissions from earthworm bodies, this program could provide continuous observations of earthworm behavior in response to environmental variables under laboratory conditions. In the future, this standardized method will be applied to other animals, and the quantified earthworm movement will be incorporated into models of soil material flow dynamics or behavior in response to chemical substances present in the soil.

5.1 Introduction

Earthworms, the so-called ecosystem engineers, are important soil macro fauna belonging to the subclass Oligochaeta. They inhabit various types of soil ranging from 10 to 2000 individuals per meter squared, resulting in a large biomass of between 0.5 to 305 g dry mass m^{-2} [34]. They significantly change soil properties and fertility around their huge burrowing areas [66]. Their important role in soil formation has been already emphasized by Darwin [28]. Earthworms are one of the main components of the drilosphere, one of the biological systems regulating mineralization of soil organic matter, where they activate microbes by mixing the litter and soil, breaking the physical protection of soil organic matter and developing soil aggregates during gut transit [70]. The changes in soil pore structures and the extent of aggregations also alter soil gas diffusion coefficient physically, which could increase global warming gas emission, such as CO_2 , CH_4 or N_2O . Because of the behaviors and features, earthworms are also regarded as important biological indicators of chemical toxicity in soil ecosystem and many studies have examined the effects on population dynamics of earthworms such as fecundity, reproductive activity and mortality [6]. Their behaviors, thus, affect not only below-ground material flow dynamics as well as indicate “soil health” conditions.

Evaluation of the functional role of earthworms on soil ecosystems has been, however, insufficient because of the methodological difficulties [25]. Traditional research methods including descriptive observation of their behavior, gut content analysis, choice tests, and litter bags have increased our understanding of their ecological importance. Recent advances of molecular tools provide information on enzyme activities of microorganisms which interact with earthworms. Isotope labeling technique is also useful to quantify how much materials are transferred between earthworms and soils or atmosphere, or earthworms and microorganisms. These new techniques are powerful but still limit our knowledge on the soil biogeochemical processes regulated by earthworms because they are indirect and static methods. In combination with existing methods, additional development of the tools helping quantitative and mechanistic evaluation of their movements and exploring factors controlling their feeding behavior would be helpful to further understand their ecological roles. Such information will also enable us to establish more realistic model of soil ecosystems because many of current models do not take the impact of soil animals into account [6]. Thus, observing earthworm feeding behavior such as in response to some stimuli (i.e. light, temperature, and vibration and odor cues of food) in vitro without soil will be of importance albeit under very simplified artificial condition. The understanding of their physical activities resulting from their feeding behavior will advance quantifying earthworm’s contribution to whole below-ground biogeochemical processes. To determine earthworm activity, it is necessary to quantify the extent to which they move and stretch their bodies and relate this to gas emissions from the soil. To confirm the activities, therefore, direct observation is a powerful tool and the combinations of conventional methods with direct observation such as using web camera or scanner will be of great merit in this field.

In addition to the importance of earthworms on ecosystem functions, they are also regarded as an important biological indicator of chemical toxicity in the soil. Ecotoxicology has traditionally used biological indicators to quantify or predict the changes in soil

“health” or “quality” [6]. To date many studies have found the serious effects of chemicals and heavy metals on reproductivity, growth rate and mortality of earthworms (i.e. [2] [48] [83]), and experimental protocol and earthworm culture techniques in vitro have been developed [121]. However, observation of earthworms in response to such chemical pollutant has been conducted in relatively long term span with lower resolution time (i.e. on a minute scale), and less attention has been paid to the signs in early stage, such as their irregular behavior or morphological alterations by gaining the pollutions. However, since the sudden changes of their activity and feature might be also tightly connect to the damages and types of the pollutant, observations at high time resolution, i.e. on a second time scale, have to be also conducted.

To date, automated systems have been used to track movement with high time resolution continuously using a web camera for nematodes, ants, honeybees, and drosophila, and to subsequently analyze the continuous image data [23] [38] [61] [63] [76] [95] [107]. Image analysis is an efficient method for quantifying the tracking data. Previous studies have attempted to track earthworms directly in the soil by means of X-ray or minirhizotron [54] [67]; the time resolution, however, was low to track their dynamic changes over a long time period on a time scale of seconds. Revealing their habits without soil but with a high time resolution would be a first step in investigating their behavior. However, those tools cannot be applied directly to earthworms as some of their traits are different from other animals in terms of the extent to which they can stretch their bodies (their body stretch can reach 1.5 times that of their default body length) [73].

A number of automated tracking systems for a model worm, *Caenorhabditis elegans*, have not considered changes in the extent of body stretch [73]. The WormScan is indeed a useful tool to observe population dynamics such as fecundity, reproductive activity, or mortality along with the growth rate that occur over a long time span. However, we still lack a program that can track dynamic change in their activities to observe a fast response to chemical pollutants, environmental changes or toxic substances. WormScan’s default settings are for the nematode as a target animal. The features of nematodes, such as the movement and body size, vary widely from those of earthworms, therefore it requires intensive modification of the parameters of the program to apply it to earthworms. In addition, a standardized tracking system is required so that reliable comparisons can be made between species or phenotypes when tracking is done by different users. Standardized measurements will reduce misestimation and will output more reliable quantification.

Although existing methods such as WormScan [73] are candidates that may have been applicable in this study, the current WormScan program, which was originally developed for tracking nematodes, was not capable of following the changes in morphology of earthworms because earthworms move more quickly and over a wide area compared with nematodes. WormScan has been developed for observing population dynamics such as reproductive activity, fecundity or mortality which occur over a longer term span (several days to weeks), and the behavior is also relatively static. WormScan, therefore does not suit observations at a continuous and high time resolution, which captures dynamic and rapid movements. This is a remarkable property of earthworms and is an important factor in quantifying their physical activity. We believe that WormScan can track some objects of varying sizes, however we found that earthworms’ movements are too fast

and dynamic, and WormScan could not cope with this. The observation of dynamic changes would help to understand the ecological background of earthworm behaviors; chemical assays in vitro without soils could also be conducted. The existing application ImageJ (Fiji) can provide a plug-in to manually track the two-dimensional positions and lengths of objects. Even when using this application, analyzing large data sets is labor intensive; therefore, a more reliable and automated quantification method is required for continuous image data.

In the present study, we developed a high-throughput program that tracks two dimensional coordinates of multiple points and body lengths of objects in continuously acquired image data from earthworms that achieved to process approximately 1 frame per second. Following user initialization, the program tracks the head, central position and tail of a single earthworm. This program provides a means to track animals that change their body lengths over time both accurately and efficiently. The output was subsequently used to calculate the velocity of those points. Finally, in the future, the quantified behavior could be incorporated into soil material flow dynamic models or applied to measure their reactions to chemical inputs in the soil.

5.2 Materials and Methods

5.2.1 Study animals

Two different species of earthworm were used in this study; *Eisenia japonica* [73] with a body size ranging from 4-17cm [77], and *Metaphire hilgendorfi* [73] with a body size ranging from 9-30 cm [120]. The lifespan of both is usually half a year from spring to fall. Both species are common in Japanese mountains and fields, and both genera have a wide global distribution [52]. *M. hilgendorfi* is epigeic because it primarily inhabits the litter layer and *E. japonica* is polyhumic-endogeic because it mainly lives in the topsoil layer and has more inorganic content in the gut [10].

5.2.2 Continuous recording of earthworm movement in glass containers (image capture)

Adult earthworms were captured from a field at the National Institute for Agro-Environmental Sciences, Tsukuba, Japan on the 28th October, 2012. Earthworms were kept in a plastic box (inner diameter 15 cm, height 6 cm) with approximately 100 g of alluvial soil at 60 % of its water holding capacity until the experiment. The weights of *E. japonica* and *M. hilgendorfi* used in the experiment were 1.45 and 4.75 g, respectively. *E. japonica* and *M. hilgendorfi* were transferred from the plastic box to glass containers for network-camera recording inside an incubator, where the mean ambient temperature was set at 15°C and the ambient air humidity was saturated at the given temperature (Fig. 1). A fiber scope light was used to maintain constant light conditions (MHAB-150W, Moritex, Tokyo) at approximately 500 lux outside of the container and app. 250 lux inside the container, this was measured with a radiometer (T-10A, Konica Minolta Optics, Inc., Tokyo, Japan). The glass containers were covered with 1-mm mesh plastic cloths and fixed with double-sided sellotape to prevent the earthworms from escaping during the experimental period. A small fan (OD2510, Orion Fans, Dallas, USA) was placed on the

top of the glass container to ventilate the air inside and wet tissue was put in to keep the humidity constant during the measurement period. A network camera (VB-M40, Canon Inc., Tokyo, Japan) was fixed to the bottom of the glass container approximately with 60cm distance between the camera and the glass container (Figure 5.1). The network camera was fixed in parallel to the glass container firmly while capturing the image. The network camera uses a lens with 40mm in diameter that can cover 60.4° from the horizontal angle. The captured image data was 640 x 480 in size. To obtain the coordinates of the glass container, marks were fixed at three positions on the edge of the containers with red tape. The round glass containers were 85 mm inside diameter and 2.5 mm in thickness. The image data were recorded as a movie using a network camera continuously recording every second and saved as JPEG files. One pixel was equivalent to 0.2 mm in the captured image data.

To estimate local image distortions, checkerboard (8 rows and columns) was located for image capturing system on the same position as the glass container. The length of the diagonal lines were measured using a vernier caliper with a 0.1 mm precision and calculated the errors. The maximum error in mm was 0.2 mm that resulted the precision less than 0.1%.

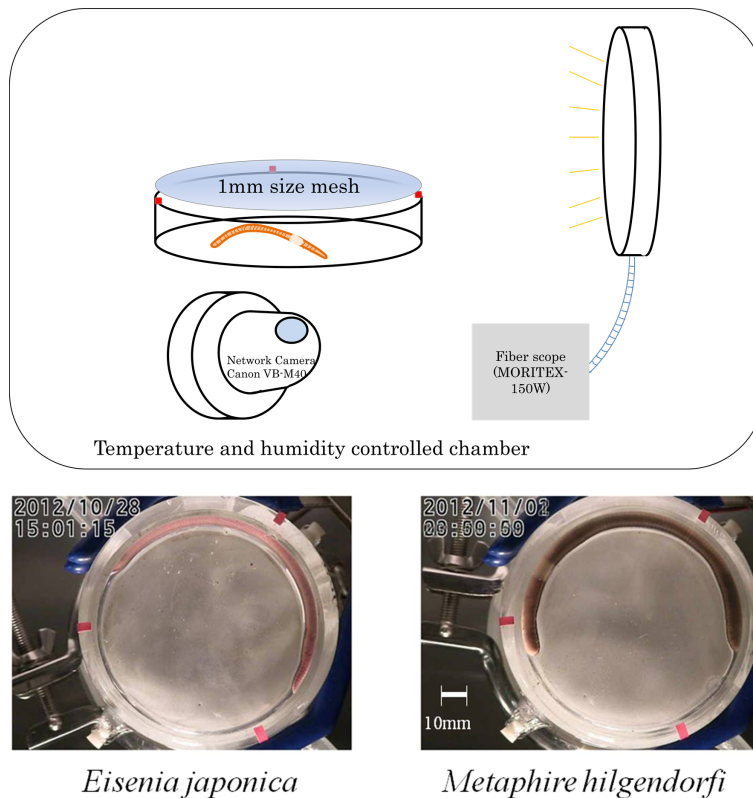


Figure 5.1: Webcam set up and study animals. The upper diagram shows the system for webcam recording in a controlled chamber. The earthworms used in this study are shown in the bottom panel. The diameter of the glass petri dish was 85mm (inside diameter) and the height of the petri dish was 20mm.

5.2.3 Image analysis

Two methods are implemented: a manual tracking method and a new automated tracker. Both of which are explained in more detail below. ; The first method is manual tracking which relies on the Fiji implementation of ImageJ with an additional plugin, Manual Tracking. Currently the manual tracking is a standard method to track animal behavior using image data. Secondary, we then used a new method which is a newly developed program (MimizuTrack) that is comparable to Manual Tracking in Fiji.

5.2.4 Manual tracking

Using the Manual Tracking plug-in on the Fiji implementation of ImageJ, head and tail of the body were tracked separately. The static image data (JPEG: Joint Photographic Experts Group) captured at 1 second intervals were converted to movie data, using a function in Fiji to convert from JPEG into AVI(Audio Video Interleave) formatted data. Each coordinate per one second was tracked to obtain the velocity of the movement. We then handled the output data as ground truth for further evaluation since the data were input by expert users. The output data were used for evaluation of the program described in the next section. Our program was benchmarked against user input only. This was because no automated tracking system currently exists that is directly comparable to our tracker in this specific setting.

5.2.5 Automatic tracking - Initialization Process

The image data obtained were first processed to separate the objects, i.e. earthworms, from the background. The steps are shown in Figure 5.3. In the original image, contrast between the earthworms and the background was highest in the red channel so only this component was used for the process. Earthworms were assigned a value of 255 and the non-earthworm areas were eliminated by applying a value of 0. The procedural details are as followings and following procedures are made manually as an initial step to proceeds the image data by using a program (image shown in Figure 5.2); Since we have already developed several programs to track moving objects over time such as honeybees or plant roots in the previous studies [61] [113] whose source code is original not derived from elsewhere, we have, therefore, developed a new program (MimizuTrack) for earthworms, ‘Mimizu’ in Japanese, based on the previous program. The steps are shown in Figure 5.3 and detailed below. The detailed initialization process are described as follows:

1. The outline of the inside of the glass container was determined using a tool for drawing around the shape and cutting the outside of the image.
2. A color threshold was determined to highlight the color of the earthworm body and split the color channels into three colors (green, blue, and red). The red image was then used for further analysis. Earthworms were assigned a value of 255 and the non-earthworm areas were eliminated by applying a value of 0.
3. A threshold to extract the earthworm body was determined and a binary image with white for the object and black for the background was generated.

4. The noise was eliminated by removing small fragments from the background.

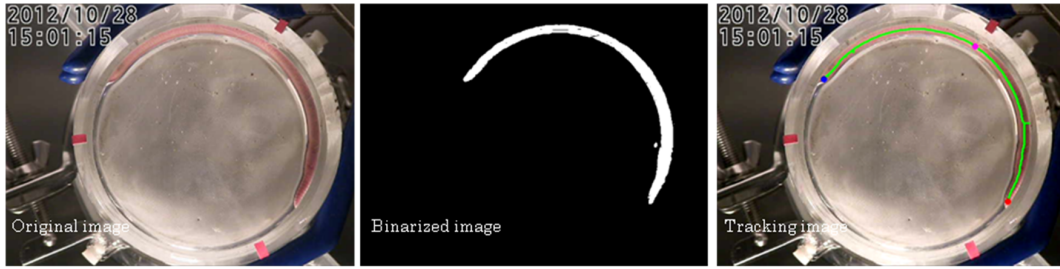


Figure 5.2: Earthworm body thinning procedure and determination of the head, tail, and central points. The captured image data were used for the latter.

5.2.6 Automatic tracking - Continuous Tracking

The rest of the image processing was carried out by our newly developed program. The procedures are shown in Figure 5.2. We developed a prototype program using Microsoft Visual Studio 2010 (Visual C++ 2010) and OpenCV 2.31 on a computer with an Intel Core i5 2.50 GHz (CPU), 16 GB (Memory), 640 GB (HDD) and running Microsoft Windows 7 Professional 64-bit (OS). Our developed software is executed as a Win64 console application on Windows.

The tracking method is as follows;

- Thinning of earthworm body areas

To extract feature points, such as the head, tail, and center points, the program was tasked with identifying the end-points (tails) of the line segment corresponding to the worm body. That is as thin as possible whilst remaining fully connected and centered. Using binary images of the earthworm body, the program was able to get three feature points on each frame. First, the Hilditch algorithm is used to reduce the pixel region representing the earthworm to its skeletal form [62]. The end-points of the center line are recognized as candidates of earthworm feature points.

- Extract the head, tail, and center point of individuals (feature extraction)

The next step was to determine the head, tail, and center point. The program extracted the feature points of the earthworm body by first identifying the head and tail using the different features between the two points (i.e. tail points always move slower than the other points), then the center point was determined. To initialize the program, the tail point was determined manually. By doing this, the program automatically calculated four-neighbor distances of the center line from the tail point and extracted the point at the furthest distance. The program defined this as the head point of the earthworm. Then, the program recognized the

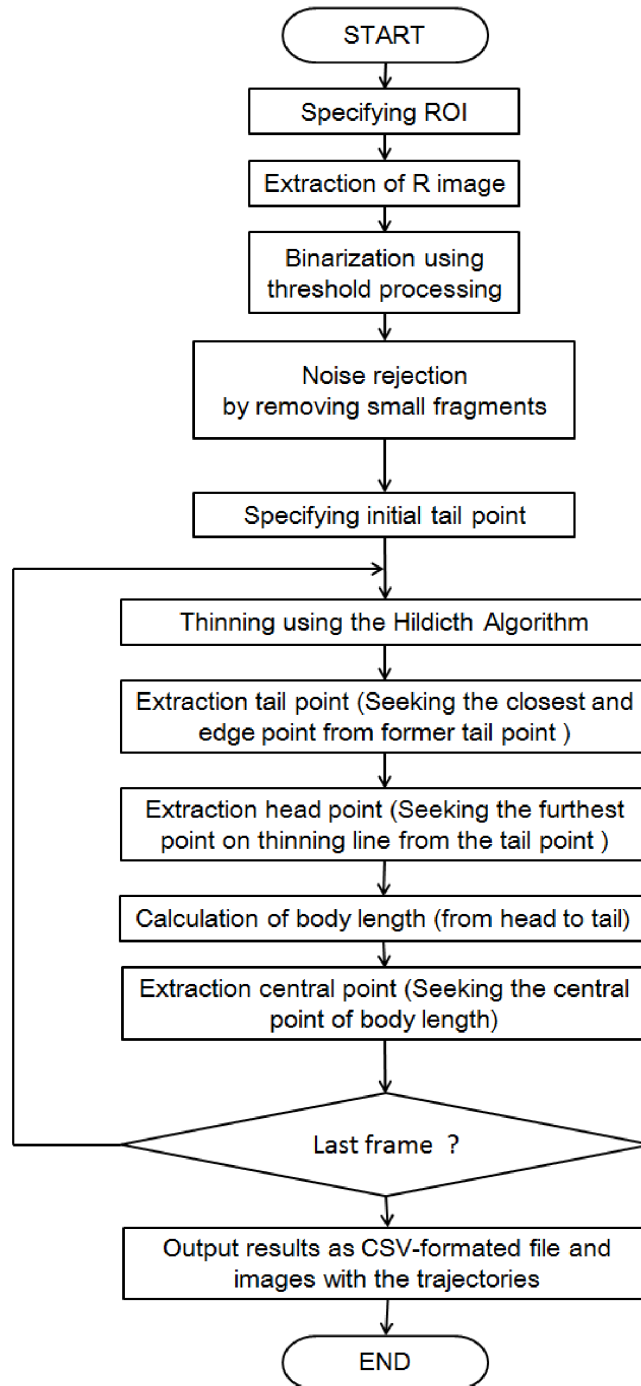


Figure 5.3: Work flow of the proposed method in the program.

half-way point between the head and tail as the central point of the body. Using this process, the program could obtain the initial feature points of the earthworm (i.e. head, tail, and center points). From the second frame, the program calculated new feature points using the previous ones. First, the program extracted new tail points based on the fact that the tail points of the earthworm move slower than the other points. Using this feature, the program recognized the edge points as new tail points with the shortest distance from the previous points. Both head and center points were obtained by the same procedure, which were extracted from the initial head and center points. These processes were carried out on all of the images. The program outputs positional data in each frame to a CSV(Comma-Separated Values) formatted file, allowing trajectories over time to be visualized (Figures 5.4 and 5.6)

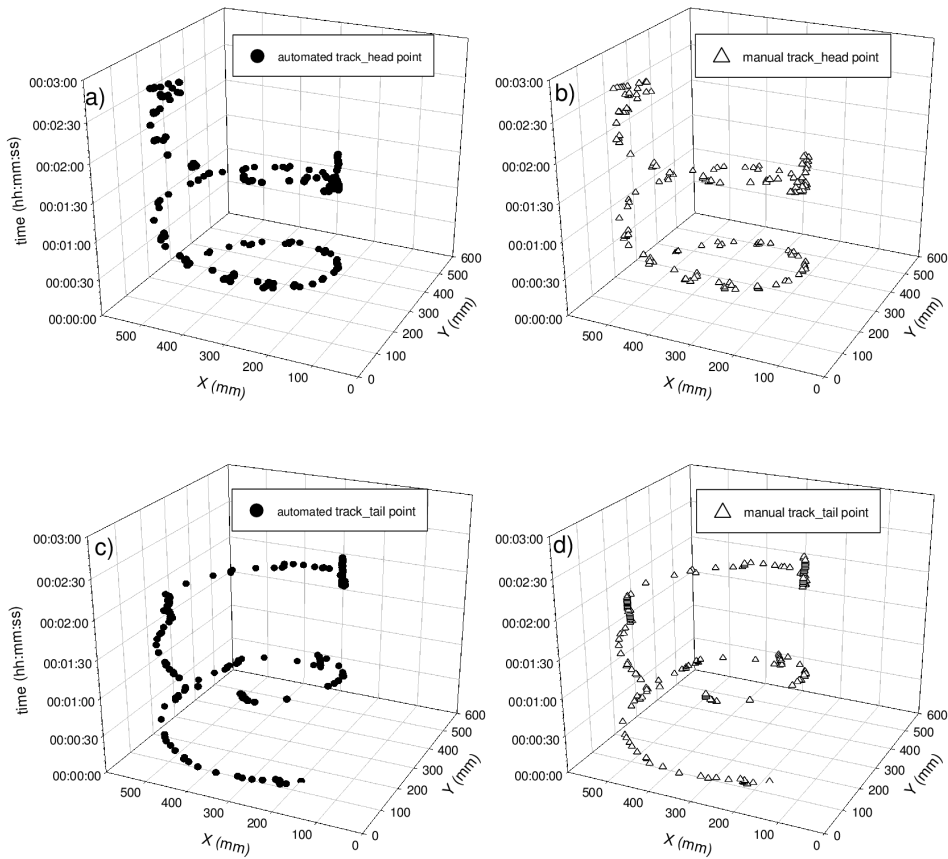


Figure 5.4: The automatically and manually estimated two dimensional coordinates of *Eisenia japonica* over time. Each panel shows the head point in the upper panel and the tail point in the bottom panel.

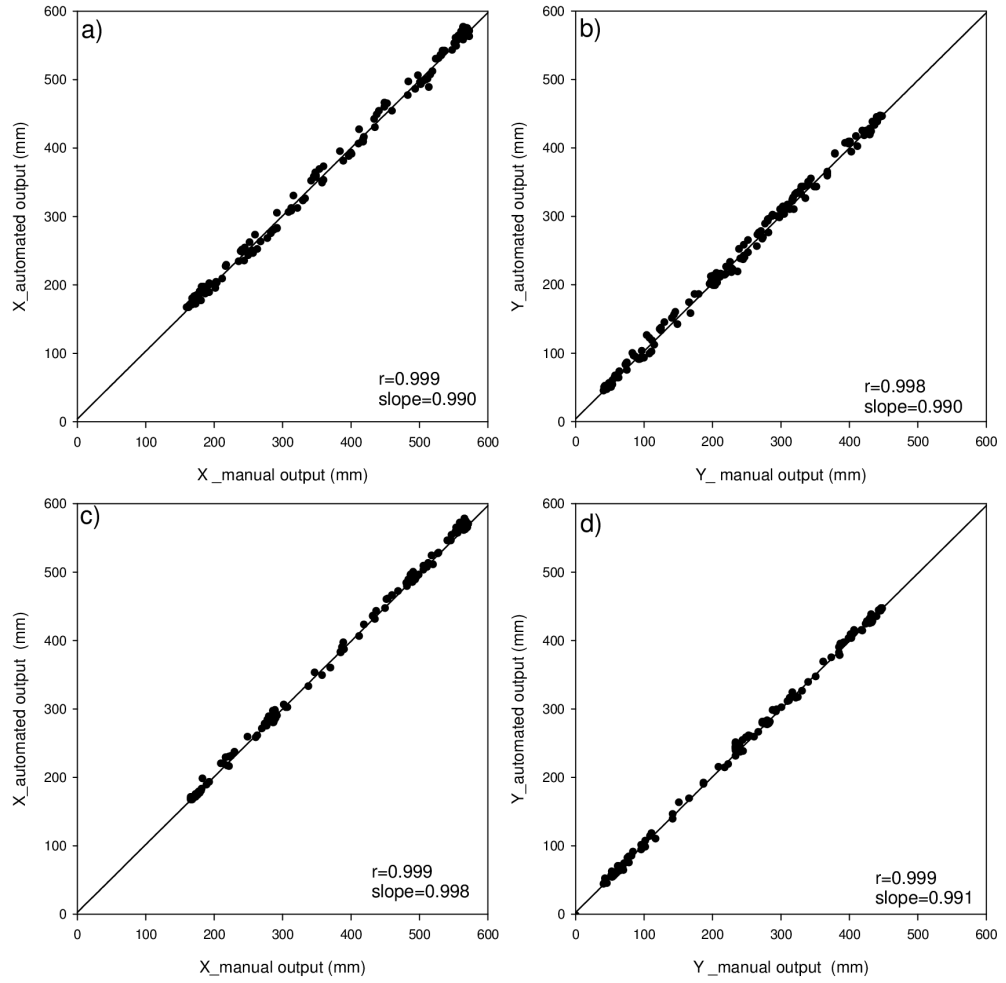


Figure 5.5: Correlations between the automatically and manually estimated two dimensional coordinates of *Eisenia japonica*. Each panel shows the head point in the upper panel and the tail point in the bottom panel.

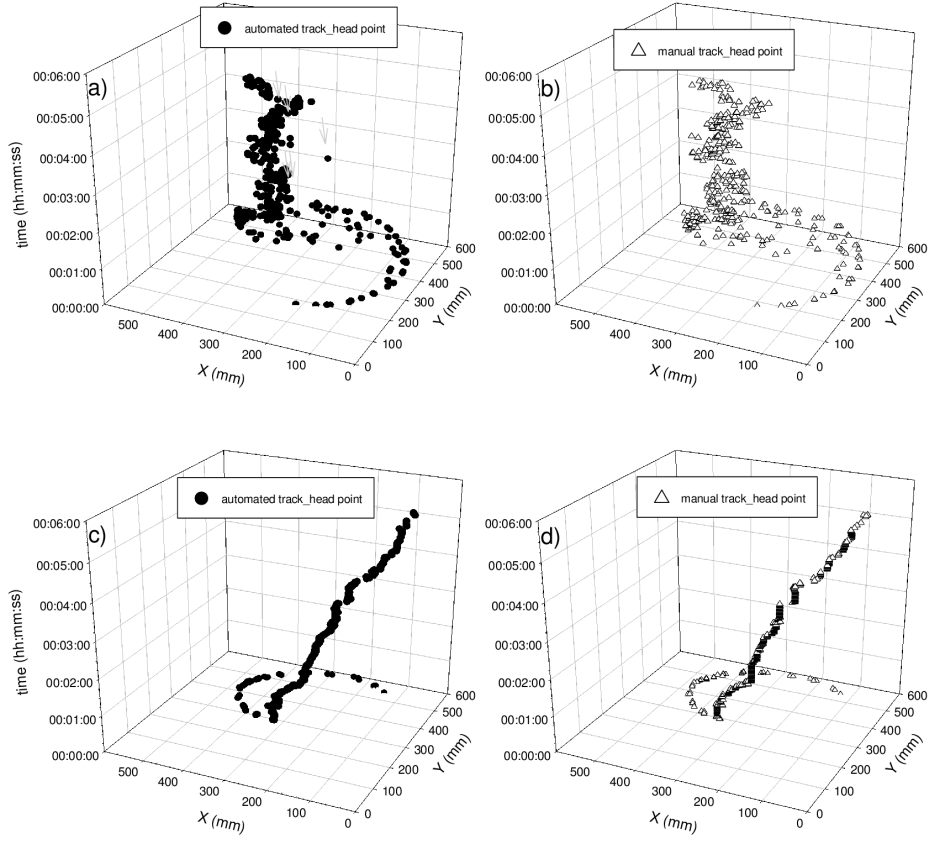


Figure 5.6: The automatically and manually estimated two dimensional coordinates of *Metaphire hilgendorfi* over time. Each panel shows the head point in the upper panel and the tail point in the bottom panel.

5.2.7 Statistical Analysis

All statistical analyses were performed using SPSS 10.05 (SPSS Inc., Chicago, IL, USA). Relationships between two variables (program output and manual output) were assessed using standard bivariate correlation procedures. The correlation coefficient was shown in the text below and figures.

5.3 Results

5.3.1 Evaluations of automatic output using the manually estimated values.

- Tracking and extraction of each point

First, we were successful in tracking the three target points, namely the head, center, and tail of the body on a second time scale continuously followed by body length estimations using the newly developed program. We used a part of obtained data for the evaluation of the program which shows typical movement of 2 species of earthworms each 150sec. and 300sec for *E. japonica* and *M. hilgendorfi* respectively (Figures 5.5 – 5.11). The tracking of the head and tail with the automatic and manual programs over time are shown in Figures 5.4 and 5.6 respectively. All of the regression lines between the automatically and manually estimated coordinates were close to 1 (> 0.98), especially in the case of *E. japonica* (Figure 5.5), in contrast, some outliers were observed in *M. hilgendorfi* as indicated by the arrows in Figure 5.7. There were no significantly different estimations observed in *E. japonica* between different body parts; however, significant differences were observed between the different body parts of *M. hilgendorfi*. In general, the errors in estimation of the head point ($r=0.981$, $r=0.984$, X and Y coordinates, respectively) were larger than those for the tail point ($r=0.996$, $r=0.996$, X and Y coordinates, respectively). One reason for this was that the head moved more frequently while the tail stayed in the same position. The program predicts the following steps and determines the feature points, the program, therefore, may have failed to track the head point in this case. In the case of *M. hilgendorfi*, when they looped, coiled, or crossed their bodies, the program faced difficulties in recognizing the edges, and thus failed to detect the head positions.

- Calculations of velocity of each point and body length.

Using their coordinates, the velocity of each point was estimated. The output data obtained with the automated program were further confirmed by comparing it with the manually acquired data (Figure 5.9 and 5.10). The accuracy of each output was satisfactory for the application to quantify earthworm activity over time. Some outliers in head velocity in *M. hilgendorfi* were due to the misestimation of head points by the automated program, in which case the head was attached to a part of the body. Otherwise, the automated program satisfactorily detected the peaks in terms of timing and absolute values. As shown in Table 5.1, the mean velocities of the head and tail of *E. japonica* estimated by the automated program were 4.75 ± 4.36 and 3.44 ± 4.38 mm sec⁻¹, respectively, whilst those from the manually

estimated values were 4.81 ± 4.29 and 3.56 ± 4.44 mm sec⁻¹, respectively. The mean velocities of the head and tail of *M. hilgendorfi* estimated by the automated program were 4.38 ± 4.13 and 1.03 ± 1.92 mm sec⁻¹, respectively, whilst those from the manually estimated values were 5.09 ± 4.43 and 0.92 ± 2.21 mm sec⁻¹, respectively. The differences between the automated and manually estimated values were 0.07 ± 0.92 and 0.11 ± 0.88 mm sec⁻¹ for the head and tail of *E. japonica*, respectively and 0.71 ± 2.49 and 0.11 ± 0.80 mm sec⁻¹ for those of *M. hilgendorfi*, respectively (Table 5.2). The difference was largest in the head of *M. hilgendorfi* but the others were quite well estimated with the differences being less than 0.2 mm sec⁻¹. The tail points tended to move slower than the head points according to the estimated velocities, and the head point of *M. hilgendorfi* moved faster than that of *E. japonica* by 1.65 ± 7.07 mm sec⁻¹. The body lengths were estimated from the binary data, which were converted into a line (shown in Table 5.3).

The mean lengths of *E. japonica* and *M. hilgendorfi* were 167.02 ± 13.62 and 199.28 ± 10.05 mm, respectively, while the maximum and minimum lengths of *E. japonica* were 190.73 and 131.44mm, respectively, and those of *M. hilgendorfi* were 220.38 and 145.33 mm, respectively. Therefore, the stretch from minimum to maximum was 59.19 and 75.05 mm for *E. japonica* and *M. hilgendorfi*, respectively. The extent of the stretchiness were in agreement with previously reported values [34], which indicated that the body could reach 1.5 times longer than their default length. Moreover, our program also generated the change in length over time per sec, so that we could observe the frequency of the stretchiness for further applications (Figure 5.11).

Table 5.1: Mean, maximum, and minimum values of automatically and manually estimated velocities of each point (head and tail) of two earthworm species (*E. japonica* and *M. hilgendorfi*). The errors indicate standard deviations over time.

Species	velocity	mean (mm sec ⁻¹)	max. (mm sec ⁻¹)	min. (mm sec ⁻¹)
<i>E. japonica</i>				
program	head	4.75 ± 4.36	21.37	0.00
	tail	3.44 ± 4.38	23.09	0.00
manual	head	4.81 ± 4.29	21.45	0.21
	tail	3.55 ± 4.44	24.69	0.00
<i>M. hilgendorfi</i>				
program	head	4.38 ± 4.13	29.71	0.21
	tail	1.03 ± 1.92	13.9	0.00
manual	head	5.09 ± 4.43	28.29	0.00
	tail	0.92 ± 2.21	14.58	0.00

Table 5.2: Mean maximum values of differences between automatically and manually estimated velocities of each point (head and tail) of two earthworm species (*E. japonica* and *M. hilgendorfi*). The errors indicate standard deviations over time.

Species	mean (mm sec ⁻¹)
<i>E. japonica</i>	
head	0.07 ± 0.92
tail	0.11 ± 0.88
<i>M. hilgendorfi</i>	
head	0.71 ± 2.49
tail	0.11 ± 0.80

Table 5.3: Mean, maximum, and minimum values of body length of two earthworm species (*E. japonica* and *M. hilgendorfi*). The errors indicate standard deviations over time.

body length	mean (mm)	max. (mm)	min. (mm)
<i>E. japonica</i>	167.02 ± 13.62	190.73	131.44
<i>M. hilgendorfi</i>	199.28 ± 10.05	220.38	145.33

5.4 Discussions

To date there was no existing method to process and extract such high time resolution image data (on a second time scale) capturing 3 feature points along with tracking elastic object such as earthworms which moves quickly and widely. A number of automated systems have been developed to track insects/ movements, our earthworm tracking system might be similar to that of *Caenorhabditis elegans* (one of the model nematodes) [54] [81]. Although the existing methods such as WormScan [54] can be a candidate that could have been applicable in this study, the current program of WormScan, which were originally developed for tracking of nematodes, was not capable of following the changes in morphology of earthworms because the earthworms possibly move more quickly and widely compared to nematodes. WormScan has been developed for observing population dynamics such as reproductive activity, fecundity or mortality which occur in a longer term span, several days to weeks, and the behavior is also relatively static. WormScan, therefore does not suit to an observation at continuous high time resolution, which captures dynamic and rapid movements. This is a remarkable property of earthworms and is an important factor in quantifying their physical activity. Our newly developed high-throughput analysis program for high-time resolution image data of cyclic earthworm behavior successfully differentiated the features of 2 earthworm species. We believe that WormScan could track some object of varying sizes, however we found that the earthworm’s movements are too fast and dynamic, where WormScan could not cope with. Our program has achieved to deal with second time scale tracking albeit still problem

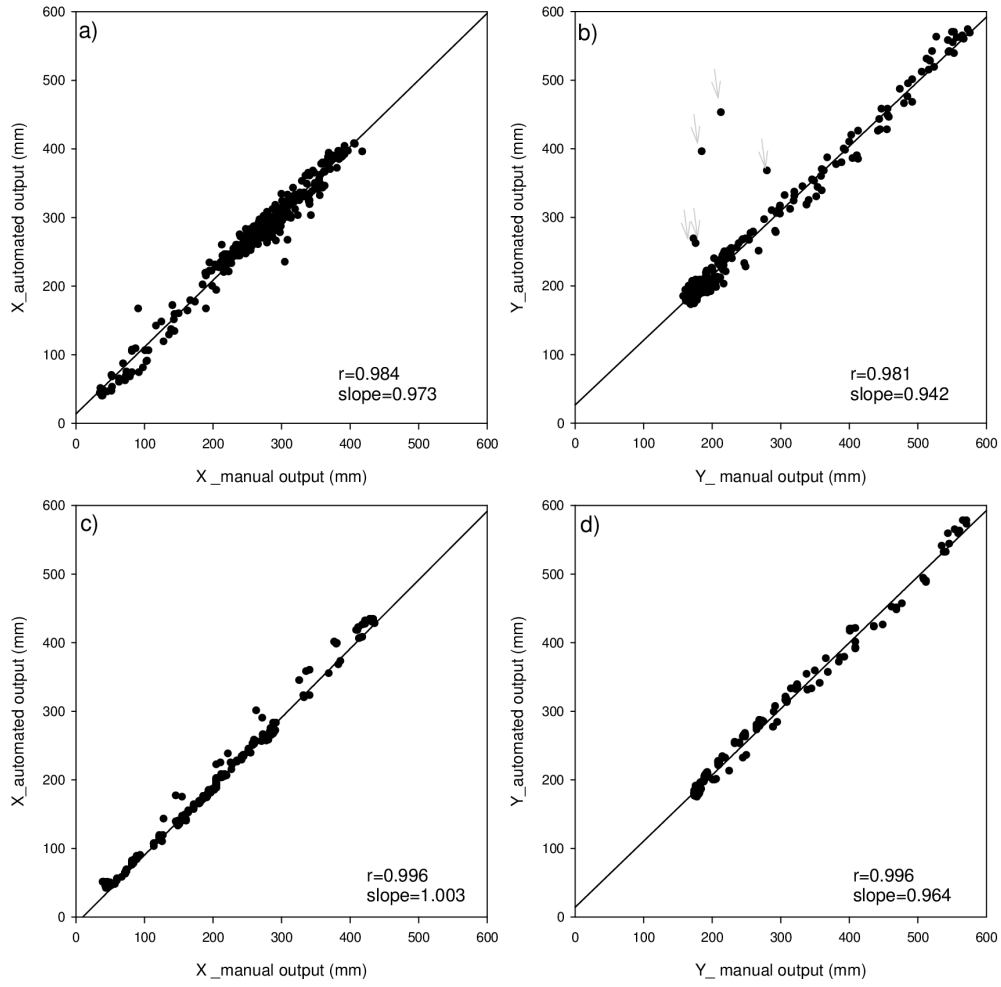


Figure 5.7: Correlations between the automatically and manually estimated two dimensional coordinates of *Metaphire hilgendorfi*. Each panel shows the head point the in upper panel and the tail point the in bottom panel.

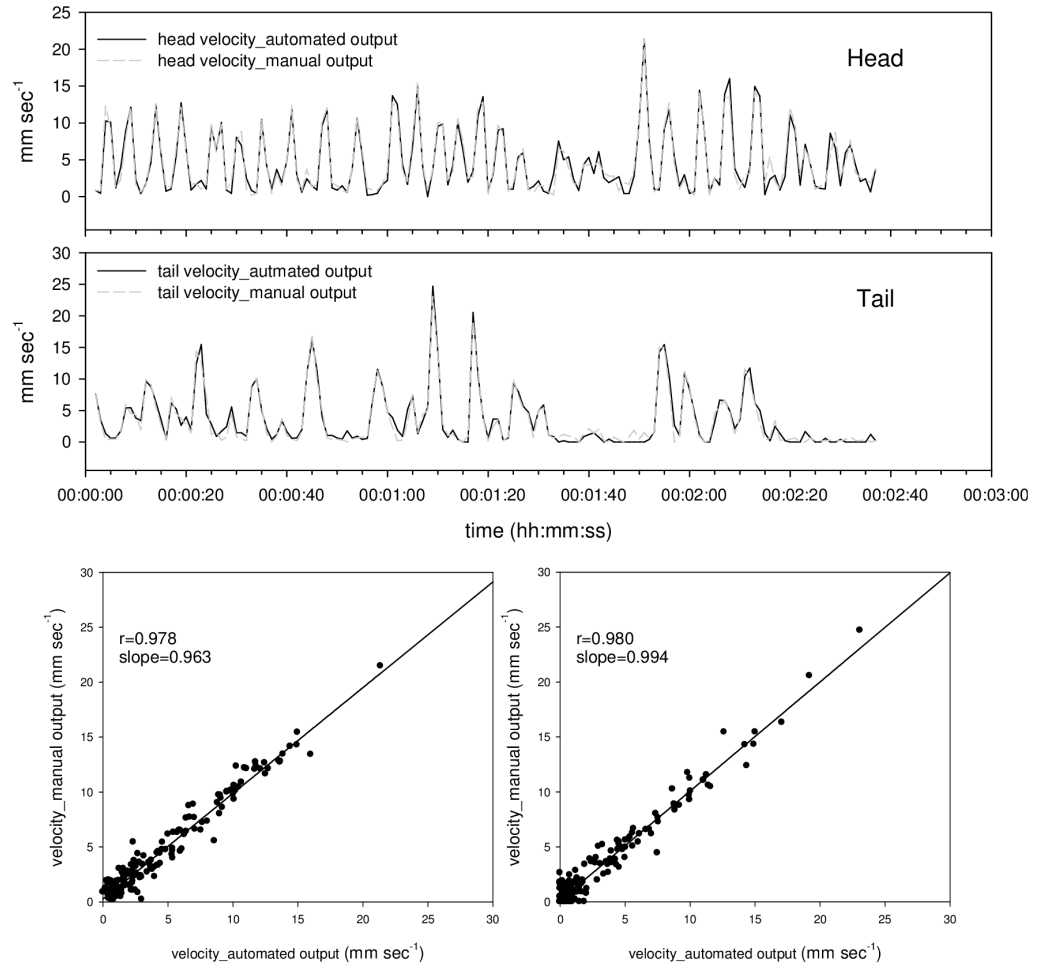


Figure 5.8: Comparison of the automatically and manually estimated velocity of each point of *Eisenia japonica*. The upper panel shows the estimated velocity of the head point and the central panel shows that of the tail point. Correlations between the automatically and manually estimated velocities of *Eisenia japonica*.

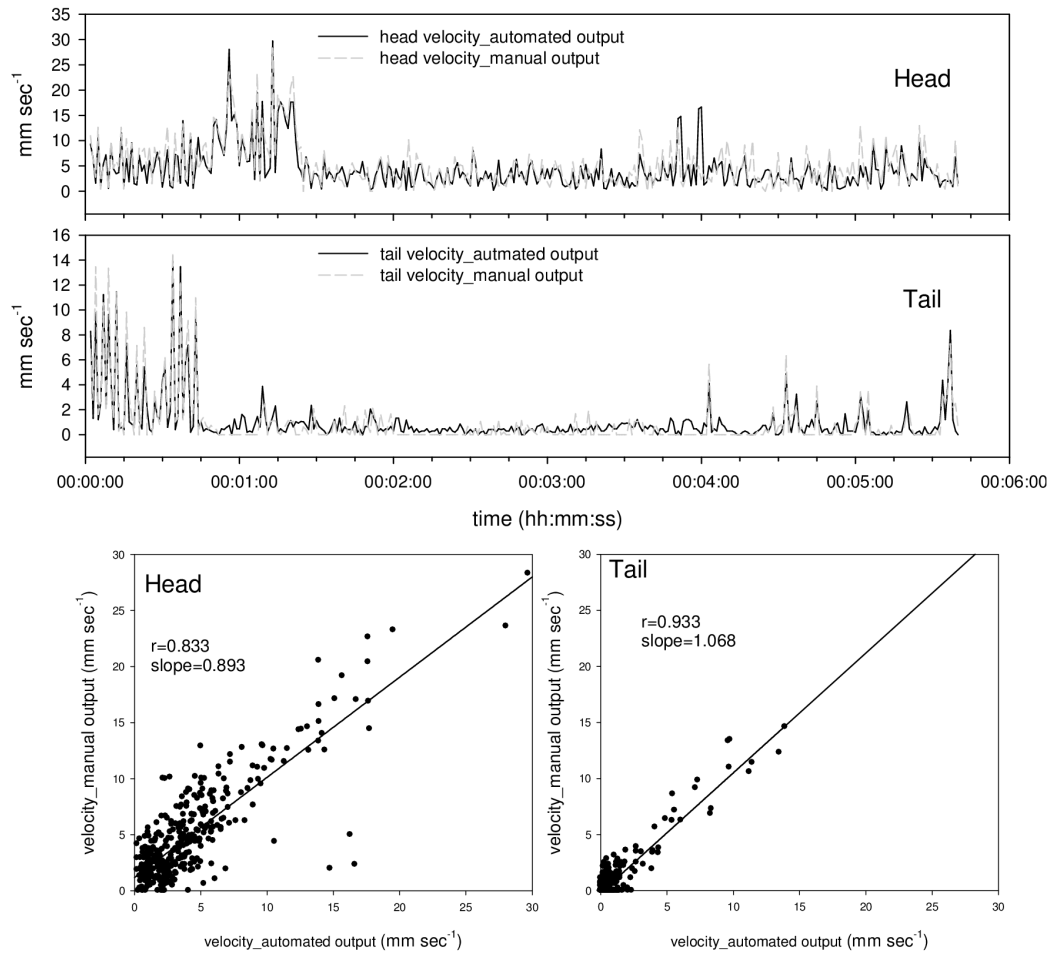


Figure 5.9: Comparison of the automatically and manually estimated velocity of each point of *Metaphire hilgendorfi*. The upper panel shows the estimated velocity of the head point and the central panel shows that of the tail point. Correlations between the automatically and manually estimated velocities of *Metaphire hilgendorfi*.

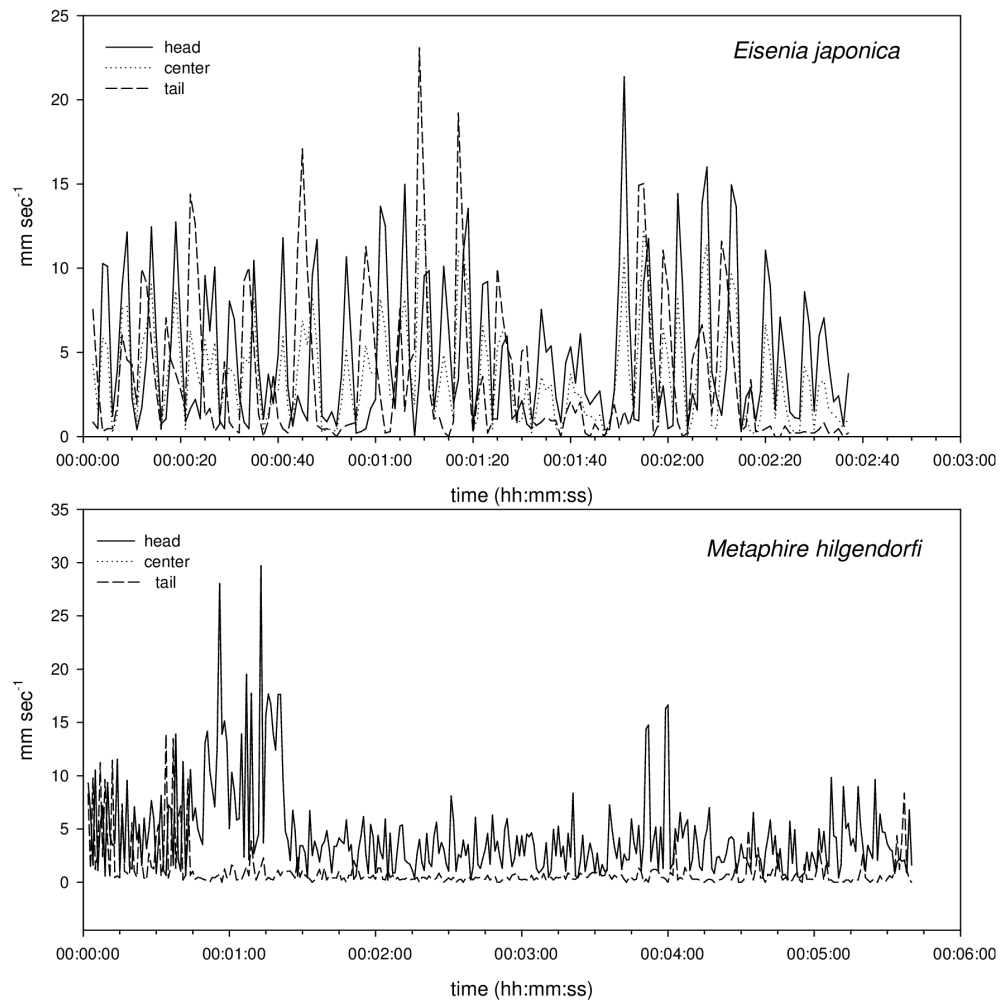


Figure 5.10: Comparison of velocities among different points (head, central, and tail points). The upper panel shows the velocities of each point of *Eisenia japonica* and the bottom panel shows those of *Metaphire hilgendorfi*.

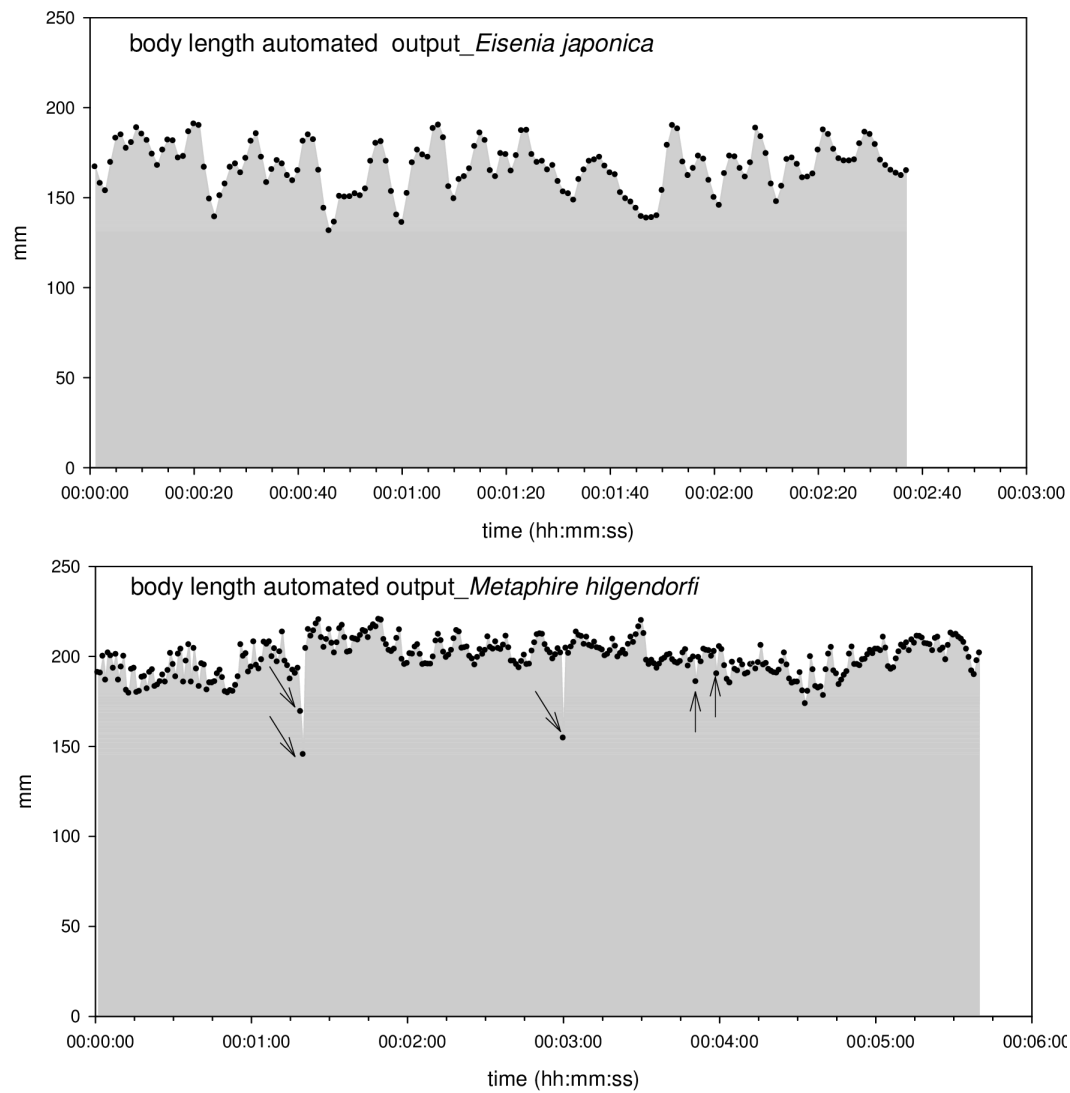


Figure 5.11: Change in body length over time. The upper panel shows the length of *Eisenia japonica* and the bottom panel shows that of *Metaphire hilgendorfi*.

remains. Especially, the program still could not cope with irregular shapes of earthworms such as coiling or overlapping. This could be achieved by modifying the program in the future study such as using the Knot Theory, or topological concept. The program also failed to follow the feature points when the head moved more frequently than general movement. Also, in the case of *M. hilgendorfi*, when they coiled or crossed their bodies, the program faced difficulties in recognizing the edges, and thus failed to detect the head positions. To overcome such problems, one solution would be to obtain image at higher resolution time image so that the program could keep tracking more frequently moving objects without losing the target points because the higher time resolution is the closer to continuous data. Increasing the contrast between the background and the objects for image analysis would also achieve more precise results. Furthermore, there would be alternative methods to detect feature points such as differentiating the shapes or brightness of head or tail which will further improve the program.

The velocity of each point of the different body parts of *E. japonica* was synchronized; in contrast, those of *M. hilgendorfi* were not. In the case of *M. hilgendorfi*, the head and center points were synchronized but the tail point was not, it tended to stay still on the same coordinate. We recognize that the velocities were under non-soil conditions; therefore, earthworms in the soil must be much slower than our estimations. However, the program picked up the features of each species when observed under the same conditions. We consider our newly developed program is sufficient to estimate earthworm activity; this method can be used to compare dynamic changes in these parameters between species or in response to environmental variables such as temperature, light or moisture as well as chemical contaminants.

Image data is powerful, direct information that can be used to quantify physical information of changes in earthworm's activities. The intention of this study is to apply the program to quantify the physical parameters to indicate earthworm's activity from their movement speed or body stretchiness. It will be more beneficial if these data are combined with other data, such as data on gas emissions from the body, enzyme activities using molecular tools, or pathway of elements using isotopic data for mechanistic understanding. The method used in this study is artificial; a small Petri dish without soil as used. However, this study will advance our understanding of the basic behavior of earthworms depending on the alteration of their living environment. This basic behavior can be incorporated in models in terms of material flow belowground or reactions in response to chemical contaminant in soil [6].

Combining different kinds of studies will help to unravel or predict the complex processes in belowground systems [25]. Furthermore, although this system could not be applied to observe their behavior directly in the soil, culturing earthworms in transparent gels or agar medium would be a useful method to observe them three-dimensionally. Furthermore, applying other rapidly developing state of the art technologies, such as MRI or radar, to observe earthworms under soil conditions, it will be theoretically possible to directly observe their behavior in soil, though the time resolution will not be high enough. In fact, radar is already being used for root growth observations in soil.

5.5 Conclusion

We demonstrated that our newly developed program successfully tracks the movement of two species of earthworms that have different features in terms of body size, mass and morphology as well as movement. Based on our data, we estimated that the program reduced the time by a 3 fold from manual estimations and the program could track the movement which is inherent in earthworm behavioral ecology studies. Huge continuous image data sets can be immediately processed and the program can determine earthworm body parts along with their changes in elastic body lengths over time. In the future, quantified earthworm behaviors could be incorporated in material flow dynamic models in soil or in models of the response of earthworms to chemical inputs to soil.

Chapter 6

Conclusion

The tracking of multiple objects is important for analyzing animal behavior. Most ethology studies involve the manual extraction of data from experimental video data, requiring considerable time and effort. Thus, software for automatically tracking behavior would have valuable applications in ethology research, improving the efficiency of behavioral analysis.

In Chapter 2, a new algorithm is introduced for examining multiple bees in an observation hive under natural light. In uncontrolled conditions, the algorithm adopts the vector quantization classification method to extract target regions. This method identifies each bee using size and spatio-temporal overlap information. The trajectories of bees are drawn by the connection of their locations frame by frame. In this dissertation, the algorithm was applied to 10-second videos with 300 frames, with approximately 700 bees in the observation hive. During all frames, the method successfully detected approximately 75% of individuals, and track approximately 50% of objects. Furthermore, the algorithm was found to be useful for classifying both active and passive areas, and for detecting particular behaviors, such as the waggle dance.

In Chapter 3, a new method is proposed for tracking multiple bees on a flat area under controlled lighting. In addition, a new software package called K-Track was developed, based on the proposed method. The software adopts the background subtraction method to extract the bees' candidate regions. It identifies each bee using size and figure information in each frame, and using spatio-temporal overlapping information and linear prediction of behavior between frames. The trajectories of bees are drawn by the connection of their locations frame by frame, as in the method described in Chapter 2. In this chapter, experimental videos with 16 walking bees on a flat circular area were analyzed. The software can detect and identify bees with high performance. Furthermore, it can track bees in approximately 91% of interactive situations. Using the obtained locational data, parameters such as velocity, acceleration speed and the distance between bees can be calculated. However, the software often cannot track bees during interactions near a wall or edge, possibly because the bees' limited movement near a wall means that linear prediction cannot function well.

To resolve this problem, an improved tracking software called K-Track-kai is proposed in Chapter 4, using the tracking trajectories from video containing both forward- and backward-playing footage. This software processes the tracking algorithm of K-Track with an experimental video with both forward and backward play, and obtains two trajectories. The software compares the maximum moving distances by frame for both trajectories, selecting the result with the shorter distance. K-Track-kai was largely successful for tracking the error results of K-Track's two-bee interaction situations. In situations with six and 16 honeybees, K-Track-kai improved tracking accuracy from 91.7% to 96.4%, and from 94.4% to 96.7%, respectively.

Chapter 5 describes the development of tracking software for an earthworm, called MimizuTrack. The software uses body color information to extract a target object region. This system detects the head and tail of the earthworm by shinning the region using the Hidlieth algorithm, and also detects the central point of the worm from the center of the shinning line. Both sides of the line are extracted as the head and tail of the earthworm, and the central point of the line is assigned as the central point of body. This system exhibits good tracking performance for these points. Based on the current data, the

program was found to enable an approximately 3-fold reduction of manual estimation. This program could be used to track inherent movement in earthworm behavioral ecology studies. Using this method, large continuous image data sets can be rapidly processed, and the program can identify earthworm body parts along with changes in elastic body length over time.

This dissertation describes the development of tracking methods and software for supporting the analysis of animal behaviors. The proposed software can provide supporting tools for analyzing animal behaviors in a range of species, such as honeybees and earthworms. However, this software is not suitable for tracking some species. Therefore, it is necessary for engineering and ethological researchers to cooperate, using and improving the software in experimental settings. These findings indicate that automatic tracking software could become a useful tool for extending our ability to analyze and understand animal behaviors.

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Chapter 2

1. Toshifumi Kimura, Mizue Ohashi, Ryuichi Okada, Hidetoshi Ikeno, "A new approach for the simultaneous tracking of multiple honeybees for analysis of hive behavior," *Apidologie* vol. 42, pp. 607–617, DOI 10.1007/s13592-011-0060-6, 2011.
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Chapter 3

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Chapter 4

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decision method to enhance the tracking performance of multiple honeybees on a flat laboratory arena,” submittd.

Chapter 5

1. Naomi Kodama[†], Toshifumi Kimura[†], Seiichiro Yonemura, Satoshi Kaneda, Mizue Ohashi, Hidetoshi Ikeno, “Automated Analysis of Two-Dimensional Positions and Body Lengths of Earthworms (*Oligochaeta*); MimizuTrack,” PLoS ONE 9(6): e97986, 1-14, DOI: 10.1371/journal.pone.0097986, 2014

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