Single-molecule in vitro reconstitution assay for kinesin-1-driven membrane dynamics

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Abstract

Intracellular membrane dynamics, especially the nano-tube formation, plays important roles in vesicle transportation and organelle biogenesis. Regarding the regulation mechanisms, it is well known that during the nano-tube formation, motor proteins act as the driven force moving along the cytoskeleton, lipid composition and its associated proteins serve as the linkers and key mediators, and the vesicle sizes play as one of the important regulators. In this review, we summarized the in vitro reconstitution assay method, which has been applied to reconstitute the nano-tube dynamics during autophagic lysosomal regeneration (ALR) and the morphology dynamics during mitochondria network formation (MNF) in a mimic and pure in vitro system. Combined with the single-molecule microscopy, the advantage of the in vitro reconstitution system is to study the key questions at a single-molecule or single-vesicle level with precisely tuned parameters and conditions, such as the motor mutation, ion concentration, lipid component, ATP/GTP concentration, and even in vitro protein knockout, which cannot easily be achieved by in vivo or intracellular studies.

Keywords Single-molecule microscopy · In vitro reconstitution assay · Autophagic lysosome reformation (ALR) · Mitochondrial network formation (MNF)

Introduction

Biological membrane dynamics, deformation, and reformation are mainly based on nano-tubular structures observed within the cell and between organelles. Most of the organelles with tubular structure rely on cytoskeleton-based movement, not only to generate but also to maintain the structure and position in the cytoplasm, such as the endoplasmic reticulum

(ER), Golgi complex, mitochondria, endosomes, lysosomes, and their interactions (Valm et al. 2017). The formation and dynamics of nano-tubes are increasingly recognized to play important roles in multiple biological progresses, such as late stage of autophagy (Yu et al. 2010), endosomal delivery in polarized T cells (Choudhuri et al. 2014), vesicle transportation between ER and Golgi (Kee et al. 2017), mitochondria network formation (Wang et al. 2015), and post-Golgi vesicles towards the plasma membrane (Ruan et al. 2016).

The dynamics of the vesicles and their nano-tubules is driven by the microtubule-dependent motors kinesin, which moves towards the plus (+) ends of microtubule cytoskeleton, and dynein, which moves towards the minus (−) ends (Vincent et al. 2017). Kinesins (KIFs) are microtubule-based motor proteins that move along microtubule cytoskeleton (Vale et al. 1985). KIFs were first discovered in giant squid axons, and more than 14 kinesin families have been identified and studied so far (Vale 2003). KIFs are required for many vital intracellular functions, such as mitosis, meiosis, and transport of a diverse array of cellular cargoes such as protein complexes, vesicles, and organelles (Lucanus and Yip 2018). In this review, we focus on the role of kinesin-1 (KIF5B) in mediating nano-tubular organelles. KIF5B, a member of the

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kinesin-1 family, is ubiquitously expressed and has an NH₂-terminal motor domain and a C-terminal cargo-binding domain (Du et al. 2016). KIF5B proteins play essential roles in axonal transport (Pilling et al. 2006). Lysosomes and mitochondria are also transported by KIF5B proteins. KIF proteins bind to their cargo through their cargo-binding domains and adaptor proteins. Various KIF proteins have been shown to directly bind to phospholipids, and this KIF-phospholipid binding is required for KIF-mediated cargo transportation (Du et al. 2016).

In order to understand the interactions of motor proteins with membranes in general, it is advantageous to be able to reconstruct the system faithfully outside the living cell with pure components and controllable parameters. The previous study showed that the formation of ER-like membrane networks can be reconstituted *in vitro* and, as is thought to be the case *in vivo*, is a two-step process involving membrane tubule extension along microtubules, followed by membrane fusion. Ron Vale et al. observed the process of network formation from two rat liver membrane fractions by an *in vitro* reconstituted system (Allan and Vale 1994; Vale and Hotani 1988). Therefore, the *in vitro* reconstitution system is a good tool to study the membrane dynamics. Using liposome as a membrane source, motor protein drives vesicle transport and deformation in the presence of ATP after binding with liposome. Roux et al. (2002) showed that lipid giant unilamellar vesicles, to which kinesin molecules have been attached by means of small polystyrene beads, give rise to membrane tubes and to complex tubular networks when incubated *in vitro* with microtubules and ATP. It is possible that organelle movement and tubular formation can be driven by motor proteins *in vivo*. Klopfenstein et al. showed that Unc104 can transport native vesicles *in vitro* after docking onto membrane cargo through a lipid binding pleckstrin homology (PH) domain (Klopfenstein et al. 2002). It is indicated that the *in vitro* reconstitution system is a good platform to study the molecular mechanism of motor protein. In this review, we will summarize the application of *in vitro* reconstitution system for KIF5B-driven membrane dynamics, especially for autophagic lysosomal regeneration (ALR) and mitochondrial network formation (MNF).

Motor-driven intracellular membrane dynamics

Powered by the hydrolysis of adenosine triphosphate (ATP), the motor domain of KIF proteins moves along microtubule filaments using the force generated (Vale et al. 1985). Through direct interactions between the cargo domain and the lipid composition, KIF proteins can also drive membrane tubulation by pulling the membrane (Dabora and Sheetz 1988; Vale and Hotani 1988). KIF5B has been shown to play important roles in maintaining lysosome homeostasis during autophagy (Du et al. 2016; Rong et al. 2012) and mediating

mitochondrial distribution during cell cycles (Tanaka et al. 1998; Wang et al. 2015). In KIF5B-knockout cells, the generation of reformation tubules from autolysosome is abolished, and the mitochondrial distribution is disrupted.

Autophagic lysosomal reformation

Autophagy, a lysosome-dependent degradation process, is essential for the maintenance of cellular homeostasis (Klionsky et al. 2016). It is an important mechanism for adaptation to stress and intracellular quality control. In the past decade, autophagy research has largely focused on the early stages of the process, namely omegasome formation, isolation membrane elongation, and autophagosome formation (Mi et al. 2015). However, most lysosomes fuse with autophagosomes to form autolysosomes during autophagy. So at the peak of autophagy, the number of free lysosomes will be consumed due to the formation of autolysosomes. Thus, a mechanism is required to restore free lysosomes once autophagy is finished (Chen et al. 2019).

In the past decade, we have discovered and studied the terminal step of autophagy, termed the autophagic lysosome reformation (ALR), which restores the level of free lysosomes to maintain lysosome homeostasis (Chen et al. 2018b; Yu et al. 2010). During ALR, tubular structures composed from lysosomal membrane components are extruded from autolysosomes. Small vesicles, also made of lysosomal membrane components, then bud from these tubular structures. These vesicles, named proto-lysosomes, are initially pH-neutral and do not contain lysosomal luminal proteins. Eventually, they mature into nascent lysosomes by gaining acidity and lysosomal luminal proteins (Yu et al. 2010).

The most noticeable step of ALR is the generation of reformation tubules. Previous studies have identified the key players in ALR at a molecular level through proteomics combined with large-scale RNAi screening (Rong et al. 2012). We found that the kinesin motor protein KIF5B is required for autolysosome tubulation and that KIF5B drives autolysosome tubulation by directly pulling on the autolysosomal membrane (Du et al. 2016).

Mitochondrial network formation

Mitochondria are multifunctional organelles in mammalian cells that play critical roles in multiple intracellular processes, including energy production, metabolism, apoptosis, and senescence (Chan 2012). Mitochondria form a network with complex and highly dynamic structure and morphology. During interphase, the mitochondria are arranged in elongated tubules, while during mitosis, the network is fragmented (Horbay and Bilyy 2016). Formation of the mitochondrial network plays a vital role in maintaining mitochondrial functions, while disruption of the mitochondrial network affects

mitochondrial DNA integrity, interchange of mitochondrial material, respiratory capacity, apoptosis, and response to cellular stress, leading to the abnormal development and several human diseases, such as neurodegenerative disease (Chan 2012; Chen and Chan 2009; McArthur et al. 2018).

The highly dynamic morphology of the mitochondrial network relies on a balance of four integrated processes: (i) motility, the active movement of mitochondrial membrane along the cytoskeleton driven by motor protein (Wang et al. 2015); (ii) nano-tubes or nano-tunnels formation, the dynamics of transient or stable tubular structures of mitochondria membrane (Vincent et al. 2017); (iii) fission, the ability of one mitochondrion to divide into two or more (Kanfer and Kornmann 2016); and (iv) fusion, the merging of two separate mitochondrial membrane to form only one (Chan 2012).

KIF5B and kinesin-3 (KIF1B) have been reported to mediate mitochondrial distribution during directional axonal and dendritic transport in neurons (Hirokawa and Takemura 2005). Inhibition of dynein affects the mitochondrial distribution and bidirectional movement in fast axonal transport (Waterman-Storer et al. 1997). It is also known that the inhibition of kinesin-1 and dynein in *Drosophila* affects the fast transport of mitochondrial movement in motor axons (Pilling et al. 2006). Dynamic mitochondrial tubulation and network formation were recently reported to be important for maintaining mitochondrial DNA integrity and interchanging mitochondrial material (Wang et al. 2015).

Single-molecule in vitro reconstitution assay

To pinpoint the exact molecules and understand the physical principles involved in the ALR and mitochondrial network formation (MNF) processes, the in vitro reconstitution system with pure components and controllable conditions is the best choice. On a microtubule filament-coated coverglass chamber, the motor protein KIF5B can drive the tubulation process by directly pulling the membrane from purified autolysosomes, mitochondria, or man-made liposomes with specific lipid. Nano-tubule protrusion and elongation can be reconstituted in vitro. Based on the in vitro reconstitution system, many more adjustments can be fine-tuned to understand the molecular mechanisms underlying ALR and MNF, such as protein mutation, small molecule concentration, lipid modification, and even in vitro protein knockout.

Single-molecule microscopy for motor protein

As one of the most widely used single-molecule microscopies, the total internal reflection fluorescence (TIRF) microscopy has been utilized to directly observe dynamics and kinetics of individual molecules. It is easy to obtain higher signal-to-noise ratio (SNR) and better localization accuracy by using TIRF than conventional epi-illumination. Using the TIRF

illumination, the fluorescent probes within the evanescent field, which is only 100–200 nm above the coverslip surface, can be selectively excited with higher SNR, lower photo-toxicity, and shorter exposure time than the conventional fluorescence microscopies (Sahl et al. 2017). In the biophysics and biophotonics field, TIRF serves as an effective imaging method for tracking molecular motors, such as myosin, kinesin, and dynein, while they walk along the cytoskeleton (Sun et al. 2007; Yildiz et al. 2003), and probing protein-protein interactions in cell plasma (Liu et al. 2014) with < 1.5-nm localization precision at video rates. In the past 5 years, we have developed multiple single-molecule in vitro assays with TIRF microscopy to characterize motor protein conformations and interactions with cytoskeleton filaments under various biochemical conditions (Guan et al. 2017; Shen et al. 2016; Su et al. 2016).

Here, we focus on ALR and MNF, which are membrane deformation processes mediated by the motor protein KIF5B (Chen et al. 2018b). Since the pulling force of KIF5B is critical during these processes, proteins with good motor activity and a high survival ratio are essential. The Bac-to-Bac expression system in Sf9 cells is optimal for the purification of full-length KIF5B (Chen et al. 2018b). The activity of the motor protein KIF5B should be characterized right after protein purification, and the purified protein has to be stored properly in aliquots to avoid freeze-thaw cycles. What's more, all other components should be freshly made or stored within acceptable times.

As shown in Fig. 1, gliding assay (Fig. 1a–c) and motility assay (Fig. 1d–f) are recommended to verify the motor activity of KIF5B, which will ensure a successful in vitro reconstitution of membrane tubulation. In the gliding assay, full-length KIF5B proteins are immobilized on the cleaned coverslip surface by incubation and in vitro polymerized microtubule filaments with fluorescent labeling are force-sheared to short filaments by multiple pipetting. The gliding motion of microtubule filaments is observed by adding an ATP working solution, with an ATP regeneration system and an oxygen scavenger system, into the system under TIRF illumination. The gliding trajectory and distance are quantified by “Z-stack max” for a certain period in ImageJ (Fig. 1c). In the motility assay, microtubule filaments were immobilized on the coverslip surface through anti-tubulin antibody, a truncated motor domain of KIF5B; K560 was biotinylated and conjugated with streptavidin-QDots-525. The motility trajectory, speed, and distance of K560 motion are observed and quantified with a similar method as for gliding assay (Fig. 1f).

In vitro reconstitution assay for ALR and MNF

When it comes to pinpointing the molecular mechanisms and verifying the role of KIF5B during intracellular membrane dynamics, we have successfully reconstituted an in vitro

Fig. 1 Single-molecule microscopy for motor protein KIF5B. Schematic diagram (a), snapshot (b), and z-stack of max intensity indicated trajectories (c) of in vitro kif5b motor-driven gliding assay, in which the short microtubule filaments (red, labeled with HyLight647) glide on the motor protein coated coverslip surface in the presence of 20 μ M ATP. Schematic diagram (d), snapshot (e), and z-stack of max intensity

indicated trajectories (f) of in vitro motor motility assay, in which the biotinylated kif5b motor was conjugated with QDots-525 (green) and move along the immobilized microtubule filaments (red, labeled with HyLight647) on antibody (purple objects in d) coated coverslip surface in the presence of 20 μ M ATP

system to mimic the physiological processes of ALR and MNF. Using artificial liposomes, purified autolysosomes, or mitochondria as the membrane source, in vitro polymerized microtubules with fluorescent labeling, together with the purified motor protein KIF5B to provide the driving force in the presence of ATP, it is able to reconstitute the membrane tubulation during ALR and MNF in vitro with single-vesicle or single-molecule sensitivity.

In the in vitro reconstitution system shown in Fig. 2, similarly as the motility assay, firstly, the truncated KIF5B is incubated with artificial liposome containing biotin-PE, the full-length KIF5B is incubated with artificial liposomes containing PtdIns(4,5) P_2 , purified autolysosomes, or mitochondria. Then, the mixture is loaded into a microtubule-coated coverglass chamber and the tubulation process is triggered by adding ATP working solution into the system, with an ATP regeneration system and an oxygen scavenger system (Chen et al. 2018b). We found that in the presence of ATP, 10–30% of biotin-liposomes (Fig. 2a), 10–30% PtdIns(4,5) P_2 liposomes (Fig. 2b), ~30% of autolysosomes (Fig. 2c), and ~100% of mitochondria (Fig. 2d) can form tubules and/or networks in vitro.

Our study reveals a novel method to study mechanisms of autolysosome tubulation and mitochondria network formation

by in vitro reconstitution system. For each of these four assays in Fig. 2, we found that (i) the tubulation frequency increases in a motor domain concentration-dependent and a vesicle size-dependent manner (Su et al. 2016); (ii) the efficiency of tubulation increases in a PtdIns(4,5) P_2 concentration-dependent manner and the KIF5B directly interacts with PtdIns(4,5) P_2 proved by a sedimentation assay (Du et al. 2016); (iii) the KIF5B motors are recruited and clustered on autolysosomes via interaction with PtdIns(4,5) P_2 -enriched microdomains in a clathrin/AP2-dependent manner (Du et al. 2016); and (iv) the mitochondrial networks form in a GTP-enhanced manner and rely on mitofusins Mfn1 and Mfn2 mediated fusion of mitochondrial membrane. What's more, the mitochondrial network in different cytoplasm regions is formed by different mechanisms (Wang et al. 2015).

It is worth to note that, with this in vitro reconstitution assay, we are also able to perform in vitro knockout for some membrane proteins, which may be difficult to perform in vivo. As an example for ALR, clathrin proteins can be stripped off from autolysosomal membrane by treating the isolated autolysosomes with trypsin. Re-incubating these stripped autolysosomes with purified rat brain cytosol with an ATP regeneration system results in the recruitment of clathrin to the autolysosomes and reconstitutes the buds structures on

Fig. 2 In vitro reconstitution assays for biotin-liposomes, PtdIns(4,5)P₂-containing liposomes, autolysosomes and mitochondria. **(a)** In vitro reconstituted liposomal tubulation process driven by 80 nM truncated and biotinylated KIF5B (K560, motor domain only) for liposomes containing 0% PtdIns(4,5)P₂, 10% rhodamine-PE and 10% biotin-PE (cross-linked through biotin-streptavidin-biotin interactions) in the presence of 20 μ M ATP. **(b)** In vitro reconstituted liposomal tubulation

process driven by 80 nM full-length KIF5B for liposomes containing 25% PtdIns(4,5)P₂ and 10% rhodamine-PE in the presence of 20 μ M ATP. **(c)** In vitro reconstituted autolysosomal tubulation process driven by 80 nM full-length KIF5B for CMD1l-labeled autolysosomes in the presence of 20 μ M ATP. **(d)** In vitro reconstituted mitochondrial tubulation and network formation driven by 80 nM full-length KIF5B for CMD1l-labeled mitochondria in the presence of 20 μ M ATP

the membrane. While adding clathrin antibody during the incubation efficiently blocked the formation of buds, which is proved by both transmission electron microscopy (TEM) (Rong et al. 2012) and the in vitro tubulation frequency assay (Du et al. 2016). The buds have been further proved as PtdIns(4,5)P₂-enriched microdomains, which serve as the linker with the cargo domain on KIF5B.

Conclusion and Discussion

The recent development of single-molecule and super-resolution imaging techniques, which were awarded the Nobel Prize in 2014, drives our understanding of the hidden mechanisms of intracellular processes with spatial resolution down to tens of nanometers resolution and dynamic information in the milliseconds sensitivity (Su and Ju 2018).

Intracellular membrane nano-tube formation and its dynamics play important roles in cargo transportation and organelle biogenesis, which are mainly driven by motor proteins. The formation and dynamics of nano-tubes are increasingly

recognized to play important roles in a multitude of biological progresses. We recently demonstrated the role of nano-tube dynamics in autophagic lysosome reformation (ALR) during autophagy and mitochondrial network remodelling with single-molecule in vitro reconstitution assay and super-resolution fluorescent microscopy.

Using purified kinesin-1 (KIF5B) motor protein, taxol-stabilized and fluorescently labeled microtubule filaments, and liposome or purified organelles, such as autolysosomes and mitochondria, we were able to set up an in vitro system in which autolysosome tubulation and mitochondrial networks are mediated by motor protein, lipid composition, membrane proteins, and also the physical size of the vesicles. The advantage of the in vitro reconstitution system is to study the key questions with single-molecule or single-vesicle sensitivity with precisely tuned parameters and conditions, such as the motor isoform, Ca²⁺ concentration, lipid composition and modification, ATP/GTP concentration, and even in vitro protein knockout, which cannot easily be achieved by in vivo or intracellular studies.

During the late stage of autophagy, lysosomes fuse with autophagosomes to create autolysosomes. Lysosomes are later regenerated via a unique process termed the autolysosome tubulation. We show that the KIF5B, through direct interactions with PtdIns(4,5)P₂ micro-domain, drives the tubulation by pulling on the autolysosome membrane, revealing a motor-based membrane deformation process that helps maintain the balance of cellular lysosome.

The formation and maintenance of mitochondrial networks play an important role in maintaining mitochondrial DNA integrity and interchanging mitochondrial material. Disruption of the mitochondrial distribution and morphology affects mitochondrial functions. We reported a new molecular mechanism for the establishment of mitochondrial membrane networks through KIF5B-driven dynamic tubulation. In vitro reconstitution assay demonstrated that KIF5B pulls thin, highly dynamic tubules out of the mitochondrial membrane. Fusion of these dynamic membrane tubes, which is mediated by mitofusins, Mfn1 and Mfn2, helps to establish and maintain the mitochondrial network remodelling.

In the in vitro reconstitution process, either purified autolysosomes, mitochondria, or man-made liposomes can be used as the membrane source. The isolated organelles are more physiologically relevant, and the artificial liposomes make it possible to precisely tune the composition of each lipid type, like the phosphorylation, to study the function of different lipids in ALR and MNF. Also, liposomes provide a more homogeneous system for measuring different parameters involved during the membrane dynamics. The morphology of the purified autolysosomes, mitochondria, and man-made liposomes can be checked by negative staining and TEM. Otherwise, the procedures for cleaning the coverslips and assembling the flow chamber for single-molecule detection are very important in in vitro system. Since the detection method is single-molecule fluorescence level, all the procedures should be carried out in a mega-level ultra-clean hood to avoid contaminations of auto-fluorescent particles. All reagents should be of molecular grade, and all solutions must be filtered through a 2-μm syringe filter to remove impurities which could influence the single-molecule fluorescent signal.

Collectively, the in vitro reconstitution assay demonstrated that kinesins are able to drive membrane tubule formation and KIF5B plays important roles in forming membrane tubules and membrane networks. The in vitro system described in this review is less sophisticated than the in vivo physiological conditions; it can be used to characterize the essential biological components, biophysical parameters, and molecular mechanisms underlying ALR and MNF. It has a huge potential application for the other intracellular membrane dynamics, such as ER-mitochondria interactions, endosomal transportation, and Golgi-derived vesicles. However, it cannot precisely mimic the cytoplasm condition of cells or the viscosity of the microenvironment in which membrane deformation occurs.

Also, the in vitro system can only reveal the roles of a few essential proteins and regulatory proteins. New imaging modality and novel fluorescent probes are also needed for long-term imaging and tracking with high spatio-temporal resolution (Chen et al. 2018a). Ultimately, the combination of in vivo physiological and in vitro pure assays will preferably support the study of KIF5B in autolysosome tubulation and mitochondria network dynamics.

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Author contribution W.D. and Q.P.S. contributed equally, prepared figures, and wrote and edited the manuscript together.

Compliance with ethical standards

Conflict of interest Wanqing Du declares that he has no conflict of interest. Qian Peter Su declares that he has no conflict of interest.

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