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Endocytic vesicle movement in a cytoskeletal network revealed by numerical analysis

(数値解析に基づいた細胞骨格ネットワーク内小胞の輸送様式の解明)

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Abstract

The process of cellular uptake of molecules is called endocytosis, which sustains the intracellular homeostasis. During the endocytosis, the extracellular molecules are engulfed by the cell in the form of vesicle, and the vesicle starts to navigate the cytoplasmic area from the plasma membrane to the center of the cell. The movement of endocytic vesicle in a living cell contains significant information that can be broadly utilized in the biomedical applications, such as drug delivery. Therefore, many researches have been conducted hitherto as focusing on the mechanism of vesicle transport in the molecular level *in vitro*. The precise movement of a vesicle in the intracellular area, however, has been considered as one of the challenging tasks in biophysics. The reason why is that the vesicle inevitably interacts with complex cytoskeletal network in a living cell, which appears as a complicated movement trajectory, and this hinders the accurate detection and analysis of the vesicle movement. Here, this dissertation aims to provide a complete set of vesicle movement analysis method for understanding the vesicle movement in a complex cytoskeletal architecture, and to present the actual features of the three-dimensional vesicle movement detected in a living cell, in terms of the interaction between the vesicle and cytoskeletal network. As a prerequisite for the accurate detection of vesicle position data, the development of axial position stabilization system is also proposed.

In chapter 1, the overall background of the vesicle movement in cytoplasmic area is introduced, with the importance of the information acquired from the vesicle movement in a living cell and the history of the related studies. In addition, the complexity in analysis of the movement trajectory of vesicle is explained.

In chapter 2, the enhancement in three-dimensional imaging optics is presented for achieving high accuracy in vesicle position detection, by developing and installing the external axial position stabilization system. Because live-cell microscopy imaging system typically suffers from thermal and vibrational fluctuations, it is imperative to secure absolutely stable imaging condition for acquiring precise three-dimensional position data of the target. Based on the capacitive sensor, the axial position stability was achieved as keeping the constant distance between the objective lens and microscope stage, using the feedback control.

In chapter 3, a novel numerical method for analyzing the detailed movement of vesicle is introduced, as a cornerstone for understanding the vesicle movement in a complex cytoskeletal network in living cell. In contrast to the hitherto analysis methods, the numerical method features intuitiveness and practicality as a combination of geometrical and statistical approaches. Treating the three-dimensional trajectory of vesicle as a data point set in a space, the local curvature of the trajectory is detected by angle correlation function after the noise reduction. Since the interaction between the vesicle and cytoskeleton appears as the linearity and persistency in the trajectory, the location of cytoskeletons were estimated by principal component analysis. The precise angular and translational movement of vesicle on the estimated cytoskeleton was presented as a vector calculation utilizing the relationship between the consecutive data points and projected points.

In chapter 4, the endocytic vesicle movement acquired by live-cell imaging is analyzed by the numerical analysis method, and the newly revealed detailed features of three-dimensional vesicle movements in a microtubule network are presented. In the experiment, the vesicle-quantum dot movement in GFP-tubulin expressing KPL4 human breast cancer cell was tracked. The transfer angle of vesicle between

two crossed microtubules was measured in three dimensions, which turned out to be either very acute (10–60°) or obtuse (100–180°), but with similar time scale, 0.5 s. This result reflects the actual angles of microtubule crossings in living cell. Particularly, vesicles on their long-range transports (> 400 nm) showed a unique rotational movement around the axis of microtubule with high probability of occurrence (> 50 %), which consists of quick dodging and gentle walking, regardless of the direction of rotation. Additionally, the angular intervals between the adjacent quick dodgings appeared to be 180° in almost all rotational movements. These characteristic angular motions suggest the reaction of the vesicle when it encountered an obstacle on the microtubule.

In chapter 5, the conclusion of this dissertation is presented. This study proposed a pioneering understanding of the detailed feature of vesicle movement which navigates in a complex cytoskeletal architecture. This dissertation covered from establishing the stable and reliable three dimensional live cell imaging condition to developing the practical analysis method and revealing the actual vesicle movement in terms of precise angular and translational motion. Therefore, it is expected that this work will inspire the related studies for better understanding the vesicle movement in a living cell, as an initiative platform.

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Contents

List of Figures

List of Abbreviations

GFP	**G**reen **F**luorescent **P**rotein
fps	**f**rame **p**er **s**econd
STORM	**S**tochastic **O**ptical **R**econstruction **M**icroscopy
NA	**N**umerical **A**perture
MSD	**M**ean **S**quared **D**isplacement
ATP	**a**denosine **t**ri**p**hosphate
HMM	**H**idden **M**arkov **M**odel
SPT	**P**oint **S**pread **F**unction
ROI	**R**egion **O**f **I**nterest
STD	**ST**andard **D**eviation
SVD	**S**ingular-**V**alue **D**ecomposition
PCA	**P**rincipal **C**omponent **A**nalysis
SRRF	**S**uper **R**esolution **R**adial **F**luctuation
SNR	**S**ignal to **N**oise **R**atio
MSAD	**M**ean **S**quared **A**ngular **D**isplacement

Chapter 1

Introduction

This dissertation focuses on reporting the precise movement of endocytic vesicle in a complex cytoskeletal network, which is analyzed by a newly developed numerical method. Particularly, characteristic rotational movements of vesicles around the axis of estimated cytoskeletons are described in detail. Each chapter in this dissertation describes following respective contents: In chapter 1, the overall background and motivation for the vesicle movement analysis are introduced, and the main purpose of this research is clarified. In chapter 2, the three-dimensional imaging microscopy assisted by the newly developed axial position stabilization system is described as a prerequisite condition for vesicle position data acquisition with high spatiotemporal resolution. Chapter 3 presents a novel numerical analysis method for the vesicle movement in terms of vesicle-cytoskeleton interaction, which enables us to recognize the precise dynamics of vesicle in a complicated interconnected cytoskeletal network structure. In chapter 4, the applications of numerical analysis method to the actual vesicle movement data acquired in a living cell are illustrated. Particularly, characteristic rotational vesicle movements detected in active transport sections are reported and analyzed in detail. In addition, the discussion about the biological significance of the observed vesicle movement is addressed. Finally, the conclusion of this dissertation is summarized in Chapter 5.

1.1 Background and motivation

1.1.1 Endocytic vesicles in cells

Living cells are required to continuously intake extracellular molecules in order to maintain their intracellular homeostasis, through the biochemical process called 'endocytosis' (Conner and Schmid, 2003a; Alberts, 2017). The extracellular molecules engulfed via endocytosis are delivered into cytoplasmic area, as a form of vesicle. The vesicles created by the endocytosis can be called endocytic vesicles, and the transport of them are critical for the life of cells, as they contribute to the delivery of the nutrients as well as to the intracellular signal cascade (Sorkin and Zastrow, 2002). Also, the homeostasis of the plasma membrane is sustained by the transport of vesicles in that the lipid bilayers surrounding the vesicle are recycled back (Maxfield and McGraw, 2004).

A diagram of cell cross section shown in Fig. 1.1 illustrates the geometry of vesicle as an intracellular organelle with respect to the entire feature of a eukaryote cell. Fig. 1.1 (A) describes the endomembrane system of a cell where vesicle is in the initial stage of being budded at the plasma membrane (LadyofHats, 2006). The process of endocytosis, where the vesicle is now a separated sac from the plasma membrane is shown as a zoomed view in Fig. 1.1(B). Therefore, as shown in Fig. 1.1 (C), the outer shell of the vesicle is lipid bilayer which comes from the plasma membrane

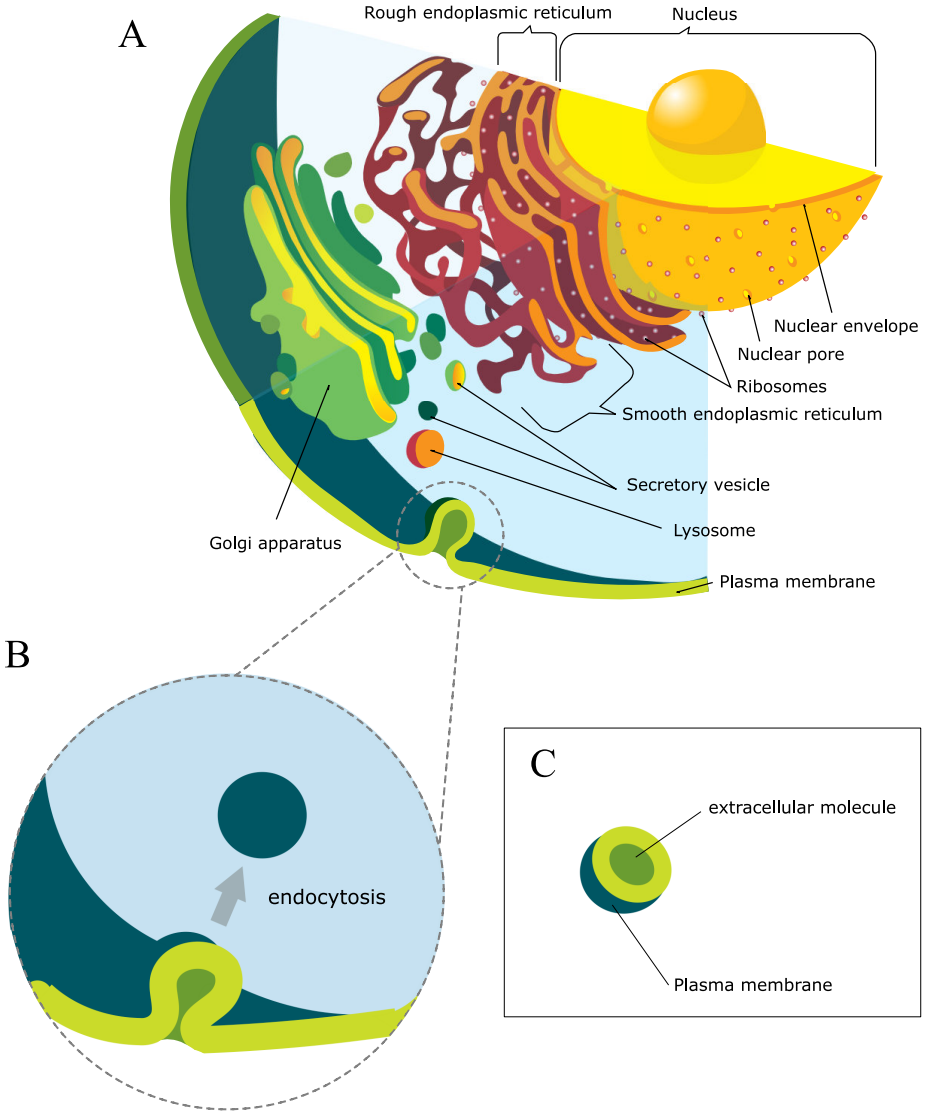

FIGURE 1.1: Cell cross section and endocytic vesicle (LadyofHats, 2006). (A) Cross section of a cell. The budding of plasma membrane is the initial stage of endocytosis which produces vesicle. (B) Zoomed view of the bud at plasma membrane and vesicle, which explains the process of endocytosis. (C) Cross section of a vesicle. The shell of vesicle is the lipid bilayer comes from plasma membrane, enclosing the extracellular molecules.

and encloses the molecules inside. Note that the plasma membrane of cells is lipid bilayer which consists of outer hydrophobic domain and inner hydrophilic domain, and this prevents the vesicle from being dissolved instantly while protecting the inner materials (Simons and Ikonen, 1997).

1.1.2 Vesicle transport and its complexity

The importance of vesicle transport has been recently emphasized, since many biomedical applications for therapeutic purposes such as drug delivery are expected to take advantages from the core information regarding the intracellular vesicle transport mechanisms (Chou, Ming, and Chan, 2011; Matveev et al., 2001; Hess and Vogel, 2001). Fundamentally, the drugs can be considered as a chemical agents surrounded by protection barrier until they reach the biological target, the idea of loading drugs on nanoparticles is reasonable as well as promising, provided that subtle and selective control for the nanoparticle delivery is achievable. In fact, cellular level biochemical techniques are in progress, but still the ability and performance of such applications are primitive. The reason why the task has been challenging is largely because our understanding of the particle transport, which is vesicle transport in case of natural condition, is still not yet elucidated in detail.

For example, although the vesicles are started to be formed by budding of the plasma membrane and recruited by motor proteins to be transported on the cytoskeletons, each step is only conceptually known, or observed in the limited condition such as via electron microscope. Also, the precise movement of the vesicle on the cytoskeletons had been thoroughly studied in terms of the physical properties of single motor protein – such as the amount of force generated or the length of step size – which mediate the interaction between vesicle and cytoskeleton, but only in *in vitro* system and only for a single molecule. However, since the vesicles navigate their destination in cytoplasmic area by being transported on the cytoskeleton, the information about the movement of vesicle as a result of the interaction between vesicle and cytoskeleton in a living cell condition is the core for understanding this biological process.

One of the main hurdles which prevent us from investigating the precise movement of vesicle on the cytoskeleton in a living cell is that the endocytic vesicle inevitably interacts with complex cytoskeletal network structure after it entered the cytoplasmic area, rather than interacting with only a single cytoskeletal filament as frequently exploited in hitherto *in vitro* experiments which indeed had oversimplified the reality. In the actual intracellular condition, vesicles experience higher probability of interacting with multiple motor proteins while repeatedly attaching and detaching to multiple cytoskeletons, in a complicated interwound architecture of the cytoskeletal network. Such inherent complexity in the vesicle movement led to the lack of standard methods or models for analyzing the precise motion of the vesicle in the intracellular area.

Therefore, it is a critical task to establish a reliable analysis model for understanding the detailed feature of vesicle movement observed in a living cell condition, which is robust against not only to the noise of the practical imaging system but also to the inherent complexity of the vesicle movement trajectory caused by the multiple-cytoskeleton involvement.

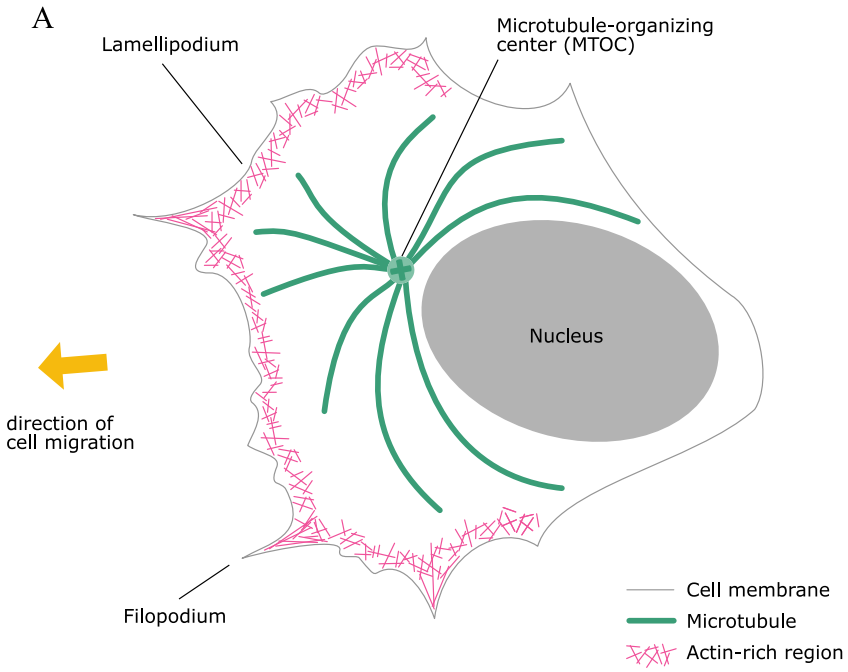

FIGURE 1.2: (A) Diagram of intracellular cytoskeletons. In the itracellular area, microtubules are spread out from microtubule-organizing center (MTOC) which is located perinuclear area. On the other hand, actin filaments are located cell periphery where cell membrane experiences frequent fluctuation in morphology. The actin-rich area is often called as a leading edge of a cell, in that cells migrate in the direction where the density of actin is high. Lamellipodium (*pl.* lamellipodia) refers to the ruffling structure of leading edge, and filopodium (*pl.* filopodia) indicates the extended lamellipodia by bundling of actin filaments. (B) Image of actin filament in a living cell. LifeAct-labeled actin filaments are imaged via common fluorescent microscopy. Note that the actin filaments are distributed densely near the leading edge. White dashed line indicates the entire cell shape. (scale bar = 10 µm) (C) Image of microtubule in a living cell. GFP-labeled microtubule network is imaged via common fluorescent microscopy. Note that the microtubules forms complicatedly interconnected architecture all over the intracellular area. (scale bar = 10 µm)

1.2 Interaction between vesicle and cytoskeleton

The term 'cytoskeleton' covers several types of filament-like structure in the cytoplasmic area that sustain the shape of a living cell. The most representative types of cytoskeleton can be considered as microtubule, actin filament, and intermediate filament (Chang and Goldman, 2004). Microtubule shows a hollow tube-like shape which consists of α-β tubulin heterodimers as a structural unit (Li et al., 2002), and actin filament forms a dense network at the leading edge of a cell by spreading extensive branches (Svitkina and Borisy, 1999). Intermediate filaments are rod-like bundles of filament, and play the main role in supporting the shape of a cell and cytoskeletal crosstalk (Chang and Goldman, 2004).

In the intracellular area, as shown in Fig. 1.2, microtubules are spread out from a microtubule-organizing center (MTOC), which usually locates near the nucleus, and plays a role as a main track in the cytoplasmic area. Actin filaments are observed near the cell edge, especially the leading edge of a cell, where the membrane shows a ruffling structure caused by frequent actin polymerization. The microtubule and actin filament are often referred to as tracks for vesicle trafficking, in that motor proteins such as myosin, kinesin, and dynein recruit the vesicle as a cargo and carry them on these cytoskeletal filaments, by interacting both cargo and track simultaneously (Vale, 2003).

Although the diagram shown in Fig. 1.2 (A) depicted the cytoskeletons in a sparse manner, actin filaments and microtubule filaments in living cells usually form a dense network structure. Figure 1.2 (B) is the image of actin filament labeled by SiRActin. Since the actin filaments actively polymerize near the cell edge, it is recognizable that the leading edge contains a large area of actin network, while the trailing edge accommodates a long and thick bundle of actin filament. Note that the actin filament network near the cell edge is so dense that a single filament is not even distinguishable under common fluorescent microscopy. Also, when microtubules in a living cell are labeled by GFP and imaged, as shown in Fig. 1.2 (C), the microtubule shows an interwound structure which is interconnected together covering the entire cytoplasmic area.

1.2.1 Myosin molecules and actin filaments

Since the actin filaments are distributed near the cell edges, where the vesicle starts to enter the intracellular area, it is expected that actin filaments are involved in the initial stage of the vesicle transport. Although the specific mechanism of vesicle transport in the network structure of actin filaments has not yet been fully explained, the type of motor proteins which gives the locomotion on the actin filament when the vesicle is transported at the actin-rich area is known as unconventional myosins, that are myosin V and myosin VI (Cheney and Mooseker, 1992; Titus, 1997; Woolner and Bement, 2009).

While conventional myosins are referred to be the type of myosin which forms a filament, such as myosin II, and mediate the contraction and relaxation of muscle, unconventional myosins including myosin V and myosin VI are known to be involved in the vesicle transport without forming filament-like structure. In *in vitro* experiments, the single molecule of actin-based processive motor myosin V shows $\sim$ 35 nm in step size upon ATP hydrolysis with characteristic hand-over-hand walk (Ali et al., 2002; Yildiz et al., 2003). On the other hand, myosin VI has been reported to have a larger step size than myosin V, but to the backward direction (Wells et al., 1999; Rock et al., 2001). In *in vivo* condition, the speed of myosin V is known to be

200 nm s^{-1}, while myosin VI is - 60 nm s^{-1} (Ron Milo, 2016). Note that the negative sign implies the inward direction. Although myosin V works alone or as a group of few molecules, the model of action of myosin VI is still largely unknown (Ron Milo, 2016). Simple diagram in Fig. 1.3 (A) depicts the movement of a vesicle along an actin filament mediated by myosin V (upper) and myosin VI (lower).

Moreover, several reported facts hinders the accurate analysis of vesicle trajectory in actin filament network. For example, the actin filament itself shows a coiled structure (Ecken et al., 2015). Also, the myosin involved in intracellular vesicle transport is known to walk in a spiral manner along the actin filament, in case of *in vitro* (Ali et al., 2002). In addition, the fact that there exist mixture of forward and backward movement regardless of the type of myosins according to the affinity between myosin and actin filament makes the study in depth over the vesicle trajectory in the cell leading edge more complicated.

1.2.2 Kinesin, dynein and microtubule

In the case of microtubule, kinesin and dynein are the representative motor proteins related to the vesicle transport (Hirokawa, 1998; Goldstein and Yang, 2000; Vale, 2003; Ross, Ali, and Warshaw, 2008).

Kinesin is one of the most studied motor protein families, the physical properties and functions of which are vastly researched (Courty et al., 2006; Hirokawa and Noda, 2008). The most important function of kinesin is that this motor protein carries a cargo in the direction of where the microtubule filament polymerizes, which is named as a plus end (Howard and Hyman, 2003). The velocity of kinesin for vesicle tranport was measured as near 600 nm s^{-1} (Courty et al., 2006). Since the microtubule spreads out as polymerizing in the radial direction toward the plasma membrane, kinesin might be considered as the motor protein which only concerns the exocytosis, which mediates the movement of secretory vesicles. However, because the vesicle interacts with multiple cytoskeletons while frequently changing direction, kinesins also can be considered to be involved in endocytic vesicle movement. Figure 1.3 (B) shows the cargo movement on a microtubule when kinesin molecule is involved.

Dynein also has been substantially studied since several decades (Aniento et al., 1993; Mallik et al., 2004; He et al., 2005). Since cytoplasmic dyneins are known to walk along the microtubule toward the minus end, which is the direction to the center of a cell, dynein can be considered as a direct mediator of endocytic vesicles that are transported on the microtubules. In terms of physical property, dyneins showed around 8 nm of stepsize and 800 nm s^{-1} of velocity in *in vitro* condition (Toba et al., 2006). In the case of *in vivo* the mean velocity was measured as - 1000 nm s^{-1} (Ron Milo, 2016). Note that the negative sign implies the inward direction. Figure 1.3 (B) represent the movement of vesicle along a microtubule filament when dynein carries the cargo.

Unlike the case where myosin V and actin filament are involved in vesicle delivery, it has been known that the cargos are carried along microtubule linearly without spiral motion based on simple *in vitro* assay. However, one of the recent researches using three dimensional bridge assay showed that dynein also show a helical movement along the axis of microtubule, but without preference in the direction of spiral motion, in *in vitro* experiment (Can, Dewitt, and Yildiz, 2014). This discovery enlarged our understanding about the precise motion of vesicle on the microtubule, and simultaneously leaves a question whether the shape of vesicle movement on the microtubule in a living cell would show a similar feature.

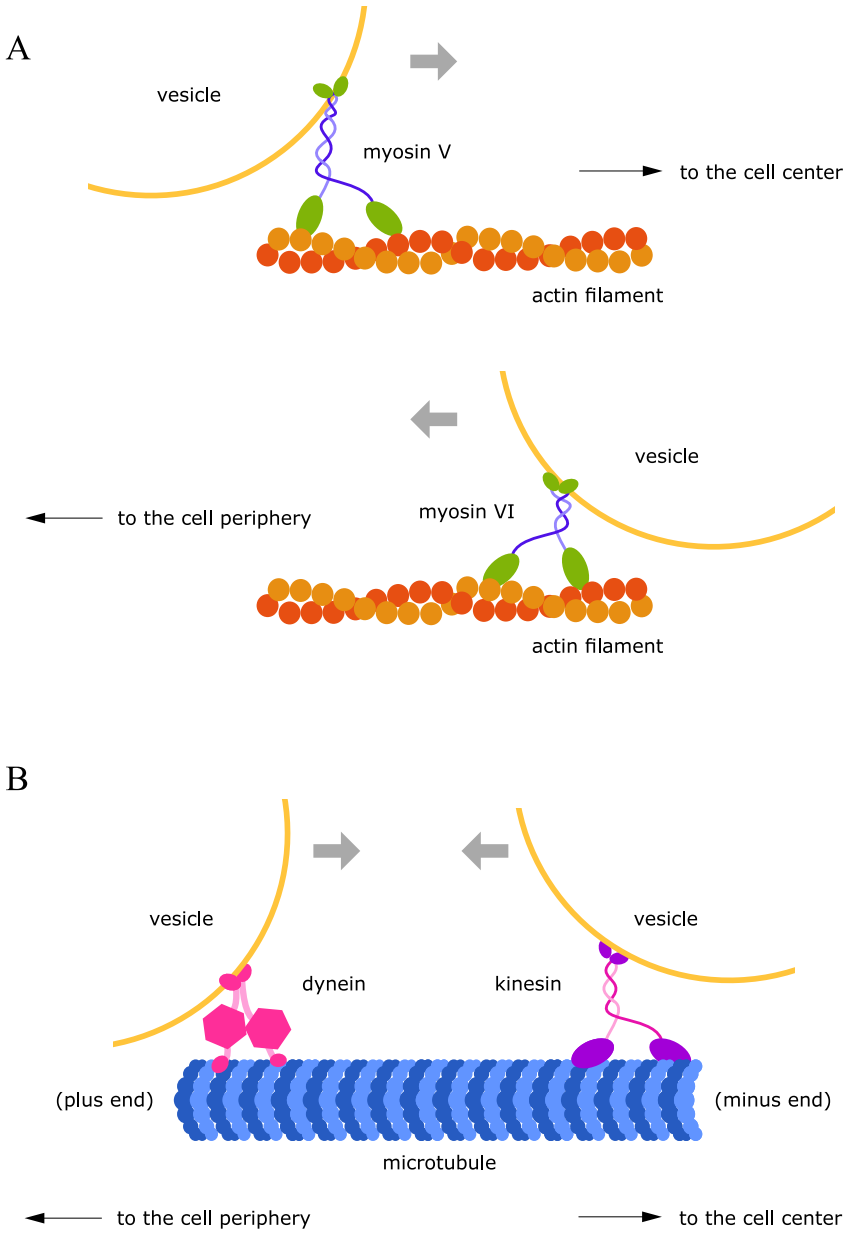

FIGURE 1.3: Cytoskeleton and motor proteins (A) The diagram of cargo carriers myosin V and VI when walking on actin filaments. (B) Dynein and kinesin are the mediator of vesicle transport on microtubule to the direction of plus end and minus end, respectively.

1.3 Vesicle movement in a cytoskeletal network

If we confine our attention to the principle of vesicle movement, it might seem that almost all of the major information is already obtained, since the origin of the locomotion that vesicle gains while it navigate the intracellular area is unveiled by discovering the existence of motor proteins. As mentioned in the previous section, it is known that the motor proteins such as myosin, kinesin, and dynein carry the cargo along the track such as actin filament and microtubule. Moreover, since a great number of researches on the precise step size or the amount of force generated by a single molecule of motor protein have been accumulated via extensive *in vitro* experiments and data collections, one might consider that we now can completely analyze the vesicle movement observed in living cell condition by such numbers.

1.3.1 Reasons for complexity

Strikingly, however, the movement of vesicle in a living cell still remains largely elusive. In fact, there are not even many reported vesicle trajectories acquired in three dimensions with high spatiotemporal resolution, which is essential for the analysis. The reason why we hardly understand the actual movement of vesicle trajectory also owes to several particular conditions that make significant difference from the *in vitro* condition: First, unlike a precisely controlled single-molecule assay system, a vesicle is recruited by multiple numbers and multiple types of motor proteins in cytoplasmic area, due to the high concentration of motor proteins in living cell condition. Second, since the microtubule and actin filament form a dense network structure that are interconnected in three dimensions, the trajectory data of a vesicle in such area are hardly distinguishable in terms of the interaction between the vesicle and the involved cytoskeletons.

The aforementioned circumstance, where multiple numbers and multiple types of motor proteins are involved in a single vesicle movement in a living cell has actually been anticipated and concerned by many researchers, together with the progress in the motor protein studies conducted in *in vitro*. In fact, the situation in which multiple numbers and multiple types of proteins interact with a single vesicle on a cytoskeleton has been called 'tug-of-war', imaging several motor proteins with opposing directionality pull simultaneously the identical vesicles (Okada, Higuchi, and Hirokawa, 2003; Welte, 2004; Nan et al., 2005). For example, as shown in Fig. 1.4, kinesins and dyneins can interact a vesicle at the same time, since both of them can interact with microtubule. In addition, since the actin filament and microtubule can be closely located in the cytoplasmic area, pulling between myosin molecules and kinesin or dynein molecule over the same cargo also cannot be completely ruled out.

In contrast to the problems regarding the involved motor proteins, the latter reason which concerns the complex architecture of cytoskeleton network is rather structural and intuitive. Regardless of the motor proteins interacting with cytoskeletons, vesicle is expected to interact with cytoskeletons in an intermittent manner, from the statistical point of view. Since it can be visually proved if the cytoskeletons and vesicle are imaged at the same system with high spatiotemporal resolution, many approaches have been made to elucidate the detailed feature of vesicle movement in terms of the precise interaction between the vesicle and cytoskeletons.

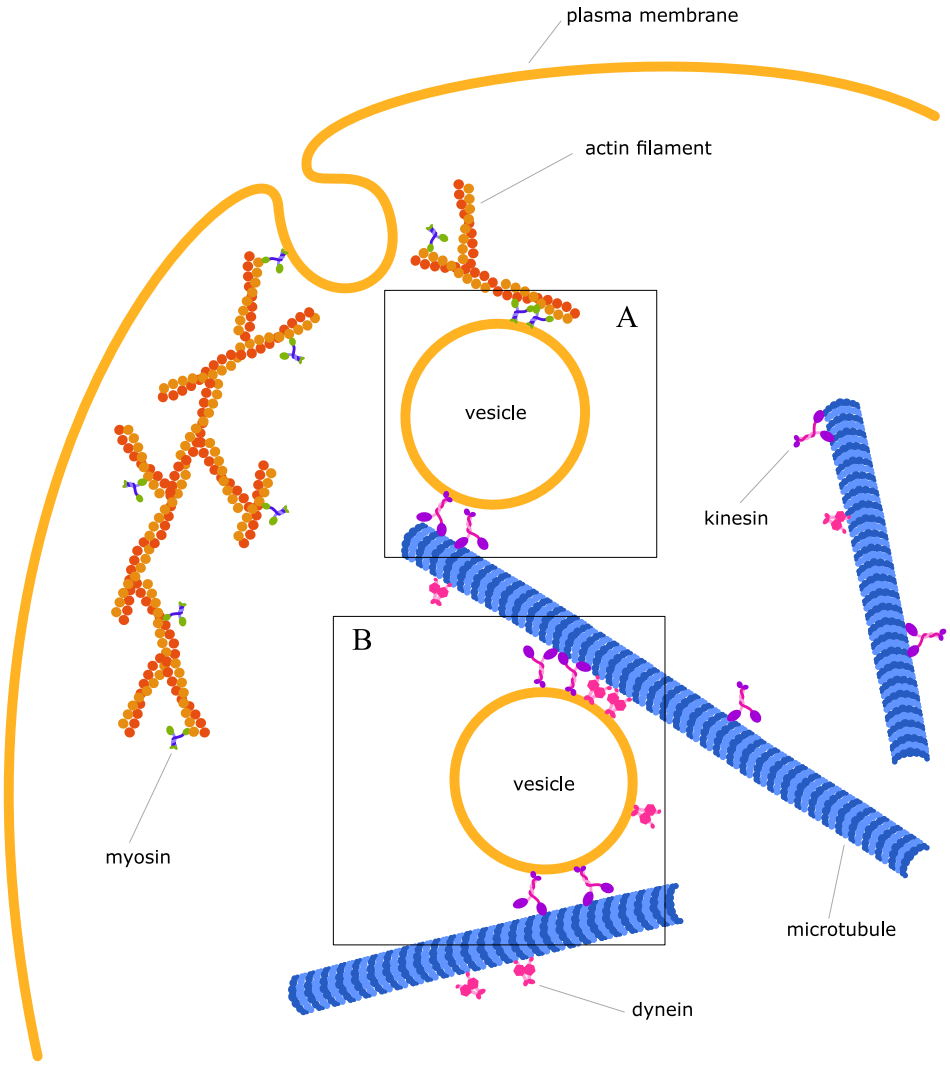

FIGURE 1.4: Vesicle movement in a cytoskeletal network (A) Vesicle moving between actin filament and microtubule. Conceptually, endocytic vesicle travels from the actin-rich area to microtubule dominant area can experience the tug-of-war between myosin molecules and microtubule-binding molecules such as kinesin or dynein. Note that the actin filaments form a branched structure in living cell condition (Mullins, Heuser, and Pollard, 1998). (B) In a microtubule network structure, the vesicles also can be pulled by different types of motor proteins that might work as a group.

1.3.2 Recent approaches

In order to understand the movement of vesicle navigating in a complex cytoskeletal network, various approaches have been made. Among these approaches, one of the most popular topics is to visualize and analyze the vesicle movement at the intersection of two crossing microtubules, which is a highly probable situation for an endocytic vesicle traveling in a microtubule network. Since the microtubules organize a complex three dimensional network structure, vesicles are inevitably required to interact with multiple microtubule filaments, which is accompanied with the interaction between the vesicle and the microtubule intersections. Figure 1.5 shows the fluorescent image of actual network structure of microtubule near cell periphery. Microtubules labeled by GFP in Fig. 1.5 (A) shows a complex and interconnected feature of microtubule, which are not always radially located from the center of the cell where MTOC is located. In fact, it is recently known that not only MTOC but other organelles, such as Golgi apparus, can be the center where the microtubules anchor to (Martin and Akhmanova, 2018), which aids the network structure of microtubules in living cell to be more complicated as shown in Fig. 1.5 (B) and (C).

Earlier studies concerning the shape of vesicle motion at the microtubule – microtubule intersection have categorized the types of movement of the vesicle, such as pass, pause, switch, dissociate, and reverse, according to the decision of the vesicle on the intersection (Vershinin et al., 2007; Ross et al., 2008). Although these studies enlarged our understanding about the movement pattern of vesicle when multiple microtubules are involved, still the information concerning the actual vesicle trafficking in a microtubule architecture was not enough, because the experiments have been conducted in *in vitro* environment, mainly focusing on the numbers or the types of the motor proteins that take part in carrying the cargo. In contrast to the limited *in vitro* condition, vesicles are carried more dynamically in more complicated architecture of microtubules in a living cell condition.

As one of the initial approaches for detecting the movement of vesicle in living cell condition, a switching motion of the vesicle between two microtubule tracks were observed in living neuronal cells (Mudrakola, Zhang, and Cui, 2009). Although they succeeded in detecting and analyzing the detailed motions of vesicle when it switched from a microtubule filament to another, the question whether the vesicle actually traveled on two different microtubules remains, because the vesicle trajectory analysis was presented without the microtubule images. The initial observation of the pattern of vesicle movement at the microtubule intersections with correlative image of microtubule was conducted recently (Bálint et al., 2013; Verdeny-Vilanova et al., 2017), using superresolution imaging technique, Stochastic Optical Reconstruction Microscopy (STORM). They detected and analyzed the movement of lysosomes on the stabilized microtubules, and categorize the pattern of movements into several groups: pause, pass, switch, and reverse, at the microtubule intersection. Also, they suggested that such pattern of lysosome movement at the microtubule intersection can be originated from the separation between the microtubules, by comparing the rate of occurrence of pause or switch according to different separation. Since the separation of microtubules are meaningful criteria considering the average diameter of vesicles ($\sim$ 100 nm), the movement patterns of the cargo at the microtubule intersection having different separation were modeled in *in vitro* condition using beads and *de novo* microtubules (Bergman et al., 2018).

Although previous researches have enlarged our knowledge about the movement of the vesicle where multiple number of cytoskeletons are involved in cargo transport, there exist limitations which hinder us from accessing the information

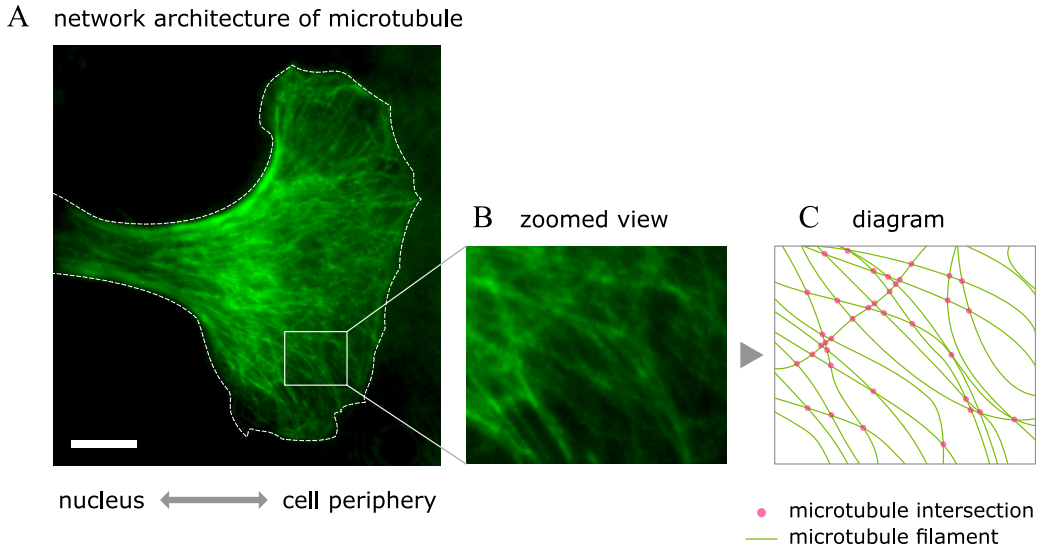

FIGURE 1.5: Network architecture of microtubule in a living cell. (A) Microtubule labeled by GFP was imaged via fluorescent microscopy. The microtubules are organized a complex network covering the entire intracellular area. (scale bar = 10 μm) (B) Zoomed view of a small patch in the microtubule network image. The microtubule filaments spread from the center of the cells are not parallel but form a complicated network structure as crossing one another. (C) Diagram sketched based on the extracted information about the microtubule geometry from the zoomed view. The pink dots represent the position of microtubule intersection. Note that the intersections are determined based on two dimensional information, which includes the cases of crossed microtubule separated axially. However, such intersections are also counted as the points where vesicle possibly experience transfer or switching, considering the common size of endocytic vesicles ($\sim$ 100 nm in diameter).

about detailed features of vesicle movement in a complex cytoskeletal network. Most importantly, the resolution of microtubule images are still between the range of several tens of nanometers and a hundred nanometer, in axial direction, which plays a critical role in detecting the precise geometry between the carried cargo and the cytoskeleton. Also, since the vesicles are imaged in time-lapse condition, the movement of vesicle occurring in a short time span, for example at 100 fps (frame per second), has not yet been fully explained in terms of the interaction between the vesicle and cytoskeletal architecture.

1.4 Advances in microscopy imaging techniques

Achieving high spatiotemporal resolution microscopy imaging is the prerequisite in investigating the precise movement of vesicle, and have been widely studied and developed. In fact, our understanding about the shape of small particle movement including the vesicle movement in a cell has been deepened in accordance with the development of the microscopy imaging technique, in that the image is the major source of the information. The recent advent of superresolution microscopy, which refers to the microscopy imaging techniques that overcome the limit of the optical diffraction which is classically known as Abbe's formula shown below, has directly accelerated the related studies which allowed us to discover the unknown phenomena actually have happened in the molecular world.

$$d = \frac{\lambda}{2nsin\alpha} \tag{1.1}$$

where d indicates the diffraction limit and $nsin\alpha$ corresponds to the numerical aperture (NA) of the imaging system. Since the diffraction is the original property of light, it has been considered that the imaging resolution limit is determined by the wavelength of the light and the number of numerical aperture equipped in the imaging system. However, considering the range of wavelength usually exploited in microscopy, which is the visible light, the structure smaller than several hundreds of nanometers could have not been clearly observed, before the scheme of superresolution microscopy has appeared. Initiated from single molecule imaging (Funatsu et al., 1995), the superresolution microscopy imaging techniques have extensively studied (Yildiz et al., 2003; Huang et al., 2008; Dertinger et al., 2009; Chen et al., 2014; Gustafsson et al., 2016). These studies directly enabled superresolution imaging by dramatically lifting up the spatial resolution beyond the conventional imaging limitations.

Although the recent advances in superresolution imaging technique has shed light on the observation of the single molecules, adopting the superresolution scheme and directly installing the optics to existing system might not always be the practical solution. This is because the limitation of temporal resolution of respective superresolution method might not be suitable to the customized imaging plan, and the superresolution itself does not guarantee the imaging system stability which greatly influences to the quality of image that is directly related to the reliability of the acquired information.

Most importantly, the axial position stability in three-dimensional microscopy imaging system is primarily required (Speidel, Jonáš, and Florin, 2003; Neuman and Nagy, 2008; Hayazawa, Furusawa, and Kawata, 2012), regardless of which types of high spatiotemporal resolution scheme is adopted. The reason why the z-axis stability is crucial is because the imaging system can be influenced by the external

environment, which cannot be completely removed within optical system. Since such surrounding conditions affect the axial position stability the most significantly, considering the direction of gravity, establishing the robust imaging system in terms of axial stability is imperative.

1.5 Vesicle movement analysis methods

Provided that the perfect data sets of vesicle trajectory are acquired with sufficiently high spatiotemporal resolution in absolutely stable imaging system, it is not the end of the story when it comes to whether all the information in such trajectory data is now understandable and all the questions are solved. In fact, even if the precise data point sets of vesicle movement are obtained, there is not much information we can take directly from then, in that the vesicle trajectories are basically acquired as a lump of position data which is seemingly rambling inside somewhere in a cell (Huet et al., 2006; Racine et al., 2007). The direction of movement is frequently changing rather than persisting in one direction, and some area in the trajectory shows quite random motion with a large variance in position while other area shows intermittent pauses with a small variances. This is a natural outcome of the vesicle trajectory data as a result of the interaction between the vesicle and other organelles in cytoplasm, including a complex cytoskeletal network, not caused by any uncontrolled conditions we need to deal with. In fact, as two distinguishable representative movement patterns of a vesicle inside a cell, diffusion and active transports have been considered.

1.5.1 Diffusion of vesicle

One of the basic movement patterns in vesicle trajectory that can appear in a cytoplasmic area is a simple diffusion. In fact, since a vesicle is budded from the plasma membrane as a small sac, the amphiphile property of the surface of vesicle which is originated form the cell membrane plays various roles in the cytoplasmic area (Discher and Eisenberg, 2002). However, provided that the vesicles are not yet anchored to any organelle by motor proteins or other protein, the probability is high that the vesicles simply diffuse into intracellular area according to the physical properties of vesicle and cytoplasm (Luby-Phelps, 1999; Brangwynne et al., 2008). Although it spontaneously occurs, the vesicles can be delivered to some extent in cytoplasm by diffusive motions, the diffusion is also called as passive transport.

In case of this passive transport, the vesicle position at time t is randomly determined with respect to the initial position, considering the vesicle acts as a random walker due to the Brownian motion. For defining the diffusivity, mean squared displacement (MSD), the variance in the position of the vesicle at time t compared to its previously detected position at $t = 0$, is often calculated (Metzler and Klafter, 2000; Howse et al., 2007; Meijering, Dzyubachyk, and Smal, 2012). The equation of MSD can be expressed as following.

$$\mathrm{MSD}(\tau) = \langle \Delta \mathbf{r}(\tau)^2 \rangle = \langle [\mathbf{r}(t+\tau) - \mathbf{r}(t)]^2 \rangle \tag{1.2}$$

where $\mathbf{r}$ indicates the three-dimensional position of the vesicle at time t, and τ refers to the time difference between the position $\mathbf{r}(t)$ and $\mathbf{r}(t+\tau)$ (Crocker and Grier, 1996; Frenkel and Smit, 2001; Savin and Doyle, 2007). Using this equation, the diffusion of vesicle can be easily detected as the MSD curve for diffusive movement is drawn to be approximately linear, in that the probability of being traveled to

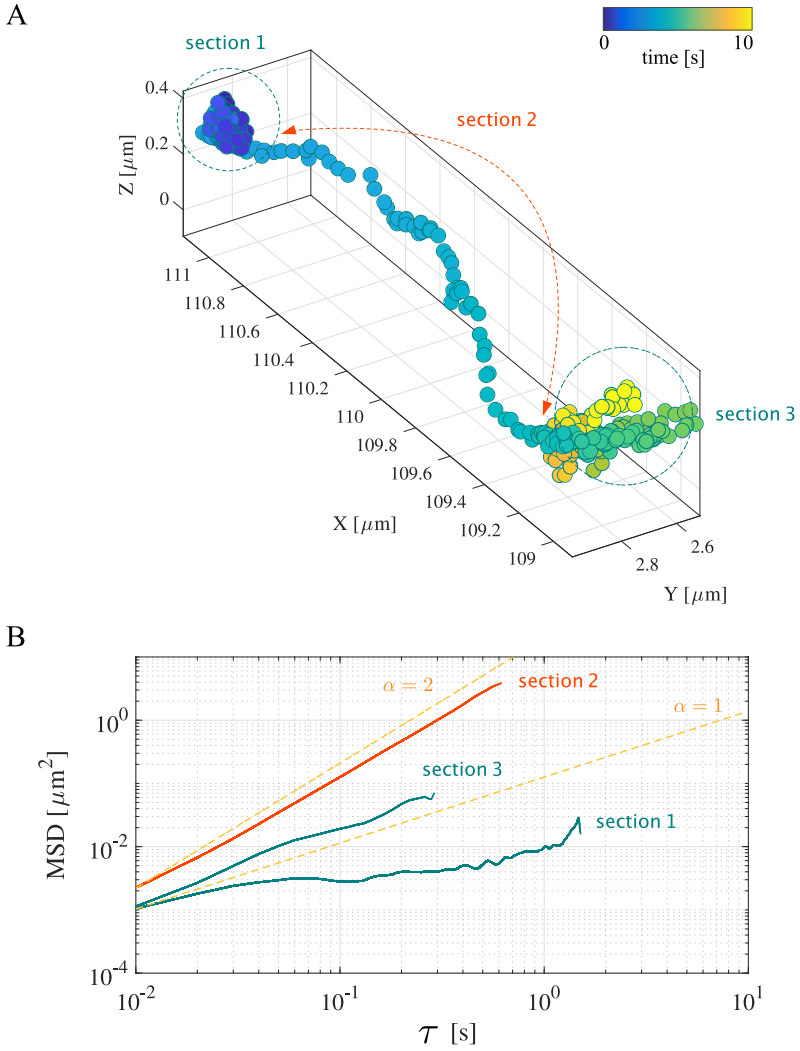

FIGURE 1.6: MSD plot for an example vesicle trajectory. (A) A representative trajectory of vesicle movement acquired in the cytoplasmic area. Three distinguishable domains are manually selected based on the fact whether the movement appeared to be persistently linear or not. While section 2 shows apparently linear, the other section 1 and section 3 are seemingly neither linear nor persistent in the direction of travel. (B) MSD plot for each section recognized in the vesicle trajectory. The number of order $\alpha = 1$ and $\alpha = 2$ are depicted in the orange dashed lines, as the representative cases of active transport and passive transport, respectively. The slope of MSD plot is close to $\alpha = 2$ in case of section 2, while the section 1 and section 3 are close to or smaller than $\alpha = 1$.

certain extent is time-dependent in case of random walk. For such simple diffusion, the diffusion coefficient is determined by the slope in the MSD plot (Chandrasekhar, 1943; Qian, Sheetz, and Elson, 1991). More specifically, for the diffusive movement as a result of Brownian motion, the MSD can be interpreted as following, assuming the medium is isotropic.

$$\mathrm{MSD}(\tau) = 2nD\tau^{\alpha} \tag{1.3}$$

where D denotes the diffusion coefficient, n refers to the number of dimensions, and α is determined by the power of MSD plot over τ (Dupont et al., 2013). The details about the meaning of MSD and the use of α is explained in the later section comparing with the concept of active transport.

1.5.2 Active transport of vesicle

In contrast to free diffusion, active transport in the intracellular area specifically refers to the vesicle movement on the cytoskeleton, such as actin filament and microtubule, mediated by respectively binding motor proteins. Although the free diffusion occurs spontaneously without requiring energy, the active transport requires energy to be occurred. The kinetic energy for the active transport is supplied by the motor protein, mainly as a chemical energy from adenosine triphosphate (ATP) hydrolysis which is involved in the force generation of the motor proteins (Vale, 1987; Howard, 2001). Since the motor proteins such as myosin, kinesin, and dynein transform the chemical energy into mechanical energy, the cargo can be effectively delivered along the respective cytoskeletal filament.

Therefore, the shape of vesicle trajectory when it is actively transported by the motor protein appears highly linear and direct along the cytoskeleton, as revealed in the ATP-addition experiment in vitro (Vale, 1987). In the case of living cell, the concentration of ATP is maintained at a physiological level as functioning crucial roles such as a chemical switch related with cell death (Leist et al., 1997). Using the ATP existing in the cytoplasmic area, motor proteins bound to the vesicle can deliver their cargo along cytoskeleton showing a persistently linear and direct movement, as long as the motor proteins are interacting with both vesicle and cytoskeleton at the same time.

Besides such strong visual evidence–the linear and direct vesicle movement–about the active transport, the higher value of MSD of the active transport compared to the passive transport has played a role as one of the major criteria to determine whether the obtained vesicle trajectory is active or passive transport. Conventionally, the slope acquired from the power-law fits of MSD over the lag time τ is close to the first order when the vesicle experiences passive transport, while the active transport produces the slope close to the second order in the same MSD graph. This number of order is defined as α in Eq. 1.3.

Figure 1.6 (A) shows an example trajectory of a vesicle obtained in cytoplasmic area. Apparently, the trajectory consists of partial diffusions and partial linear and persistent movement, even in a single trajectory. The vesicle did not move much in the direction of travel in section 1 and section 3, while the active transport is recognizable in section 2 as it shows persistently linear movement compared to the initial and final pauses. When these three visually distinguishable sections are divided and the MSD for each section is plotted in log–log graph, as shown in Fig. 1.6 (B), the value α of the section 2 is close to $\alpha = 2$ while others were close to or smaller than $\alpha = 1$. The conventional threshold value of α for the active transport is determined

when $\alpha = 2 \pm \delta_\alpha$, and $\alpha = 1 \pm \delta_\alpha$ refers to the diffusive movement, and $\alpha < 1 - \delta_\alpha$ indicates that the vesicle experiences sub-diffusive motion or confined in some area (Dupont et al., 2013), where δ_α indicates the standard deviation which may vary upon the measurement systems.

Although the MSD efficiently provides the information about the state of vesicle movement, depending on MSD solely to determine the initial position and final position for dividing the sections in vesicle trajectory according the value α involves entire scanning of the position data, which is time-consuming and cost-inefficient. Also, since the visual information is not exploited in MSD calculation, the valuable visual information that can be acquired from the geometry of vesicle trajectory is neglected. Therefore, various modified analysis methods have been suggested to understand the precise movement of vesicle, in more effective ways.

1.5.3 Various analysis methods

First of all, many mathematical approaches start with the idea that is related to precisely determining the partial domain of active transport, because the core information about the vesicle delivery is considered as the specific section where the vesicles are delivered on the cytoskeleton mediated by motor protein. Therefore, as a modification of MSD dependent method, some studies suggest the geometry-assisted approach in determining the local domain for efficient MSD calculation and comparison.

The angle correlation function which is related to the local curvature of vesicle trajectory is one of the representative case in such modified suggestions (Arcizet et al., 2008; Harrison et al., 2013). In this case, since the geometry information in vesicle trajectory is exploited, the methods gain more persuasiveness in that the active transport sections are determined as relatively alined linear domains. The angle correlation function is often customized according to the system characteristics, such as following equation (Arcizet et al., 2008).

$$\Delta\theta_i(\delta(i)) = \langle(\theta(i' + \delta i) - \theta(i'))^2)\rangle^{1/2}_{-(T/2)<i'<(T/2)'} \tag{1.4}$$

where i indicates the time index, and T refers to the size of the scanning window. θ can be exploited in determining the local curvature, for recognizing whether the movement trajectory is linear or not within the local domain. In this case, the active transport section in the vesicle trajectory is determined when the calculation result of both angle correlation function and the α value satisfies the predetermined conditions, linear and direct movement and $\alpha \sim 2$, respectively. Although the angle-correlation assisted MSD evaluation method have provided new insight into the efficient and intuitive analysis method for complex vesicle movement, still questions remained in terms of validity and adaptability to three-dimensional vesicle trajectory including the necessity of MSD.

Another approach to identify the directional persistence in vesicle movement is based on a statistical model, and one of the most representative cases is the method using Hidden Markov Model (HMM) (Das, Cairo, and Coombs, 2009; Röding et al., 2014). Basically, HMM is a statistical prediction about some hidden states with the sequence of observation and the probability between the hidden states (Schuster-Böckler and Bateman, 2007). Since the active transport and passive transport of the vesicle movement can be considered as two hidden states, applying HMM to vesicle trajectory can be reasonable. Although this kind of statistical model is attractive and features good accuracy, the intuitiveness of the method is not much appealed

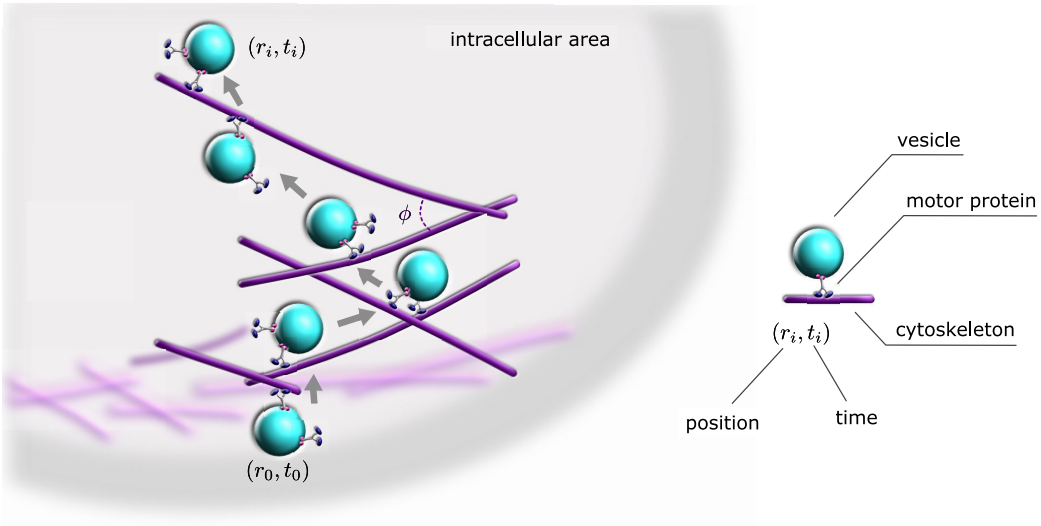

FIGURE 1.7: Concept for the vesicle movement in a complex cytoskeletal network (violet rods). The endocytic vesicle (mint cargo) inevitably interact with multiple cytoskeletons while navigating the intracellular area. Provided that the microscopy imaging system is stable, three-dimensional position of a vesicle detected somewhere in the cytoplasm, which is depicted as r_i at time t_i, can be tracked and analyzed. The numerical analysis method presented in this dissertation opens a door to understanding the precise movement of vesicle on the cytoskeleton after recognizing the linear section from the vesicle trajectory and estimating the accurate position of the cytoskeleton in three dimensions. The information about the cytoskeletal network, such as an angle ϕ between the cytoskeletons, can be also investigated.

because the visual information from the geometry still was not exploited as in the case of MSD calculation alone.

Various analysis methods stated above have been suggested hitherto for understanding the complex vesicle movement. However, there have not yet been a complete set of analysis method, which practically provides the guideline for distinguishing the active transport and passive transport, with robust evidence. Most importantly, the precise vesicle movement in the active transport section has not been clearly explained in terms of the geometry between the vesicle and cytoskeleton, which contains a core biological information about the vesicle delivery occurring in a complicated cytoskeletal architecture in a living cell. Therefore, an intuitive and reliable analysis method for vesicle movement has been highly required.

1.6 Purpose and outline

The major purpose of this dissertation is to provide a complete set of numerical analysis method for understanding the vesicle movement in a complex cytoskeletal network, and to report the three dimensional movement of the vesicle interacting with the interwound cytoskeleton filaments, in terms of angular and translational movement along the axis of the cytoskeleton. The numerical analysis method presented here treats the continuous vesicle trajectory as a discrete data point set that is grouped according to the property of interaction between the vesicle and surrounding, as shown in Fig. 1.7. As a prerequisite condition for securing the high spatial resolution in three dimensions, establishing the axial position stabilization system for the microscopy imaging is also explained as one of the accomplishments.

In this introductory chapter, the importance of understanding the vesicle movement in a living cell was emphasized, and the inherent complexity of vesicle movement trajectory was explained. Also, the two state of vesicle transport – active transport and passive transport – were described to introduce why many studies hitherto have focused on recognizing the active transport, which is the persistent and linear movement along the cytoskeleton.

In chapter 2, the details of imaging microscopy system for acquiring three dimensional vesicle position data is described. Besides the evaluation of x, y coordinates as a center of the point spread function (PSF), the intensity information acquired via the scheme of dual focus optics (Watanabe et al., 2007) is exploited to obtain the z-coordinate. Since the axial stability is crucial for the data reliability, as a newly developed equipment, the axial position stabilization system achieved by feedback control is presented (Lee, Kim, and Higuchi, 2018a).

In chapter 3, the concept and details of novel numerical analysis method for a complex vesicle trajectory is explained (Lee, Kim, and Higuchi, 2018b). Gaussian filter and modified angle correlation function is suggest to calculate and determine the linear domain from the local curvature of the vesicle trajectory, and the validity is tested with simulation. Most importantly, the method for the cytoskeleton position estimation in the recognized linear section is proposed based on Principal Component Analysis (PCA). Also, the vector analysis method is described for analyzing the feature of interaction between the vesicle and cytoskeleton.

In chapter 4, as a result of numerical analysis method application, three dimensional motion of vesicles in a complex microtubule network in living cell condition, in terms of the angular and translational interaction between the vesicle and microtubule. Since the unique rotational motions around the axis of microtubule were discovered in the analysis, the characteristics in rotational movement of vesicle is illustrated. In addition, the causes of the rotational movement are discussed.

Finally, in chapter 5, the overall summary is presented. The diagram for the outline of this dissertation is illustrated in Fig. 1.8.

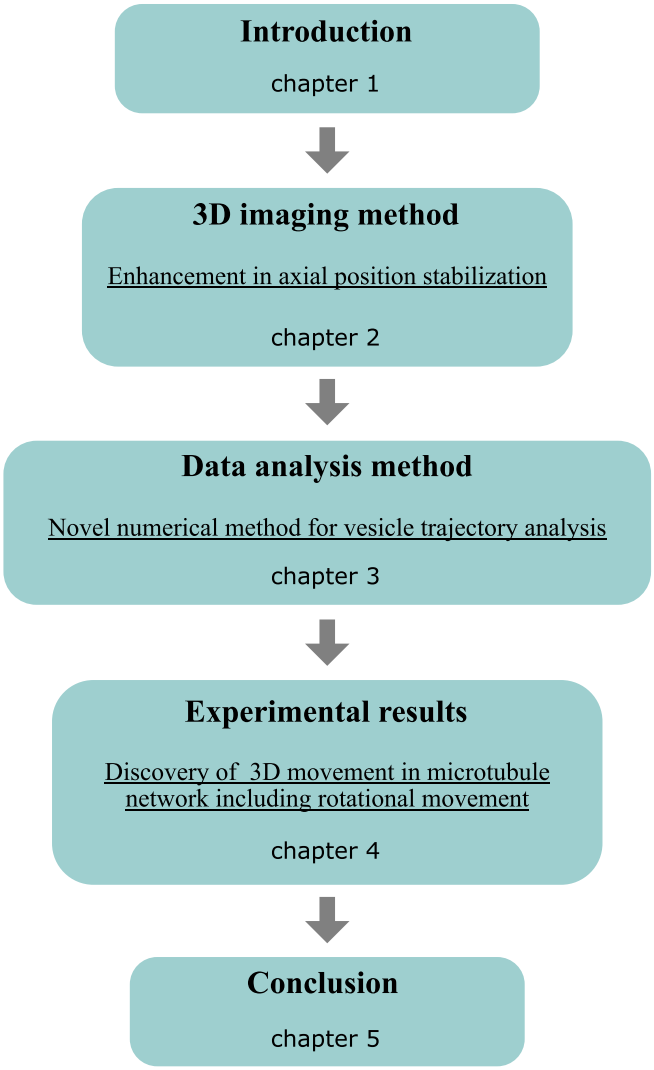

FIGURE 1.8: The outline and flow of this dissertation illustrated in a diagram.

第２章

本章については、５年以内に雑誌等で刊行予定のため、非公開

第３章

本章については、５年以内に雑誌等で刊行予定のため、非公開

第４章

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Chapter 5

Conclusion

This dissertation presented the three-dimensional vesicle movement in a complex cytoskeleton network in a living cell, based on a novel numerical analysis method. Since the movement of endocytic vesicle in the intracellular area contains key information for various biomedical applications which exploit the mechanism of vesicle delivery, understanding the precise movement of vesicle in a living cell condition has been considered as one of the momentous topics in biophysics. Although recent advances in microscopy imaging techniques enabled us to detect more accurate position of the vesicle with high spatiotemporal resolution, extracting biological meaning from the observation was not simple, because there exists few applicable analysis method for the vesicle trajectory. With above background, this study reported the endeavors to understand the detailed feature of vesicle movement in a three-dimensional network of cytoskeletons.

In chapter 1, the background of this study was explained with the introduction of basic molecular mechanisms involved in the vesicle transport. The history of related studies which have mainly focused on the motor proteins were introduced, and the complexity of vesicle movement in living cell condition, where the vesicle inevitably interacts with multiple cytoskeletons during its navigation, was emphasized in comparison with the purified experimental condition. Since our understanding about the vesicle movement is closely related with the accuracy in the vesicle position acquisition via microscopy imaging, the contribution of enhanced imaging techniques including superresolution microscopy to the study of vesicle movement was described. In addition, the importance of axial position stability was underlined as a remaining work for accurate reconstruction of vesicle trajectory in three dimensions. Additionally, the existing analysis methods developed for understand the vesicle movement were introduced with respective strong and weak point, in order to urge the importance of developing appropriate analysis method. The purpose and the outline of the dissertation was proposed at the end of the chapter.

Chapter 2 was dedicated to explain the improvement of three-dimensional microscopy which was exploited in the microscopy imaging experiment to obtain reliable three-dimensional position data acquisition. In particular, the development of axial position stabilization system was explained with the proof of its performance. Basically, the imaging system for live-cell experiments were established following the concept of dual focus optics (Watanabe et al., 2007), which utilizes the intensity information of quantum dot-labeled endocytic vesicle. In order to track the vesicle with high accuracy in dual view, affine transformation-based image mapping scheme was applied with self windowing auto-tracking method. Most importantly, the development and application of the axial position stabilization system to the imaging system was described, which compensate external fluctuation by axial position feedback control (Lee, Kim, and Higuchi, 2018a).

In chapter 3, a novel numerical analysis method which was devised to detect and analyze the detailed movement of vesicle from its complex trajectory data was presented (Lee, Kim, and Higuchi, 2018b), as a core of this dissertation. Since the vesicle trajectory appears complicated as a result of irregular interactions between the vesicle and the interwound cytoskeleton network, appropriate analysis method has been highly required. The proposed analysis method, which was developed for meeting the need, was introduced as a series processes from linear section finding algorithm to the estimation on the cytoskeleton location. Because the active transport section where the vesicle is transported by motor proteins on the cytoskeleton appears linear in intracellular area due to the nature of linearity of cytoskeletons, the proposed analysis method started with finding the linear sections in the vesicle trajectory, after removing noise with Gaussian filter. The liner sections were screened by the curvature of trajectory using the threshold angle to define the local linearity. Principal component analysis was applied to detect the direction of travel for the local linear domain, and the location of cytoskeleton for the linear section was estimated using the eigenvector which shows the largest eigenvalue. Additionally, based on simple vector calculation, the detection method for angular and translational movement of vesicle on the estimated cytoskeleton was also described.

Chapter 4 reported the actual three-dimensional movements of vesicles in a complex microtubule network (Lee and Higuchi, 2018: to be submitted), which were imaged with axial position stabilization system explained in chapter 2 and analyzed by the numerical analysis method introduced in chapter 3. The vesicle trajectories provided the information about the angles between the microtubules in their network structure, and the time taken for the vesicles at their transfer between the adjacent microtubules. The transfer of vesicle could be divided into abrupt direction change and smooth transfer, because the transfer angles were turned out to be either very acute (10–60°) or obtuse (100-180°). However, the distributions of time taken for the transfer showed similar time scale, which was approximately 0.5 s. Particularly, the vesicle showed rotational movement on the estimated location of microtubules, in both left-handed and right-handed direction. Since the rotational movement of vesicle showed with high probability (> 53 %) for the long-range transport and there existed incomplete rotations such as turning-back motions, it is highly probable that the rotational motion of vesicle were induced by obstacles they encountered on their path.

Consequently, this dissertation provides the initiative analysis method and its result of the three-dimensional movement of vesicle during its navigation in a complex cytoskeletal network. For establishing a reliable imaging condition, which is the prerequisite of the accurate observation, axial position feedback control system was developed (Lee, Kim, and Higuchi, 2018a). As a vesicle movement analysis method, a novel numerical algorithm was devised based on principal component analysis and vector calculation, which plays a significant role in detection of precise vesicle movement in terms of the interaction between the cytoskeletons (Lee, Kim, and Higuchi, 2018b). The actual vesicle movement trajectory was analyzed by the numerical method, and it was possible to detect the pattern of vesicle movement in a complex cytoskeletal network in terms of the transfer angle and time scale. In particular, unique rotational motion of vesicle around the microtubule axis was discovered, which suggest the reaction of vesicle when it encountered an obstacle during the transport (Lee and Higuchi, 2018: to be submitted). As an initial result of vesicle movement analysis including the detection of detailed motions, this study is expected to open a door for understanding the precise movements of vesicle in a living cell.

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