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Detergent-Assisted Reconstitution of Recombinant Drosophila Atlastin into Liposomes for Lipid-Mixing Assays

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February 20, 2019

Stephanie R. Weldon, PhD
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Dear Dr. Weldon,

Please find attached our manuscript entitled “Detergent-Assisted Reconstitution of Recombinant Drosophila Atlastin into Liposomes for Lipid-Mixing Assays”, which we submit for consideration for publication in the Journal of Visualized Experiments. Atlastins are conserved endoplasmic reticulum GTPases that mediate homotypic fusion. In this article we describe in detail a protocol for purifying, reconstituting, and measuring fusion activity of recombinant Drosophila atlastin. We also describe quality control measures for reconstituted proteoliposomes, including, analysis of the protein orientation by protease cleaving, and quantifying reconstitution efficiency by floatation assays. This protocol can be extended to other membrane and fusion proteins making it an accessible system for studying protein-lipid interactions and fusion.

We report no competing interests as described in the instructions to authors.

Thank you for your consideration.

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TITLE:

Detergent-Assisted Reconstitution of Recombinant Drosophila Atlastin into Liposomes for Lipid-Mixing Assays

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KEYWORDS:

membrane fusion, reconstitution, fluorescent resonance transfer (FRET), lipid bilayer, phospholipid vesicle, detergent removal, transmembrane domain, protein purification, lipid mixing assay, atlastin, recombinant protein

SUMMARY:

Biological membrane fusion is catalyzed by specialized fusion proteins. Measuring the fusogenic properties of proteins can be achieved by lipid mixing assays. We present a method for purifying recombinant Drosophila atlastin, a protein that mediates homotypic fusion of the ER, reconstituting it to preformed liposomes, and testing for fusion capacity.

ABSTRACT:

Membrane fusion is a crucial process in the eukaryotic cell. Specialized proteins are necessary to catalyze fusion. Atlastins are endoplasmic reticulum (ER) resident proteins implicated in homotypic fusion of the ER. We detail here a method for purifying a glutathione S-transferase (GST) and poly-histidine tagged Drosophila atlastin by two rounds of affinity chromatography. Studying fusion reactions in vitro requires purified fusion proteins to be inserted into a lipid bilayer. Liposomes are ideal model membranes, as lipid composition and size may be adjusted. To this end, we describe a reconstitution method by detergent removal for Drosophila atlastin into preformed liposomes. While several reconstitution methods are available, reconstitution by detergent removal has several advantages that make it suitable for atlastins and other similar proteins. The advantage of this method includes a high reconstitution yield and correct orientation of the reconstituted protein. This method can be extended to other membrane proteins and for other applications that require proteoliposomes. Additionally, we describe a FRET based lipid mixing assay of proteoliposomes used as a measurement of membrane fusion.

INTRODUCTION:

Membrane fusion is a critical process in many biological reactions. Under biological conditions, membrane fusion is not spontaneous and requires specialized fusion proteins to catalyze such

reactions[1]. ER homotypic membrane fusion is mediated in animals by the dynamin related GTPase atlastin[2]. Atlastin's role in homotypic fusion is fundamental for three-way junctions in peripheral ER, which constitutes a large interconnected network of tubules that extend throughout the cell. Atlastins have a conserved domain morphology consisting of a large GTPase, a three helix bundle middle domain, a hydrophobic membrane anchor, and a short cytoplasmic C-terminal tail[3]. In vitro studies with recombinant Drosophila atlastin have shown that when reconstituted to liposomes, it maintains its fusogenic properties. Other atlastins, including human homologs have not been able to recapitulate fusion in vitro. We describe here a methodology for purifying a GST and poly-histidine tagged recombinant Drosophila atlastin, reconstituting it to liposomes, and assaying fusion.

Studying membrane fusion in vitro presents a challenge as fusogenic proteins usually have a membrane anchor. In order to study them, it is necessary to reconstitute them into model lipid bilayers. Large unilamellar vesicles (LUV) are a useful tool to study lipid protein interactions. We present here a system to make LUVs of different lipid compositions for protein reconstitution and fusion assays. Reconstitution of integral proteins into LUVs can be achieved by a variety of methods including, organic solvent-mediated reconstitution, mechanical mechanisms, or detergent assisted reconstitution[4]. We present here a method for reconstituting Drosophila atlastin into preformed liposomes by detergent removal. Advantages of this reconstitution method include high reconstitution yields and proper orientation of atlastin in the lipid bilayer. Additionally, through this method, the protein is not dried or exposed to organic solvents thereby maintaining structure and function. Among its disadvantages, the presence of detergents may not be ideal for all proteins and the final proteoliposomes may have some incorporated detergent in the lipid bilayer. Further dialysis may be used to eliminate more of the detergent. However, dialysis may take a long time and can therefore lead to loss of protein activity.

Assessing atlastin's fusion activity can be determined by lipid mixing assays as previously described[2]. Here, we delineate a method for measuring atlastin mediated fusion through N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)/Lissamine rhodamine-B sulfonyl (rhodamine) labeled lipids. This assay requires fusion of donor (labeled) proteoliposomes and acceptor (unlabeled) proteoliposomes. A FRET release can be measured during the reaction as the dilution of a donor–acceptor pair from "labeled" liposomes to "unlabeled" liposomes as a result of lipid mixing during membrane fusion (**Figure 1**)[5]. While this assay serves as a proxy for membrane fusion, it is limited in distinguishing between membrane fusion and hemifusion, a state where only the outer leaflets mix. To address this issue, an alternative is outer leaflet quenching of NBD by dithionite. Following the same methodology as NBD/rhodamine lipid mixing assays, upon quenching the outer leaflet any NBD FRET release by fusion will be due to inner leaflet mixing[8].

Alternative fusion assays by inner aqueous content mixing address full fusion only[5]. Examples of this are terbium (Tb)/dipicolinic acid (DPA) assays and aminonaphthalene trisulfonic acid (ANTS)/p-xylene bis(pyridinium) bromide (DPX) assays. In Tb/DPA assays, a pool of liposomes with encapsulated Tb are mixed and fused with liposomes with encapsulated DPA; upon fusion, fluorescence is increased via internal energy transfer from DPA to Tb within the $[Tb(DPA)_3]^{3-}$ chelation complex[6]. In contrast, for ANTS/DPX assays, ANTS fluorescence is quenched by DPX[7].

While these systems address inner content mixing, more in-depth preparation of liposomes is required for removal of non-encapsulated reagents, as well as unintended interaction of the fluorophores.

PROTOCOL:

1. Purification of GST-DAtl-His8

1.1. Protein expression and lysate preparation

1.1.1. Transform BL21 (DE3) *E. coli* with the GST-DAtl-His8 construct in pGEX4-T3[2] and select on an ampicillin plate.

1.1.2. Select a single transformant and inoculate 5 mL of LB + ampicillin (5 µL of 100 mg/mL ampicillin) in a 14 mL culture tube and incubate at 25 °C with shaking at 200 rpm for 6-8 h.

NOTE: Due to leaky expression, it is not recommended to incubate at higher temperatures. Growth at 25 °C reduces aggregation of protein during this growth period.

1.1.3. Inoculate 200 mL of LB + ampicillin with 1 mL of the 5 mL culture and incubate overnight (~15-18 h) at 25 °C.

1.1.4. The next morning, harvest the cells by centrifugation (2000 x *g* for 10 min) and resuspend in 5 mL of LB.

1.1.5. Inoculate 4 L of LB + ampicillin and measure OD_{600} (0.05 – 0.15). Incubate at 25 °C with shaking.

NOTE: Reserve some media to serve as a blank for the OD_{600} measurements before adding bacteria.

1.1.6. Measure OD_{600} every hour until it reaches an OD between 0.4 - 0.5. At this point, reduce the temperature to 16 °C.

1.1.7. Induce with 0.2 mM IPTG (800 µL of 1 M stock), 10 min after the incubator reaches 16 °C. Incubate overnight (~15-18 h) at 16 °C.

NOTE: The lower temperature improves the yield of functional protein by reducing aggregation of atlastin.

1.1.8. The next morning, harvest the cells by centrifuging at 7500 x *g* at 4 °C.

1.1.9. Resuspend the cells in 200 mL of A200 (25 mM HEPES (pH 7.4) and 200 mM KCl).

1.1.10. Centrifuge the cells at 11,000 x *g* for 5 min at 4 °C.

1.1.11. Resuspend the pellet in 40 mL of breaking buffer (A200 plus 10% glycerol, 2 mM 2-mercaptoethanol, 4% Triton X-100 (TX100), 40mM imidazole, and one EDTA-free protease inhibitor cocktail tablet).

NOTE: Add TX-100 after resuspending to avoid generating bubbles and foam.

1.1.12. Pass through an 18 G needle and run the cells through a cell disrupter three times at ~10,000 psi.

1.1.13. Centrifuge the extract at 125,000 x *g* for 1 h.

NOTE: Optionally dissolve the pellet 1:2 (w:v) in 8 M Urea and nutate at room temperature overnight. Urea as a denaturant will dissolve slowly any insoluble pelleted protein for SDS-PAGE analysis. Save 1 µL for SDS-PAGE and Coomassie stain analysis.

1.1.14. Filter the extract through a 0.45 µm cellulose nitrate sterile membrane filter to remove bacteria and large bacterial debris.

NOTE: Optionally, save 1 µL for SDS PAGE and Coomassie stain analysis.

1.2. **Protein purification by affinity chromatography**

1.2.1. Load the filtered lysate onto an immobilized metal affinity chromatography (IMAC) resin column charged with Ni^{2+}, pre-equilibrated with low imidazole buffer (A100 plus 10% glycerol, 2 mM 2-mercaptoethanol, 1% TX-100, 40 mM imidazole) at a rate of 1 mL/min at 4 °C.

1.2.2. Wash the column with 25 mL of A100 plus 10% glycerol, 2 mM 2-mercaptoethanol, 0.1% Anapoe X-100, 40 mM imidazole with a rate of 1 mL/min at 4°C. Elute the protein with a 30 mL linear gradient of imidazole from 40 mM to 500 mM, and a final 5 mL wash at 500 mM.

1.2.3. Pool together peak fractions and incubate for 1 h at 4 °C with swollen GSH-Agarose beads, previously swollen in water and equilibrated in A100 with 10% glycerol, 2 mM 2-mercaptoethanol, 0.1% Anapoe X-100, and 1 mM EDTA.

NOTE: GSH-Agarose beads can be swollen in 50 mL of water the day before and incubated overnight at 4 °C, or for 1 h at RT. To remove water and equilibration buffer, centrifuge in a swinging bucket rotor at 500 x *g* for 1 min without brake. Aspirate the supernatant with a 26 G needle.

1.2.4. Pellet GSH-Agarose beads by centrifuging in a swinging bucket rotor at 500 x *g* without brake and remove the lysate supernatant by aspiration with a 26 G needle.

1.2.5. Transfer the beads to a 10 mL polyprep column and wash five times with 5 mL of equilibration buffer (A100 with 10% glycerol, 2 mM 2-mercaptoethanol, 0.1% Anapoe X-100, and 1 mM EDTA) by centrifuging at 500 x *g*.

1.2.6. Elute the protein with 1-1.5 mL of equilibration buffer supplemented with 10 mM reduced glutathione. Aliquot eluted protein and flash freeze in liquid nitrogen. Protein can be stored at -80 °C indefinitely.

NOTE: Adjust pH of elution buffer to 7.4.

1.2.7. Quantify protein concentration by amido black protein assay[9] and assess purity by SDS-PAGE and Coomassie staining.

2. Reconstitution of recombinant atlastin into liposomes

2.1. Liposome production by extrusion method[10]

2.1.1. Make lipid mix stocks in chloroform (10 mM total lipid). The necessary lipids are 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPPE), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DPPE). Acceptor liposomes consist of POPC: DOPS (85:15 molar ratio) and donor liposomes of POPC:DOPS:Rh-PE:NBD-PE (83:15:1.5:1.5 molar ratio).

NOTE: While POPC:DOPS lipid mixes have been traditionally used for in vitro lipid mixing assays, alternative lipid compositions may be adapted for different experimental purposes. POPC:DOPS liposomes are very stable and harder to fuse, therefore is a very stringent system for fusion.

2.1.2. Add 1 µCi/mL of L-α-dipalmitoyl-phosphatidylcholine (choline methyl-3H) to lipid mixes in order to determine lipid concentrations at later steps by liquid scintillation counting. Reserve at least 8 µL of this stock.

2.1.3. Transfer desired amount of lipid mixes to flint glass tubes.

2.1.4. Dry the lipid mixes under a gentle stream of N_2 gas for ~10 min until no more chloroform is visible.

2.1.5. Dry the lipid film further in a desiccator by vacuuming for 30 min.

2.1.6. Add enough aqueous A100 with 10% glycerol, 2 mM 2-mercaptoethanol, and 1 mM EDTA to the lipid film and bring back the concentration to 10 mM. Resuspend the lipid film by vortexing lightly for 15 min at room temperature. A vortexer that can accommodate flint glass tubes is recommended.

2.1.7. Freeze-thaw the hydrated lipids in liquid nitrogen ten times. After freezing in liquid nitrogen, thaw the liposomes by letting the solution sit at room temperature for 30 s, then transfer to water for faster thawing. This freeze-thaw cycling will minimize multilamellar vesicles.

CAUTION: Tubes may crack if they contain volumes larger than 0.5 mL and if they are transferred directly form liquid nitrogen to water.

2.1.8. Pass the lipid through polycarbonate filters with 100 nm pore size 19 times using mini-extruder.

2.1.9. Determining total lipid concentration of liposomes by scintillation counting

NOTE: Some of the lipid may be left behind in glass tubes and in mini-extruder.

2.1.9.1. Add 4 µL of lipid stock and liposomes in 3 mL of scintillation cocktail.

2.1.9.2. Measure average counts per minute (CPMA) for stock and liposomes. And calculate liposomes concentration with the following formula:

$$[\text{Liposomes}]=\frac{\text{CPMAliposomes}}{\text{CPMAstock}} X [\text{stock}]$$

2.1.10. Store the liposomes at 4 °C for up to a week. Longer storage is not recommended as liposomes might aggregate with time, decreasing reconstitution efficiencies.

2.2. Reconstitution by detergent assisted incorporation into preformed liposomes

2.2.1. Calculate the amount of buffer, protein, liposomes and extra detergent to be mixed together:

2.2.1.1. Determine the desired total volume. This volume is made up of buffer A100 with 10% glycerol, 2 mM 2-mercaptoethanol, and 1 mM EDTA. Volumes less than 250 µL do not mix well in 0.5 mL microcentrifuge tubes.

2.2.1.2. Calculate the volume of liposomes needed to give a final lipid concentration of about 1 mM. Subtract the volume from the buffer volume.

2.2.1.3. Calculate the volume of protein needed to give the desired protein to lipid molar ratio (usually 1:400). Decrease the buffer volume accordingly.

2.2.1.4. Determine how much extra detergent needs to be added to saturate the liposomes aiming for an effective detergent to lipid ratio (R_{eff}) between 0.64-0.8. Remember to consider the detergent that is added with the protein. The (R_{eff}) is determined by the equation R_{eff} =

$\frac{(Dtotal - Dwater)}{[lipid]}$. D_{total} is the total detergent concentration and D_{water} is the monomeric detergent concentration (0.18 mM for TX-100 and Anapoe X-100 in the presence of detergent)[4,11].

2.2.2. Mix the solutions together in a 0.5 mL tube in the following order: buffer, detergent, protein, and liposomes. Add the liposomes rapidly and vortex immediately for 5 s to homogenize the mixture.

2.2.3. Incubate the reaction in a nutator for 1 h at 4 °C.

2.2.4. Make 0.2 g/mL nonpolar polystyrene adsorbent bead "slurry" in water.

NOTE: Make the polystyrene adsorbent bead "slurry" during previous the 1 h incubation in step 2.2.3.

2.2.5. Weigh out 0.2 g of polystyrene adsorbent beads and transfer to a microcentrifuge tube.

2.2.6. Degas the beads by adding 1 mL of methanol to the tube and mix for 1 min. Degassed beads will sink.

2.2.7. Aspirate the methanol and add water to the beads. Let the beads mix with water for 5 min, then aspirate the water. Repeat four times, then bring the polystyrene adsorbent bead "slurry" to 1 mL volume with water and a final concentration of 0.2 g/mL.

NOTE: Beads should still settle to the bottom of the tube. If they do not, degas again.

2.2.8. Calculate the amount of polystyrene adsorbent beads needed to absorb all the detergent in each sample. 1 g of polystyrene adsorbent beads absorbs 70 mg of TX-100. To calculate the volume of polystyrene adsorbent bead slurry needed for each reaction, divide the total detergent in the reaction (step 2.2.1.4) by 70 mg, and then by the concentration of the bead slurry (0.2 g/mL (step 2.2.7).

2.2.9. Transfer calculated amount of polystyrene adsorbent bead slurry to a 0.5 mL tube and aspirate the water.

NOTE: Cut the end of a 20-200 µL tip to transfer the beads, vortex the tube just prior to pipetting to resuspend settled beads.

2.2.10. Add the samples to the 0.5 mL tube containing polystyrene adsorbent beads and incubate the sample nutating for 1 h at 4 °C.

2.2.11. Repeat twice leaving old beads behind and transferring the sample to fresh beads.

2.2.12. Add the sample to a fourth tube with fresh beads and incubate overnight (~15-18 h) at 4 °C.

2.3. In the morning, remove the sample from polystyrene adsorbent beads and pellet insoluble protein aggregates by centrifuging for 10 min at 16,000 x *g* at 4 °C.

2.4. Recover the supernatant and determine the final lipid concentration by liquid scintillation counting (see step 2.1.9.2). Optionally, protein concentration may be determined by amido black protein assay[9].

NOTE: For atlastin proteoliposomes, enzymatic assays should be performed with fresh liposomes. Long storage at 4 °C or freezing leads to significant loss in atlastin activity.

3. Lipid mixing assays

3.1. Bring the donor and acceptor proteoliposomes to a concentration of 0.15 mM each in A100 with 10% glycerol, 2 mM β-mercaptoethanol, 1 mM EDTA and 5 mM $MgCl_2$. Each reaction (50 µL) should be added to a well in a flat white 96 well plate suitable for fluorescence readings. Prepare at least 4 reactions including a triplicate, and a no-GTP negative control.

3.2. Place the plate in a preheated plate reader at 37 °C. Measure NBD fluorescence (excitation 460 nm and emission 535 nm) for 5 min every minute.

3.3. Induce fusion by adding 5 mM GTP (5 µL of 50 mM GTP).

3.4. Measure NBD fluorescence every minute for 1 h.

3.5. Add 5 µL of 2.5% w/v n-Dodecyl β-D-maltoside to dissolve the proteoliposomes and measure the maximum NBD fluorescence. Read NBD fluorescence for 15 min every minute.

4. Liposome floatation on iohexol discontinuous gradient[12]

4.1. (optional) Analyze the efficiency of reconstitution by floatation assays[13].

4.2. Prepare an 80% and a 30% w/v iohexol in A100 with 10% glycerol, 2 mM 2-mercaptoethanol, and 1 mM EDTA. Iohexol does not dissolve readily, so this must be prepared a day before by nutating overnight at 4 °C.

4.3. Thoroughly mix 150 µL of 80% iohexol stock with 150 µL of proteoliposomes sample to bring it to a 40% iohexol. Add this to a 5 x 41 mm Ultra-clear tube avoiding bubbles.

4.4. Layer 250 µL of 30% iohexol stock slowly on top of the sample to make a middle layer. Avoid any bubbles and disturbing the bottom layer. On top of the middle layer, slowly add 50 µL of A100 with 2 mM 2-mercaptoethanol and 1 mM EDTA.

4.5. Centrifuge the gradient in a swinging bucket rotor at 220,000 x *g* for 4 h at 4 °C with slow acceleration and no break.

4.6. Harvest the layers of the gradient and analyze by SDS-PAGE and Coomassie stain, quantification may be done by densitometry. Reconstituted protein should float to the top layer, while protein and lipid aggregates will sediment at the bottom or in the middle layer.

5. Analysis of orientation of reconstituted protein by thrombin proteolysis

NOTE: The atlastin construct reported here has a thrombin cut site between the end of the N-terminal GST tag and the beginning of atlastin. Atlastin in the correct orientation will have this cut site accessible to the protease, while protein in the wrong orientation will be protected by the lipid bilayer.

5.1. To assay atlastin proteoliposome orientation, reserve at least 8 µL of fresh reconstituted proteoliposomes.

5.2. Add 8 µL of proteoliposomes and 1 µL of 1 U/µL thrombin. Incubate at 37 °C for 1 h.

5.3. Quench the protease with 1 µL of 5 mg/mL of EDTA-free protease inhibitor cocktail and incubate for 30 min at 37 °C.

5.4. Analyze the sample by SDS-PAGE and Coomassie stain. Proportion of cleaved and uncleaved protein may be determined by densitometry.

REPRESENTATIVE RESULTS:

The efficiency of atlastin reconstitution is presented in **Figure 2**. Reconstituted proteoliposomes were floated in an iohexol discontinuous gradient. Unincorporated protein was sedimented in the bottom layer (B) or in the middle layer (M). Reconstituted protein would float to the top layer (T). Samples of the gradient were harvested and analyzed by SDS-PAGE and Coomassie staining. The quantification of the gel by densitometry shows a very high efficiency of reconstitution with negligible loses; 96% of the total protein was found as proteoliposomes that floated to the top layer (T). Less than 1% of protein was unreconstituted and found in the middle layer (M), and only 3% was unreconstituted or aggregated and sedimented to the bottom layer (B).

In addition to describing the extent of reconstitution, analyzing the orientation of atlastin after reconstitution was quantified by thrombin cleavage assays[14]. Reconstituted protein could potentially be in the wrong orientation, that is, facing the lumenal space of the liposome. Protein in the wrong orientation should be protected from proteolysis by the lipid bilayer. A thrombin cleavage site is coded between the end of the N-terminal GST tag and the beginning of atlastin. Floated proteoliposomes were incubated with thrombin for 1 h at 37 °C, followed by a 30 min inactivation with EDTA-free protease inhibitor. Samples were analyzed by SDS-PAGE and Coomassie stain and quantified by densitometry **Figure 3**. As a negative control, a sample of

untreated proteoliposomes is shown in the left lane. Detergent solubilized proteoliposomes (right lane) served as a positive control and to show the extent of thrombin cleavage, with only 1% left un-cleaved. In all, this assay shows that most of the reconstituted protein was cleaved with only 7% being protected from the protease (middle lane). It is worth noting that the remainder of uncut protein may be a result of reconstituted protein aggregates that may not have the cut site accessible. In all, these results describe a robust system for reconstituting atlastin.

The kinetics and the extent of atlastin-mediated proteoliposome fusion were analyzed by lipid mixing assays (**Figure 1**). A sample of atlastin fusion kinetics and quantification are shown in **Figure 4**. The full kinetic run is depicted in **Figure 4A**. A 5 min incubation was done before inducing fusion with GTP at the zero timepoint and after 1 h, n-Dodecyl β-D-maltoside was added to solubilize the proteoliposomes and get the maximum FRET release. The fusion maximum in the run was of 11% of maximum fluorescence (**Figure 4B,C**). Uninduced (No GTP) controls can be used to determine the background baseline.

FIGURE AND TABLE LEGENDS:

Figure 1. Fusion assay model of liposome fusion. A population of labeled liposomes with fluorescently tagged fluorescent donor lipids NBD-phosphatidylethanolamine (represented as green spheres) and the acceptor rhodamine- phosphatidylethanolamine (represented as red spheres) is mixed with an unlabeled pool of liposomes. Before fusion, NBD's fluorescence is low due to the proximity with the acceptor fluorophore, rhodamine. Upon fusion with unlabeled liposomes a surface area increase leads to dilution of the probes and a FRET release of NBD can be measured.

Figure 2. Reconstitution efficiency analyzed by floatation assays. Reconstitution efficiency of atlastin can be measured by floatation of proteoliposomes in an iohexol discontinuous gradient. Reconstituted protein should float as proteoliposomes to the top layer (T), while unreconstituted and aggregated protein should sediment to the bottom layer (B) or the middle layer (M). A Coomassie-stained SDS-PAGE gel was quantified by densitometry and measured 96% of the total protein floated as proteoliposomes with negligible loses (~4%).

Figure 3. Analysis of reconstituted atlastin in proteoliposomes by protease digestion. Recombinant atlastin has a N-terminal GST tag followed by a thrombin cut sequence. Proteoliposomes of recombinant atlastin were treated with the serine protease thrombin to analyze the orientation of the reconstituted protein in respect to the lipid bilayer. After thrombin treatment the protease is inhibited and the samples are analyzed by SDS-PAGE, Coomassie stain, and quantified by densitometry. The presented proteoliposomes have a low proportion of protected protein, with only 4% that remained undigested (middle lane). As a positive control some proteoliposomes were solubilized with detergent (0.5% TX100) (right lane); the negative control, untreated proteoliposomes, was not cleaved (left lane).

Figure 4. Lipid mixing assays of atlastin proteoliposomes. (A) Sample kinetic trace of fusion reaction with a 5 min incubation time before inducing fusion with GTP at 0 min. After 1 h run,

detergent solubilization (black arrow) was used to determine the maximum FRET release of NBD. **(B)** Zoomed in view of the trace from timepoint 0 to 1 h with and **(C)** average fusion (n=3) of 11.3%.

DISCUSSION:

The methods here delineate an efficient method for purifying, reconstituting, and measuring fusion activity of recombinant atlastin. To ensure high yields of functional atlastin some critical steps must be considered. Expression of atlastin must be done at low temperatures (16 °C) to avoid aggregation and one should aim for a final concentration between 0.4-1.5 mg/mL. Very dilute protein will not be reconstituted optimally at a 1:400 protein to lipid ratio. Reconstitution efficiency can be optionally analyzed by floatation assays described here (**Figure 2**). One of the advantages of liposomes floatation assays is that they can be extended to analyze soluble proteins that associate with lipid bilayers[13]. Additionally, orientation of the reconstituted protein may be also analyzed by protease digestion[14], however, this may not differentiate between reconstituted misfolded or aggregated protein or reconstitution in the wrong orientation (**Figure 3**). The proteoliposomes developed by this method can be applied to assay for fusion or for studies of atlastin in a model lipid bilayer. While making the reconstitution procedure is relatively simple, small deviations from optimal detergent and lipid concentrations may reduce efficiencies of reconstitution. For example, an excessive amount of detergent may lead to liposome solubilization.

Lipid mixing assays require a pool of donor and acceptor liposomes (**Figure 1**). This method uses radioactivity by tritiated lipid for quantification of lipid concentration at every step. However, alternative quantification methods have been reported using the intrinsic fluorescence of donor liposomes and acceptor liposomes with dansyl-phosphoethanolamine[15,16]. Sizing of liposomes can also be modified during extrusion, as the polycarbonate membrane pore size can be adjusted accordingly.

While lipid mixing assays are an efficient way to analyze fusion, it may not differentiate between fusion and hemifusion. Several studies visualizing atlastin proteoliposomes after fusion by electron microscopy[15] and by lipid fluorescence[16,17] further support full fusion of atlastin. However, when analyzing specific atlastin mutants or other fusion proteins, it is of interest to ensure full fusion. Additional steps to troubleshoot this could be done by quenching the outer leaflet with dithionite and measuring inner leaflet lipid mixing. Alternative fusion assays, such as inner aqueous content mixing may be employed for this, however, additional steps and dialysis of liposomes will be necessary.

It is of interest that this method can be applied to other membrane proteins for in vitro studies of proteins in model lipid bilayers. Other membrane proteins have been reported to reconstitute with this method[16,17]. This method can therefore be applied to a variety of membrane and fusion proteins.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

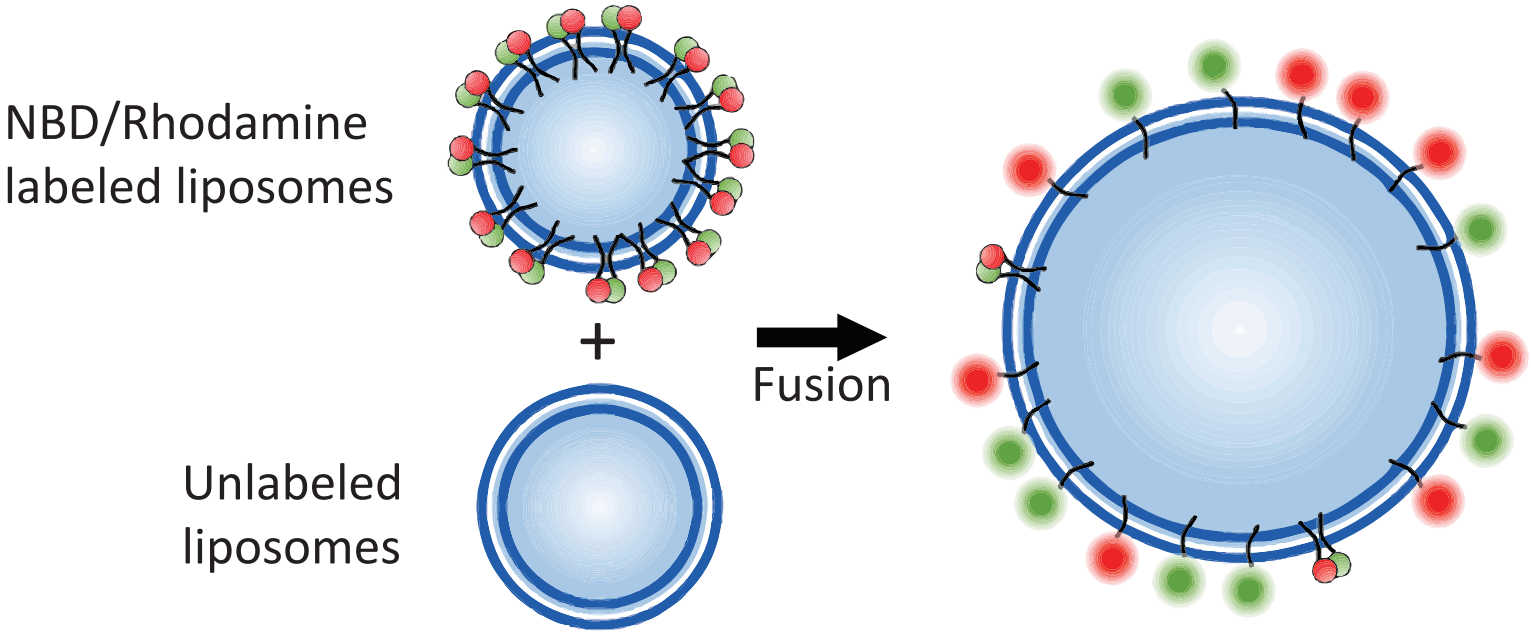

Figure 2

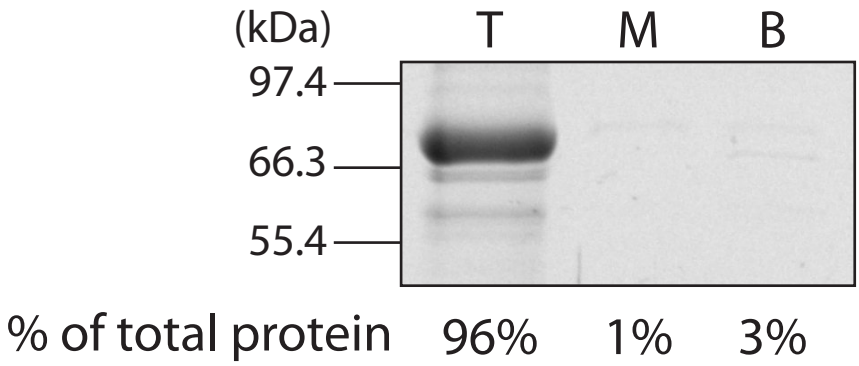

Figure 3

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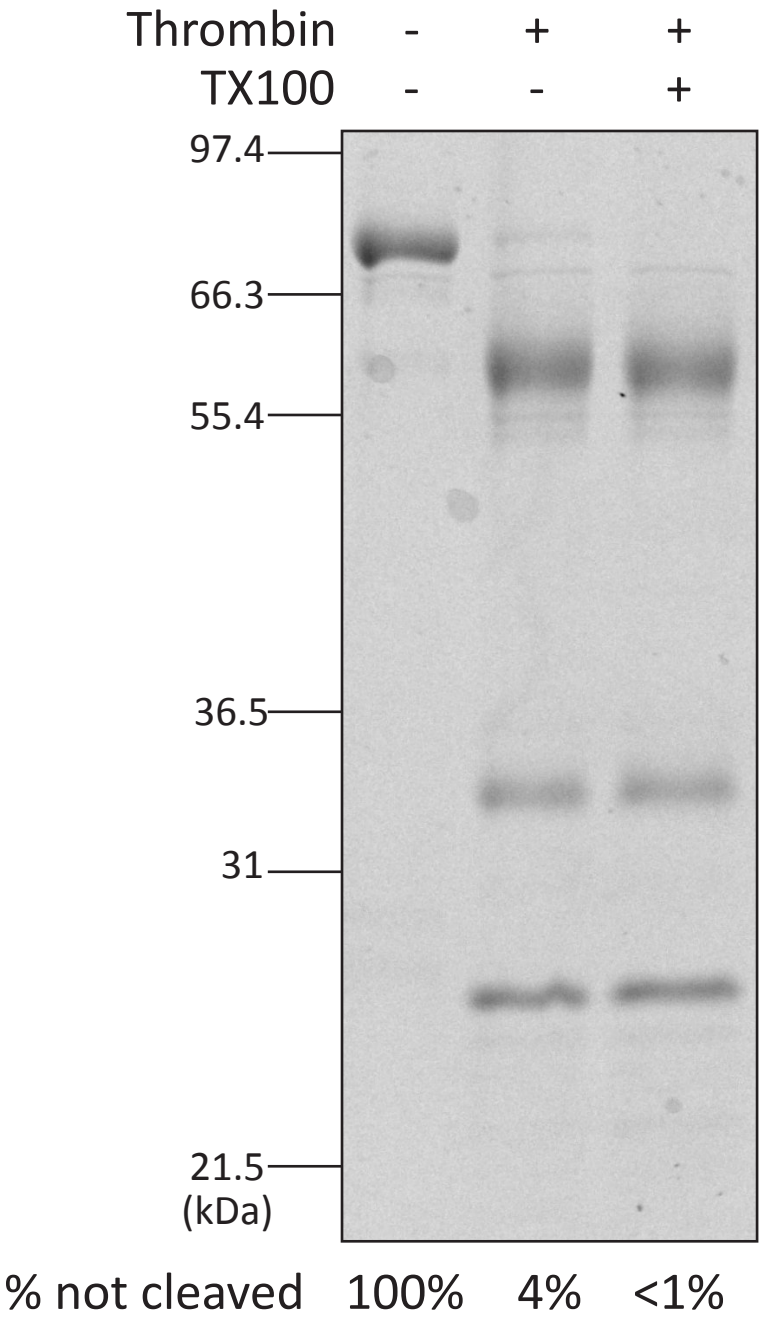

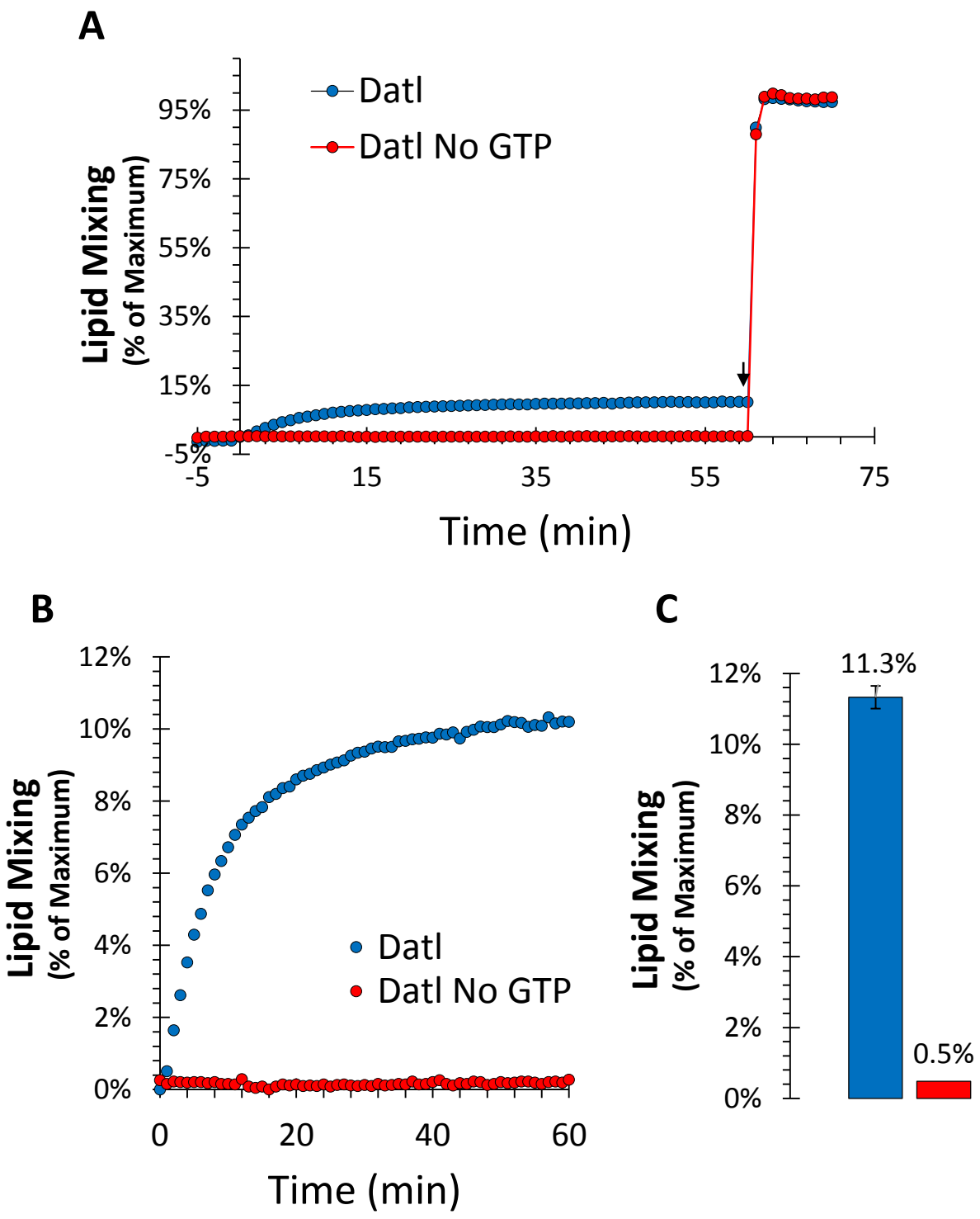

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10 mL poly-prep chromatography columns	Biored	731-1550	
10 x 75 mm Flint glass tubes	VWR	608225-402	
47 mm diameter, 0.45um pore whatman sterile membrane filters	Whatman	7141 104	
96 well white plate	NUNC	437796	
Anapoe X-100	Anatrace	9002-931-1	
Cell disrupter	Avestin		Avestin Emulsiflex C3
DOPS (1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt))	Avanti	840035P-10mg	DOPS
EDTA	Research organics inc.	6381-92-6	Ethylenediaminetetraaceti c acid
EDTA-free protease inhibitor cocktail	Roche	11873580001	Complete protease inhibitor
Extruder	Sigma Aldrich	Z373400	Liposofast Basic Extruder
GE Akta Prime liquid chromatography system	GE Pharmacia	8149-30-0006	
Glutathione agarose beads	Sigma aldrich	G4510-50ml	
Glycerol	EMD	GX0185-5	
GTP	Sigma Aldrich	36051-31-7	Guanosine 5' triphosphate sodium salt hydrate
HEPES, acid free	Omnipur	5330	
Imidazole	fluka	5670	
Immobilized metal affinity chromatography (IMAC) resin column	GE Healthcare	17040801	1 mL HiTrap Chelating HP immobilized metal affinity chromatography columns
Iohexol	Accurate chemical and scientific corporation	AN 7050 BLK	Accudenz/Nycodenz

IPTG	Research products international corp.	I56000-100.0	IPTG, dioxane free
L-Glutathione reduced	Sigma-Aldrich	G4251-5g	
Magnesium chloride	Fisher	7791-18-6	
Methanol	Omnisolv	MX0488-1	
NBD-DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt))	Avanti	236495	NBD-DPPE
n-Dodecyl β-D-maltoside	Chem-Impex International	21950	
Nonpolar polystyrene adsorbent beads	BioRad	152-3920	SM2 Biobeads
Nuclepore track-etch polycarbonate 19 mm 0.1 um pore membrane	Whatman	800309	
Optima LE80K Ultra centrifuge	Beckman Coulter		
Phosphatidylcholine, L-α-dipalmitoyl [choline methyl-3H]	ARC	ART0284	Titriated lipids
Plate reader	TECAN		TECAN infinite M200 plate reader
POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine)	Avanti	850457C-25mg	POPC
Potassium chloride	MP	151944	
Rh-DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt))	Avanti	236495	Rh-DPPE
Scintillation Cocktail	National Diagnostics	LS-272	Ecoscint XR Scintillation solution for a
Scintillation vials	Beckman	592928	Fast turn cap Mini Poly-Q Vial
Thrombin	Sigma	T1063-1kU	Thrombin from human plasma
Triton X-100	Fisher	BP151-500	
Ultra-clear centrifuge tubes 5 x 41 mm	Beckman	344090	

Vortex 9 to 13mm Tube Holder	VWR	58816-138	Insert for vortexing flint glass tubes
Vortex Insert Retainer	VWR	58816-132	Retainer needed for vortex tube holde
Vortexer	VWR	2.235074	Vortex Genie 2 model G560
β-mercaptoethanol molecular biology grade	Calbiochem	444203	

queous or non-aqueous samples

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Author(s):	Miguel A. Betancourt-Solis and James A. McNew

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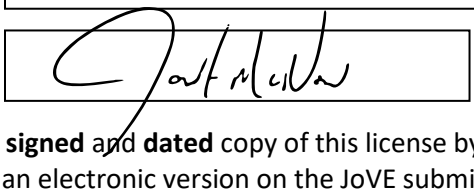
Signature:	
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We thank the reviewers and editors for their insights and appreciate their comments. We have modified the text to accommodate editorial and reviewer comments and to improve clarity and significance of each step in the protocol. Below are the comments of each reviewer (in italics) and the steps we have taken to address each.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have gone through the manuscript and made all the efforts to remove any grammatical or spelling mistakes, and modified the text to improve clarity.

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We do have any figures or data that have been previously published.

3. Please use h, min, s for time units.

We have gone through the manuscript and addressed any use of units in this notation.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

We removed any brand names and referred to any reagents or instruments in generic terms. We also added these details in the Table of Materials and Reagents.

5. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc.

We have gone through the document and made any necessary changes in the notation of SI units.

6. Please do not highlight a step without highlighting any of the sub-steps for filming.

We have gone through the protocol and removed highlighting of any step that does not require filming.

7. Step 5.1: Please write this step in the imperative tense.

We have modified the wording on this step to imperative tense.

8. Please do not abbreviate journal titles for references.

The references have been updated with the correct formatting.

9. Figure 2: Please add a unit.

We apologize for this omission. The figure was modified, we added a kDa unit for the molecular weight markers.

10. Figure 3: Please add a unit.

We apologize again for this error. We have modified the figure accordingly and added a kDa unit for the molecular weight markers.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This manuscript describes the protocol for detergent-assisted reconstitution of recombinant drosophila Atlastin into liposomes to assess its fusogenicity. It is clearly written and will be of great interest to all of the scientists working on the mechanism of membrane fusion. Apart for a few minor points (see below), the protocol clearly described the step-by-step procedure and should be easily reproducible by non-experts.

Minor Concerns:
**It would be useful to the readers to detail the rationale for a few experimental details:*
1.1.2; 1.1.3. Why are the bacteria grown at 25oC instead of 37oC?

Our construct has some leaky expression before induction with IPTG. Growth at 25ºC is mostly to reduce aggregation during this growth period.

1.1.6. Why induce at 16oC?

We have empirically determined that aggregation is minimized by inducing at 16ºC. We added this point in the text for clarity.

1.1.13. NOTE: Why use 8M Urea?

We use that to dissolve the solid pellet. It acts as a strong denaturant and should help dissolve any inclusion bodies. We modified the text to address this point.

1.1.14. Why filter extracts through 0.45µm cellulose membrane?

We appreciate this comment and it is important to clarify the rationale of this step. The 0.45 um membrane will filter out any unbroken bacteria and large debris that may not have removed by centrifugation. This also ensures that no debris is present that may clog the chromatography system. We modified the text on this step to address this point.

1.2.1. Why changing the salt concentration from 200mM to 100mM KCl?

The higher salt concentration in the breaking buffer is typical for bacterial lysis buffers. The higher salt concentration is helpful in breaking for bacteria lysis and improves the purity of recombinant proteins by decreasing non-specific binding of the expressed protein to other cellular components. However, following purification, we reduce the salt content to 100 mM to mimics physiological conditions better.

1.2.5. What is the "equilibration buffer", is it A100?

We apologize for the discrepancy in defining the equilibration buffer. It is A100 with 10% glycerol, 2 mM 2-mercaptoethanol, 0.1% Anapoe X-100, and 1 mM EDTA. The solution was defined in step 1.2.3. but we redefined it in step 1.2.5. for clarity.

2.1.9.1. What is the composition of the scintillation cocktail?

The scintillation fluid is premade. We use National Diagnostics Ecoscint XR Scintitllation Solution for aqueous or non-aqueous samples. Form the label, the composition is of Phenyl xylyl ethane, linear alkyl phenyl ethoxylates, butoxy ethanol, and primary alcohol ethoxylate. We added the brand information in the Table of Materials and Reagents.

2.2.4. NOTE: there is no step 2.5.

We apologize for this typo. We have modified the text with the correct referenced step.

2.4. The reference should be formatted.

We have added the reference in the correct format and updated our reference list.

3.1. It would be less confusing to rephrase "Make at least 4 reactions including a triplicate, and a no-GTP negative control" As it reads, it seems that the triplicate and negative control are in addition to the 4 reactions.

We apologize for this confusion, we have reworded the text for clarity.

**There is a mistake in 4.4. The top layer of the gradient should not have detergent, which would dissolve the newly formed liposomes.*

We apologize for this error. We modified the buffer composition and removed the detergent component.

**There are several typos in the manuscript. For example in 1.1.12 should be Avestin, not Avastin.*

We have made all efforts to remove all the typos. We also removed the commercial language of the instrument and will address this at the Table of Materials and Reagents.

**The buffer A100 needs to be described consistently: in 1.2.1 and at several other places it is described as 25mM HEPES(pH7.4) and 100mM KCL. In 2.1.6. A100 is described as 25mM HEPES(pH7.4) and 100mM KCL, 10% glycerol, 2mMBME, and 1mM EDTA.*

We apologize for the inconsistency. We modified the text to address the definition of A100 consistently throughout the text.

Reviewer #2:

Manuscript Summary:
In this methods paper, McNew et al., who have been publishing a number of the reconstitution studies on atlastin GTPase-mediate membrane fusion (for example, Ref. #2 and #16), describe the detailed step-by-step procedures for (i) expression and purification of recombinant drosophila atlastin protein, (ii) preparation of reconstituted proteoliposomes bearing purified atlastin proteins by a detergent-assisted incorporation method, and (iii) testing the fusogenic capacities of the prepared atlastin-bearing proteoliposomes using lipid mixing assays. Although these experimental protocols appear to be basically well-written and should be very useful for the researchers in the field, this reviewer would like to recommend the authors to amend the paper with respect to the following concerns/suggestions:

Minor Concerns:

1. Page 4, lines 189-195; it would be helpful for the readers, if the authors described why they used the POPC/DOPS lipid compositions for atlastin reconstitution.

The lipid composition of POPC:DOPS has traditionally been used in previous work addressing SNARE *in vitro* fusion. It is noteworthy that varying the lipid composition with other lipids does significantly affect fusion kinetics. POPC:DOPS proteoliposomes are very stable and harder to fuse than other lipid compositions. We use this as a very stringent way to assess fusion. We added a note on this step to address that alternative lipid composition may be adjusted at this point.

2. Page 4, lines 207-209, and Page 5, lines 253-254; the more detailed information about vortexing in these steps would be needed. For instance, regarding the equipment and the settings used.

We appreciate this comment and agree that we need to be more specific for clarity. In Page 4, lines 207-209, any vortex that can accommodate the tube with the lipid mix should suffice, also a low vortex setting is enough for resuspension. In Page 5, lines 253-254, a quick vortex for mixing the solution is enough. We added more details to the text and added the vortex model in the Table of Materials and Reagents.

3. Page 4, lines 211-212; although they described "…. to form large unilamellar vesicles (LUVs)", this reviewer believes that there must be significant amounts of "multilamellar" vesicles at this step before extrusion.

Although after resuspension most vesicles are going to be multilamellar, the ten freeze-thaw cycles minimize multilamellar vesicles. We appreciate the comment and have modified the text to improve clarity.

4. Page 5, lines 246-251; they described the Reff value (between 0.64-0.8) for the current reconstitution method, and the Dwater value (of 0.18 mM) for TX-100. It would be very helpful for the readers, if the suitable references for these numbers were cited.

We apologize for the lack of referencing, the numbers were taken from reference 4 (Rigaud, et al., 1995). We also added reference 11 (Patternostre, et al., 1988) were the values are described in greater detail.

5. Page 6, lines 273-276; what is the "calculated amount of BioBeads"? Need to indicate the typical amounts of BioBeads used in this step.

We apologize for the confusing wording. The volume of BioBead slurry is determined by the total detergent in each reaction. One gram of BioBeads can remove 70 mg of Triton X-100. A typical 250 µL reaction has around 150 µg of detergent. Using 0.2 g/mL BioBead slurry we can remove the detergent typically with 10 µL of slurry. We described the calculations for this in the text to improve clarity.

6. Page 8, lines 380-386; "Figure 3, Figure 3A, Figure 3B" should be indicated as "Figure 4".

We apologize for this typo and have corrected this on the text.

7. Figure 4; the y-axis labels for Figure 4A-C should be changed to Lipid mixing (% of Maximum).
We use lipid mixing as a proxy for fusion and agree that lipid mixing is more accurate; we modified the figures to improve clarity.

8. Discussion, Page 10, lines 438-451; in addition to the experimental evidence from lipid mixing assays (Ref. #2, #3, #14, #15, and #16), the intrinsic fusogenic capacities of atlastin-family proteins have been also tested by the prior reconstitution studies using electron microscopy experiments and fluorescence microscopy experiments (Ref. #14, #15, and #16). This would further strengthen the evidence establishing the full-fusion (not only hemifusion) activities of the reconstituted atlastin/Sey1p proteoliposomes, prepared by the detergent-assisted incorporation method described here. This would be worth noting in the Discussion section or so.

We completely agree with this comment, we have taken steps in the lab to address this question and from the literature and non-reported data we are confident that the system we describe for atlastin fusion is in fact full fusion. We have addressed this in the discussion.

Reviewer #3:

Manuscript Summary:
Fusion assays using reconstituted proteoliposome (RPL) provide a facile tool for studying the role of fusogenic proteins in membrane fusion. In this manuscript, the authors provide a detailed description of the entire procedures of protein purification, proteoliposome production, and a lipid mixing assay. Furthermore, the authors nicely show that detergent-assisted reconstitution of drosophila atlastin-1 has several advantages over other methods: high reconstitution efficiency of membrane proteins into the lipid bilayer with a proper orientation. I only have minor points that the author may want to consider.

Minor Concerns:

(1) In 2.1.10, the authors mention that LUV liposomes can be stored at 4C for up to a week. Can they be stored for a longer time? If so, mentioning the storage condition would be very informative and useful.

We appreciate this comment and agree it is an important point we need to clarify. We do not recommend longer storage time as liposome may aggregate, this may alter reconstitution efficiencies and consequent lipid mixing assays. We modified the text in the step to address this point.

(2) In 2.4, It would be also very informative and useful to describe how proteoliposomes should be stored if they are not immediately used.

Again, this is a very important point we failed to mention in the manuscript. In the case of atlastin proteoliposomes we recommend using fresh liposomes. Long-term storage at 4^{o}C lead to loss of enzymatic activity of atlastin. We also do not recommend freezing the sample again as we have found that atlastin fusogenic activity as well as GTPase activity significantly diminishes when proteoliposomes are subject to freeze/thaw cycles. We added this point in the text for clarity.

(3) Is there any difference between 'Triton X-100' and 'Anapoe X-100'? To my knowledge, they are basically same. Please stick to one to avoid confusion.

We appreciate this comment, however there are some differences between commercial sources of polyoxyethylene detergents. Most Triton X-100 sources report an average molecular weight with of 625 of the mixture of 9-10 copies of oxyethylene. Anatrace Anapoe X-100 has a bit higher purity and this formulation favors the longer 10 copy of oxyethylene with a reported average molecular weight of 647. For the initial steps in bacterial lysate preparation lower grade Triton X100 will suffice. However, for the purification and elution Anapoe X-100 is preferred. We have not systematically compared sources of detergent but prefer Anapoe X-100.

We thank the editors for their insights and appreciate their comments. We have modified the manuscript to address the concerns raised by the editors. The comments raised on this revision are listed below (in italics) and the steps we have taken to address them.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have reviewed the manuscript and modified any spelling and grammatical mistakes that may hinder clarity.

2. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Examples of commercial language in your manuscript include BioBeads, HisTrap, Roche, Nycodenz, etc.

We have renamed the following reagents by generic names to avoid any commercial language. BioBeads have been renamed to polystyrene adsorbent beads, HisTrap columns to immobilized metal affinity chromatography (IMAC) resin column, cOmplete protease inhibitor to EDTA-free protease inhibitor cocktail, and Nycodenz to iohexol. The table of materials and reagents has been modified accordingly.

3. Please do not highlight notes/caution for filming.

We have removed any highlighting on notes and caution information.