Characterizing diffusion dynamics of a membrane protein associated with nanolipoproteins using fluorescence correlation spectroscopy

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Abstract: Nanolipoprotein particles (NLPs) represent a unique nanometer-sized scaffold for supporting membrane proteins (MP). Characterization of their dynamic shape and association with MP in solution remains a challenge. Here, we present a rapid method of analysis by fluorescence correlation spectroscopy (FCS) to characterize bacteriorhodopsin (bR), a membrane protein capable of forming a NLP complex. By selectively labeling individual components of NLPs during cell-free synthesis, FCS enabled us to measure specific NLP diffusion times and infer size information for different NLP species. The resulting bR-loaded NLPs were shown to be dynamically discoidal in solution with a mean diameter of 7.8 nm. The insertion rate of bR in the complex was ~55% based on a fit model incorporating two separate diffusion properties to best approximate the FCS data. More importantly, based on these data, we infer that membrane protein associated NLPs are thermodynamically constrained as discs in solution, while empty NLPs appear to be less constrained and dynamically spherical.

Keywords: apolipoprotein; nanolipoprotein particles; nanodiscs; fluorescence correlation spectroscopy; dynamic light scattering; cell-free expression; co-expression

Introduction

The biochemical and structural characterization of individual transmembrane proteins remains a difficult task given the complex cellular environment containing a large number of other receptors and structural proteins in close proximity. Reconstitution of membrane proteins (MP) by addition of lipids during dialysis for detergent displacement allows sustaining the structure and activity of proteins after detergent-based

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Figure 1. Schematic of single step cell-free expression of fluorescently labelled membrane protein associated NLPs for characterization by FCS. (A) bR-NLP complex were formed by adding $\Delta 49\text{A1}$ and bOp encoded DNA with co-factor to a cell-free mixture. (B) Image of a tube containing the purified bR-NLP complex showing the resulting pinkish color of properly folded bR. The SDS gel exhibits protein bands of the expected sizes for the affinity-purified NLP complex indicating the presence of both the $\Delta 49\text{A1}$ and bR proteins respectively. (C) A diagram of NLPs (empty NLPs and bR-loaded NLPs) diffusing by Brownian motion in and out of the effective volume of a confocal fluorescence microscope utilizing FCS.

purification.^{1–7} However, the purified protein is now part of a proteoliposome containing multiple molecules that are difficult to define or study due to multiple interactions.⁸ Also, proteoliposomes are typically not very soluble and thus cannot be manipulated or analyzed by most traditional techniques. These problems can be addressed by combining novel spectroscopic techniques with nanolipoprotein particles (NLPs) to isolate and solubilize MP.^{9–14}

NLPs are soluble nanoparticles in an aqueous environment and formed spontaneously when apolipoproteins and a population of phospholipids self-assemble into bilayers of lipids circulated by an apolipoprotein “belt”.^{15–18} The bilayer closely mimics the cell membrane,¹⁹ allowing MP to be functional upon incorporation into NLPs.^{20–25} NLPs present distinct advantages over currently used membrane systems in terms of thermal stability,^{19,26} particle size monodispersity and consistency.^{27,28} Although NLP synthesis is now well-documented, and a few studies have described their shape (the well-studied discoidal model,^{29–31} the spherical model,³² and the saddle model³³), there remains a lack of comprehensive solution-phase physical characterization methods for understanding the structure and dynamics of an NLP with a functional protein incorporated.

A number of methods have been used to data to characterize NLP-related complexes. Size-exclusion chromatography (SEC) and native polyacrylamide gel electrophoresis (PAGE) have been the predominant techniques used to provide purification capabilities and approximation of NLP mass and polydispersity from bulk samples.^{21,27,33,34} Transmission electron microscopy (TEM) and atomic force microscopy (AFM) have also been used to examine NLP shape features upon immobilization of NLPs

onto a surface.^{27,34–36} Compared with the techniques described above, fluorescence correlation spectroscopy (FCS) may emerge as a useful tool for probing membrane protein interactions³⁷ using NLPs that are fully hydrated and freely diffusing in an aqueous environment. When FCS is typically employed in conjunction with confocal optical microscopy,³⁸ fluorescence intensity fluctuations due to diffusion,^{39–41} physical or chemical reactions, aggregation and other factors are analyzed using a temporal autocorrelation function as these species enter and exit the excitation volume.^{42–46} When an appropriate model^{42,44,47} is defined, FCS can be used to obtain quantitative information such as diffusion coefficients, hydrodynamic radii, average concentrations, kinetic chemical reaction rates, and singlet-triplet state photochemical dynamics.^{47–50}

In this study, we used cell-free protein expression for the rapid generation of fluorescently labelled NLP complexes containing bacteriorhodopsin (bR) and we characterized the dynamic structure and association between NLPs and MP in solution using FCS.

Results

bR-NLP Complex formation and purification

The cell-free protein expression and characterization process is illustrated in Figure 1. Our approach²¹ rapidly produces a functional integral membrane protein, bR (a seven transmembrane helical protein, from *Halobacterium salinarum*) co-expressed with a truncated apolipoprotein A-1 ($\Delta 49\text{A1}$) in the presence of 1,2-ditetradecanoyl-sn-glycero-3-phosphocholine (DMPC), as well as the cofactor all trans-retinal in a cell-free expression mixture. The phospholipids were

Figure 2. Gel electrophoresis of cell-free expressed proteins. (A) Denaturing SDS PAGE of purified cell-free expressed proteins. Lane 1: bOp, lane 2: $\Delta 49A1$ -NLPs, lane 3: co-expressed bR-NLP complex. (B) Native PAGE of purified NLPs. Lane 1: empty $\Delta 49A1$ -NLPs, lane 2: bR-NLP complex. All samples were loaded along with a molecular weight standard (MW).

fluorescently labelled by Texas Red[®] 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (Texas Red[®] DHPE). The proteins were labelled by Bodipy[®]-FL (Promega) through adding FluoroTect[™] GreenLys into the reaction mixture for *in vitro* translation.⁵¹ Since fluorescently labeled lysine residues were incorporated into nascent proteins during translation, only one Bodipy dye would be labeled to one $\Delta 49A1$ or bR respectively at the most, dependent on the labeling efficiency, which was not disclosed from the Bodipy[®]-FL provider.

Assembly of the soluble bR-NLP complex was observed within 4 h after addition of plasmids to an *E. coli* cell-free lysate [Fig. 2(A)]. Purification of the soluble fraction in a 1 mL cell-free reaction yielded 0.70 mg/mL (protein concentration) of bR-NLP complex. Cell-free expression and assembly of empty

NLPs yielded a protein concentration of 0.95 mg/mL after purification. The levels of bR obtained were comparable to or better than previously published data.²¹ Native gel electrophoresis was used to compare the molecular weight of empty NLPs to bR-NLP complex [Fig. 2(B)]. The results clearly indicated a shift in size of empty NLPs versus bR-NLP complex with a larger mass. As previously reported, the size range of $\Delta 49A1$ NLPs was ~ 240 kDa with a smear on the gel that represented a heterogeneous size distribution, where bR-NLP complex were slightly larger than empty NLPs.²¹ bR-NLP complex heterogeneity was also observed by native gel electrophoresis. This heterogeneity may have been due to multiple bR forming oligomers within the NLPs and/or generation of NLPs with varying diameters, as has previously been reported.²¹

Figure 3. Diffusion curves of standard samples measured by FCS. The light green, cyan, dark green, blue, red, and purple curves are corresponding to Alexa Fluor 488, ATTO 655, Polystyrene bead sample 1, 2 and DMPC vesicle sample 1 and 2 respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table I. Diffusion Times Determined by FCS and Diameters Determined by DLS for the Standard Samples

Standards	Alexa 488	Atto 655	Bead 1 ^a	Bead 2 ^b	Vesicle 1 ^c	Vesicle 2 ^d
Diffusion Time (ms)	0.036	0.059	0.86	2.16	3.2	6.8
Size by DLS (nm)	1.430	4.220	16.31	32.40	61.10	108.00

^{a,b} Dye-labelled polystyrene beads.^{c,d} Texas Red labelled DMPC vesicles made by extrusion.

Size calibration for FCS

To apply FCS for entities significantly larger than fluorescent molecules, we first generated a calibration curve that correlated FCS and dynamic light scattering (DLS) data for a range of molecules/particles to compare their bulk fluorescent and light scattering properties for comparison to NLP size information. This was necessary, because FCS is typically used to determine changes in diffusion behavior to a single standard, not to absolute values and not in the size range necessary for large protein-lipid complexes. To fit the data from these samples, we simplified the analysis by assuming that all particles/aggregates are spherical in shape (Supporting Information). Diffusion times were measured by FCS for all the standards (concentrations adjusted to $\sim$ nM). The respective diffusion curves are shown in Figure 3. We then fit FCS diffusion curves for the same samples using a 1-species translational diffusion model (Supporting Information). The fitted data resulted in the following diffusion times: $(36 \pm 1) \mu$ s, $(59 \pm 1) \mu$ s, $(860 \pm 50) \mu$ s, $(2160 \pm 80) \mu$ s, (3.2 ± 0.3) ms, and (6.8 ± 0.7) ms, respectively (Table I).

For all these particles, we independently measured their hydrodynamic diameters using DLS, which resulted in the following size distributions based on population histogram: $(1430 \pm 1) \times 10^{-3}$ nm, $(4220 \pm 2) \times 10^{-3}$ nm, (16.31 ± 0.02) nm, (32.40 ± 0.03) nm, (61.10 ± 0.05) nm, and 108.00 ± 0.06 nm for the corresponding standards (Table I). None of the standards absorb at the 780 nm (DLS source) wavelength and their concentrations were adjusted to $\sim$ μ M to meet the measurement requirement for the DLS particle sizer.

We plotted the diffusion times measured by FCS against the particle sizes (hydrodynamic diameters) measured by DLS. Because both FCS and DLS measured the same solutions (albeit at different concentrations), the diffusion times and the particle sizes were expected to be directly proportional for spherical species based on the Stokes-Einstein model (Supporting Information). As evidenced by Figure 4, these parameters exhibited a significant linear relationship. Assuming $\tau = A \times d + B$ (with τ being the diffusion times and d the diameters of the particles), the result of the linear fit gave $A = (63 \pm 3) \times 10^{-3}$, and $B = -(0.14 \pm 0.16)$, with $R^2 = 0.995$ (correlation coefficient) and S.D. = 0.28 (standard deviation). These data illustrated that particle diffusion time

was directly proportional to particle size even for a wide range of particle sizes. Using our assumptions, the linear correlation demonstrates that FCS can be used to directly measure particle sizes based on diffusion times in solution.

FCS demonstrates dynamic differences between lipids, proteins, and NLP complexes

The diffusion curve for the fluorescently labelled apolipoprotein Δ 49A1 fit a 1-species translational diffusion model quite well, while bacterioOpsin (bOp, protein expressed in the absence of the retinal co-factor) required fitting with a 2-species translational diffusion model (Fig. 5 and Supporting Information). The slightly more complex decay curve for bOp is likely a result of a combination of factors such as improper folding, insolubility or aggregation. To account for this, we only reported the fast diffusion time for bOp (Table II). In contrast, DMPC vesicles labelled with Texas Red, exhibited a much slower diffusion constant due to the fairly large size of the vesicles (Fig. 5). Cell-free NLPs with or without bR were tagged with Bodipy[®]-FL and included Texas Red labels within the complex. We separated Bodipy and Texas Red emission in two detection channels using a dichroic mirror and appropriate bandpass filters. The two fluorescence channels

Figure 4. Calibration curve for measuring particle size by FCS. The square dots are the experimental data and the red line represents a linear fit of the experimental data. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5. FCS diffusion curves of proteins and NLP complexes measured by FCS. The blue, green, yellow, purple, and red curves correspond to $\Delta 49A1$, bOp, bR-NLP complex, empty NLPs, and DMPC vesicles in 1X PBS, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

were cross-correlated and normalized to the maximum correlation value. This is a standard FCS analysis procedure and the resulting correlation curve is called a diffusion curve. Differences in fluorescence intensities between empty NLPs and bR-NLP complex do not affect this FCS analysis. As can be seen in Figure 5, both empty and bR-NLP complex (identified by cross-correlating Bodipy and Texas Red) diffused significantly faster than DMPC vesicles alone. However, this diffusion time was also significantly slower than $\Delta 49A1$, providing further evidence for the complex formation.

Interestingly, the shape of the diffusion curve of the bR-NLP complex was changed significantly when compared to empty NLPs and exhibited two plateaus (Fig. 5). These findings were very reproducible based on several repeat measurements (data not shown). Such a decay curve can be best understood with a two-species fit model. A potential explanation is based on the assumption that bR-loaded NLPs not only exhibit translational diffusion, but also a significant rotational diffusion, presumably due to their more anisotropic shape. Rotational diffusion is typically orders of magnitudes faster than translational diffusion and would give rise to a decay plateau on the microsecond timescale. The second plateau at slower diffusion times likely occurred due to a combination of the average translational diffusion time of both, empty NLPs and bR-loaded NLPs in the

samples. Accordingly, the bR-NLP curve was fitted to a 2-species diffusion model including contributions for both fast and slow diffusion (Fig. 6). To account for the contribution from empty NLPs in the mixture, the translational diffusion time obtained earlier for empty NLPs alone was used as a fixed fitting parameter (Supporting Information). This fit resulted in a fast (rotational) diffusion time of 5 μ s and a translational diffusion time of (350 ± 10) μ s for bR-loaded NLPs, which corresponded to a particle diameter of (7.8 ± 2.8) nm and was just slightly less than the diameter obtained for empty NLPs (10.4 ± 2.8) nm).

Figure 6. Normalized diffusion curve of bR-NLP complex. A 2-species rotational and translational diffusion model was used to fit the curve. After fixing 0.52 ms as the translational diffusion time of species 2 (empty NLPs), we found the translational diffusion time of species 1 (bR-loaded NLPs) to be 0.35 ms. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table II. Diffusion Times of Proteins and NLP Complexes

Sample	$\Delta 49A1$	bR	Empty NLPs	bR-loaded NLPs
Diffusion Time (ms)	0.027	0.065	0.52	0.35
Calculated	2.6	3.3	10.4	7.8
Diameter (nm)

Figure 7. 250 nm $\times$ 250 nm topographical AFM images of NLPs produced through cell free expression of (A) Δ 49A1 and (B) Δ 49A1 and bR in the presence of DMPC liposomes. The brighter regions in (B) indicate some NLPs were higher in height. (C) Scatter plot of NLP diameter and height for NLPs produced through cell free expression of Δ 49A1. (D) Scatter plot of NLP diameter and height for NLPs produced through co-expression of Δ 49A1 and bR. The two separate populations (black: empty NLPs, blue: bR-loaded NLPs) observed in (D) indicate that a fraction of the NLPs contained bR.

FCS and AFM infer the same insertion rate of bR in NLPs

Empty NLPs and bR-NLP complex formed by cell-free reactions were both examined by AFM to further assess NLP topology and to validate the association of bR with NLPs. bR-loaded NLPs showed a 1.4 nm increase in particle height relative to empty NLPs (Fig. 7, Table III), indicating both the association and insertion of bR into the NLPs. Empty NLPs displayed heights of (4.9 ± 0.2) nm as determined by AFM. Furthermore, bR-NLP isolated samples appeared as two distinct discoidal populations when examined by AFM cross sectional height analysis. The first population had an average height of (5.0 ± 0.3) nm, analogous to empty NLPs [black population in Fig. 7(D)]. The second population, which was not observed in prepared empty NLP samples, had a height of (6.4 ± 0.3) nm [blue population in Fig. 7(D)]. The increased height

observed in the presence of bR was located approximately in the center region of the NLPs [Fig. 7(B), bright green dot and pseudo color] and were consistent with bR being supported within the NLP lipid bilayer. Using solely the increase in height as a basis for distinguishing bR-loaded NLPs from empty NLPs, we were able to determine an overall yield of NLPs with bR insertion rate of 58% (a parameter defined as the percentage of bR-loaded NLPs relative to the total number of NLPs in the complex, Table III).

FCS data could also be used to calculate the bR insertion rates. The bR-NLP curve was fitted to a 2-species diffusion model including contributions for both rotational and translational diffusion (Supporting Information). Based on this model, the insertion rate of bR was determined to be $\sim 55\%$, which was consistent with the 58%, measured by AFM for the same samples.

Table III. Summary of AFM Data for Empty NLPs and bR-NLP Complex

Sample	Height (nm)	Diameter (nm)	% NLP
Empty NLPs	4.9 ± 0.2	18.2 ± 2.9	X
bR-NLP complex: empty	5.0 ± 0.3	17.9 ± 3.1	0.42
bR-NLP complex: loaded	6.4 ± 0.3	25.2 ± 5.8	0.58
<i>P</i> -value: bR empty vs. loaded	$4.4\text{E-}50$	$1.28\text{E-}16$	X
<i>P</i> -value: empty vs. bR empty	0.009	0.55	X

%NLP, percentage of the number of empty or loaded NLPs relative to the total numbers of NLPs in the mixture; X, not applicable. *P*-values were determined using a Two-tailed student *t*-test.

Discussion

We used NLPs as a complex model system to demonstrate the versatility of FCS for obtaining dynamic information regarding NLP complex formation when a membrane protein is incorporated into the lipid nanoparticle. Importantly, FCS was able to directly compare and contrast the expressed NLP complexes after cell-free self-assembly. This is the first study to attempt to characterize fully hydrated, freely diffusing membrane protein–NLP complexes using FCS. The wealth of information that FCS provided, as shown by our data, included particle diameter, diffusion times, insertion rate, and demonstration of protein–lipid interactions. Thus the utility of FCS for characterizing MP in native environments such as presented by inserting the MP in to a NLP scaffold should be considerable.

FCS studies may be contrasted directly with other biophysical techniques such as SEC and PAGE measurements. SEC and PAGE are the standard for identifying complex size differences of proteins, but are not very sensitive for observing extremely low concentrations of molecules that can be measured by FCS analysis. The techniques of TEM and AFM require immobilization of molecules on a solid surface, which might change the chemical or biological environment of MP inserted in NLPs. FCS avoids this complication by making measurements in solution phase. DLS is very simple and useful to characterize particle size, but it requires a homogeneous sample with monodispersed sizes, which means it requires strict purification to provide accurate particle size information. FCS avoids these requirements by utilizing cross-correlating measurements, which allows us to isolate complexes even in heterogeneous mixture.

By plotting normalized correlation versus lag time, FCS generated diffusion curves and inferred diffusion times for corresponding samples. Compared with bR-NLP complex, the bump in the curve at longer diffusion times for empty NLPs (Fig. 5) indicates that a larger size of them may be caused by some degree of aggregation. Also the simple 1-species translational diffusion model fits empty NLPs well (Supporting Information). The scattered points in the short lag time region represent the higher inaccuracy with which fast diffusion times can be determined, but their importance is somewhat amplified by the logarithmic scale of their overall contribution to the FCS fit analysis. Significantly less scattering along the y-axis was obtained for bR-NLP complex and their curve exhibited a clear bump at a diffusion time of ~ 5 μ s diffusion time (Fig. 5). These findings can be best explained by including a fast (rotational) diffusion term into the fit model for bR-NLP complex describing a stable discoidal shape, while empty NLPs may be more dynamic due to changes between discoidal and spherical shapes faster than the time

resolution of our FCS set up, when solubilized and freely diffusing in solution. This result obtained in an aqueous environment is in disagreement to the typically discoidal shape of empty NLPs observed, for example, from our AFM results (Fig. 7) and previously reported TEM studies.³⁵ If solubilized empty NLPs were contained as stable discs in solution, they should have equally presented a fast (rotational) diffusion term as observed for bR-NLP complex, and thus have significantly deviated from the simple 1-species translational diffusion model. The aggregation seen at longer diffusion times for empty-NLPs would not be able to compensate for the anisotropic property of individual empty NLPs either, if they were truly stable discs in solution. However, the experimental data were not consistent with these predictions and did not confirm a stable disc-like structure for empty-NLPs present in solution.

To explain the diffusion behavior of bR-NLP complex (a mixture of empty NLPs and bR-loaded NLPs) we had to resort to a fit model that accounts for a fast decay, likely due to rotational diffusion of disc-like particles (diffusion time: 4.9 ± 0.3 μ s), as well as additional terms describing two species with different translational diffusions (diffusion times 520 ± 70 μ s, and 350 ± 10 μ s). It was impossible to fit this diffusion curve with a simple 1-species translational diffusion model or a simple 2-species translational diffusion model. The adjusted 2-species translational and rotational diffusion model fit particularly well, when we fixed one of the translational diffusion terms as determined for empty NLPs (350 ± 10 μ s), while the second faster translational diffusion term also included the rotational term for bR-loaded NLPs. The faster translational diffusion time assigned to bR-loaded NLPs indicates that their average hydrodynamic diameter was about (2.6 ± 0.8) nm smaller than that of empty NLPs. This observation, combined with the additional fast rotational diffusion required to describe bR-loaded NLPs, indicates that bR-loaded NLPs are not as dynamic as empty NLPs in solution. The incorporation of the transmembrane protein to NLPs suggests that the lipids form a planar bilayer and the NLPs are more constrained as discoidal shape. This resulted in a more homogeneous size distribution for bR-loaded NLPs without the levels of aggregation as was seen for empty NLPs.

As shown in Figure 7 and Table III, the inclusion of the membrane protein leads to a larger height and a larger hydrodynamic diameter when measured by AFM (empty NLPs height/diameter: 4.9 nm/18.2 nm; bR-loaded NLPs height/diameter: 6.4 nm/25.2 nm). The discrepancy between AFM diameter data and FCS diameter data (empty NLPs: 10.4 nm, bR-loaded NLPs: 7.8 nm) can be readily explained since the solution-based model for FCS is not accessible to AFM measurements because NLPs

have to be immobilized on a surface, and will either collapse in the dry state or are compressed by the AFM tip. Combining the height data measured by AFM and the hydrodynamic diameter data measured by FCS (empty NLPs height/diameter: 4.9 nm/10.4 nm; bR-loaded NLPs height/diameter: 6.4 nm/7.8 nm), empty NLPs appear to be more disc-like than bR-loaded NLPs, because of an overall larger aspect ratio between the height and diameter. This, however, is not consistent with the observation of a fast (rotational) diffusion for bR-loaded NLPs in the hydrated state, which demonstrates their anisotropic property as discs but was not seen for empty-NLPs. Based on our AFM and FCS data, bR-loaded NLPs seem to retain their discoidal shape both, on a surface, and in solution, as evidenced by the additional fast diffusion component. Their translational diffusion time is faster than that of empty NLPs, because bR-loaded NLPs have a smaller hydrodynamic diameter. Empty NLPs has a larger hydrodynamic diameter while their shape/structure is more dynamic and less constrained in solution.

Summary

We have provided evidence that empty, hydrated NLPs adopt a different dynamic shape in solution, while membrane protein associated NLPs are more thermodynamically constrained as discs as illustrated by our FCS hydrodynamic measurements. The disc-like nature of bR-loaded NLPs can be inferred from both, a faster translational diffusion rate when compared to empty NLPs, and the observation of a fast rotational diffusion. This result was unexpected based on AFM height measurements, but may be readily explained by hydration effects of these nanoparticles. Importantly, bR-insertion rates obtained by both FCS and AFM techniques were found to be in excellent agreement, which further confirms the validity of FCS findings. This also illustrates the usefulness of FCS for characterizing NLP complex interactions.

In the future, since the kinetics of the NLP formation and membrane protein insertion is of great interest and FCS is capable of time-dependent measurement of *in situ* solution reactions, we expect FCS to observe insertion of different MP dynamically and to study their functions and interactions with ligands in a hydrated NLP system. We also should be able to determine subpopulations of NLPs more quantitatively and their effects on receptor-ligand interactions by utilizing more methods, such as emission anisotropy,⁴⁸ fluorescence lifetime,⁵² and accurate single particle size measurements.^{53,54}

Materials and Methods

Delta49A1 and bOp sequence, which encodes bR, were described previously.²¹ Preparative 1 mL reac-

tions were carried out using RTS 500 ProteoMaster Kit (Roche). A total of 5 μ g of delta49A1 encoding plasmid DNA was added to the lysate mixture along with added DMPC (Avanti Polar Lipids) vesicles to form the empty NLPs. For membrane protein co-expression, a total of 0.2 μ g of delta49A1 plasmid DNA and 1 μ g of bOp plasmid DNA were added to the cell-free mixture along with added DMPC vesicles and all-trans retinal. Preparation of 20 mg/mL DMPC stock solution was based on the instructions from Avanti Polar Lipids. The solution was stored in 4°C refrigerator at least 4 h before use. Small unilamellar vesicles of DMPC were prepared by probe sonicating 20 mg/mL aqueous solution of DMPC on ice for ~15 min until optical clarity was achieved. Texas Red labelled DMPC was prepared by mixing 99.5% (molar concentration) DMPC and 0.5% Texas Red[®] DHPE (Invitrogen) to form a solution at a total lipid concentration 25 mg/mL. Two minutes of centrifugation at 13,700 RCF was used to remove any metal contamination from the sonication probe tip. DMPC small unilamellar vesicles were added to the cell-free reaction at a final concentration of 2 mg/mL. All trans-retinal (Sigma) solution was prepared with 100% ethanol at a stock concentration of 0.586 mM or 10 mM. The stock solution was diluted with water to achieve a final working concentration of 30 ~ 50 μ M in cell-free reactions. The proteins were labelled by Bodipy[®]-FL through adding 5 μ L of FluoroTect[™] GreenLys (Promega) into the reaction mixture for *in vitro* translation. After 4-h incubation at 30°C, the soluble fraction was obtained by centrifuging the completed reactions at 14,000g for 5 min.

Immobilized metal affinity chromatography was used to isolate the proteins of interests from the cell-free reaction mixture. The soluble fraction (~1 mL) was mixed with 1 mL Ni-NTA Superflow resin (Qia-gen) according to the manufacturer's protocol using native purification conditions with the following modifications. For washing the column, 6 column volumes of buffer, 10–50 mM imidazole in Phosphate Buffered Saline (PBS) was used. A total of 6 mL of elution buffer (400 mM imidazole in PBS) was used to elute the bound protein from the column in 1 mL aliquots. All of the elution fractions were combined, concentrated and buffer exchanged into Tris Buffered Saline (TBS) using a 100 K molecular weight sieve filters (Vivascience) to achieve a final volume of ~200 μ L. This material was used for further characterization.

Five microliter aliquots of purified bOp, empty NLPs, and bR-NLP complex were diluted with 2 $\times$ sample loading buffer with reducing agents (Invitrogen), heat-denatured at 95°C for 10 min. Samples were loaded onto 4–12% gradient precast NuPAGE Bis-Tris gel (Invitrogen) along with the molecular weight standard NovexSharp (Invitrogen) and run

using NuPAGE MES-SDS running buffer (Invitrogen). Samples were electrophoresed for 40 min at 250 V. Gels were imaged with a GE Typhoon 9410 using a 488 nm laser with a 520/40 nm band pass filter. Equal amounts of purified empty NLP and bR-NLP complex samples (<20 µg) were diluted with 2× native gel sample buffer (Invitrogen). The samples were loaded onto 4–12% gradient precast Tris-glycine gels (Invitrogen) along with the molecular weight standard NativeMark (Invitrogen). They were run using Novex Tris-glycine Native Running Buffer (Invitrogen). Samples were electrophoresed for 2 h at 125 V. Gels were imaged with a GE Typhoon 9410 using a 488 nm laser with a 520/40 nm band pass filter.

All FCS measurements were performed on a MicroTime 200 single molecule fluorescence lifetime measurement system (PicoQuant). A modified Olympus 1X71, equipped with an Olympus UPlanAPO NA 1.45 oil immersion objective, served as a base of the microscope. As excitation source, a pulsed laser diode with a repetition rate of 20 MHz was used. For calibration standards, Alexa Fluor 488 (Invitrogen) emits at 520 nm; Atto 655 (Sigma-Aldrich) emits at 684 nm; two polystyrene beads (FluorSpheres®, Life Technologies) emit at 514 nm; two extruded Texas Red-DMPC lipid vesicle samples emit at 613 nm. They were all excited with a 470 nm laser except Atto 655 (excited at a 640 nm laser) and detected through bandpass filters. Proteins labelled with Bodipy®-FL dye were excited at 470 nm for analysis based on their 510 nm emission. The beam was directed into the microscope by reflecting it off a dichroic mirror (z467/638pc). The focus point was placed 5 µm above the cover slip surface, so the FCS measurements can be performed at a defined distance to the cover slip surface. The fluorescence emission was focused on a pinhole. The pinhole size was set to 50 µm. After the pinhole, the fluorescence light was divided via a 50/50 beam splitter cube, passed an emission filter and focused on two SPCM-AQR SPAD detectors (Perkin Elmer). All measurements were performed using the SymPhoTime Software (PicoQuant). Data analysis was performed using IGOR Pro 6 and OriginPro 8.

The DLS measurements were performed on a Nanotracer Particle Size Analyzer (Microtrac). The AFM measurements were performed on Asylum MFP-3D-CF AFM. Images were captured in tapping mode with minimal contact force and scan rates of 1 Hz. Asylum software was used for cross-sectional analysis to measure NLP height and diameter. Height and diameter were measured from 182 empty NLPs produced by cell-free expression. Height and diameter were also measured for totally 440 NLPs produced by cell-free co-expression, 185 were found to be empty-NLPs and 255 were found to contain bR. Two-tailed student T-tests were run to compare

both the height and diameter of the empty-NLP population in bR-NLP complex compared to empty NLPs only. A *P*-value of <0.01 was considered significant.

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