Mechanical Tension of Biomembranes Can Be Measured by Super Resolution (STED) Microscopy of Force-Induced Nanotubes

Debjit Roy, Jan Steinkühler, Ziliang Zhao, Reinhard Lipowsky, and Rumiana Dimova*

ABSTRACT: Membrane tension modulates the morphology of plasma-membrane tubular protrusions in cells but is difficult to measure. Here, we propose to use microscopy imaging to assess the membrane tension. We report direct measurement of membrane nanotube diameters with unprecedented resolution using stimulated emission depletion (STED) microscopy. For this purpose, we integrated an optical tweezers setup in a commercial microscope equipped for STED imaging and established micropipette aspiration of giant vesicles. Membrane nanotubes were pulled from the vesicles at specific membrane tension imposed by the aspiration pipet. Tube diameters calculated from the applied tension using the membrane curvature elasticity model are in excellent agreement with data measured directly with STED. Our approach can be extended to cellular membranes and will then allow us to estimate the mechanical membrane tension within the force-induced nanotubes.

KEYWORDS: giant vesicles, membrane nanotubes, STED microscopy, micropipette aspiration, membrane tension, optical tweezers

INTRODUCTION

Cellular membranes are found to attain a multitude of morphologies and often exhibit highly curved segments with certain functionality. In particular, highly curved membrane nanotubes are involved in several cellular functions such as cell migration, signaling, remote communication and motility, and cell spreading. Tunneling membrane nanotubes also play an important role in transfer of cellular content (small molecules, proteins, prions, viral particles, vesicles, and organelles) in a variety of cell types as well as electrical signals. During migration, tubular membrane protrusions (also referred to as retracting fibers) are formed behind the migrating cell and are responsible for resealing cellular content. In all of these examples, when not supported by the underlying substrate, membrane shape is modulated by membrane tension which affects the membrane surface area and morphology. Membrane tension thus provides a link between membrane mechanics, morphology, as well as mechanical transduction in the cell, for example, via tension-sensitive membrane channels. However, how cellular tension is regulated and mechanobiological cues are perceived by the cell is poorly understood.

In principle, plasma membrane tension can be indirectly inferred from nanotube pulling experiments where the membrane diameter and force of pulling could be used to extract membrane mechanical parameters such as tension, bending rigidity, and spontaneous curvature. Tension-sensitive probes with fluorescence decay lifetimes depending on tension have also been recently introduced. However, it is unclear how curvature and local probe concentration increase due to sorting mechanisms affects the dye performance. Apart from providing the means to assess the membrane bending rigidity and tension, tube pulling experiments also allow the study of cellular processes that take place at highly curved membranes. In these experiments, the cell or vesicle is immobilized or more often aspirated by a micropipette setting the membrane tension, and a tube is typically pulled by means of optical-tweezer manipulation of a bead attached to the membrane. For a fixed bending rigidity of the membrane, the tube radius depends on membrane tension and thus measuring the radius allows assessing this mechanical parameter. However, membrane nanotube diameters are not directly accessible via diffraction-limited microscopy imaging and these limitations obstruct progress in the field.

Here, we measure for the first time the diameter of membrane nanotubes directly using stimulated emission depletion (STED) nanoscopy as a function of membrane tension in a controlled reconstituted system. To form membrane nanotubes, we employ giant unilamellar lipid

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vesicles (GUVs). GUVs represent a popular model system of cellular membranes as their response to external factors as well as thermodynamic state can be visualized under the optical microscope. In addition, they are amenable to micromanipulation (see Chapters 11 and 16 of ref 28). Pulling a membrane nanotube (also referred to as tether) provides such a micromanipulation protocol, in which a cylindrical membrane segment with a diameter ranging between 20 nm to few hundreds nanometers is extruded from the GUV. Controlled membrane nanotubes can be generated by hydrodynamic flow (both inward and outward tubes with respect to the vesicle body can be pulled), gravity, micromanipulation, and magnetic or optical tweezers (see also overview in ref 41), whereby tube formation is enforced by a localized pulling force.

We use micropipettes to aspirate and hold the vesicle in place and to modulate the membrane tension by adjusting the aspiration pressure. The imposed tension mimics cellular conditions corresponding to the cortical tension. To pull the nanotube from the vesicle, a sticky microsphere, trapped by the optical tweezers, is used as a handle. For a GUV being aspirated at a suction pressure, at which the GUV tongue inside the micropipette is longer than the micropipette radius, the total membrane tension is given by

\[ \Sigma = \Delta P_{\text{asp}} \frac{R_y R_p}{2(R_y - R_p)} + 2\kappa m \left( \frac{1}{R_p} + \frac{1}{R_y} \right) \]

where \( R_y \) and \( R_p \) are the respective radius of the vesicle and micropipette, \( \Delta P_{\text{asp}} \) is the aspiration pressure of the micropipette, \( m \) is the membrane spontaneous curvature, and \( \kappa \) is the bending rigidity of the membrane. The first term in eq 1 is the aspiration tension, for which we will use the notation \( \Sigma_{\text{asp}} \). The trapped bead, located at the terminal of an outward nanotube pulled from the aspirated GUV, experiences a force

\[ f_y = 2\pi \sqrt{2\Sigma} - 4\pi \kappa m - \frac{\pi \kappa}{R_y} \]

which contains two terms that depend on the spontaneous curvature \( m \), because the total membrane tension \( \Sigma \) depends on the spontaneous curvature as well, see eq 1. In our experiments, the spontaneous curvature is so small that we can ignore these two \( m \)-dependent terms; further below, we will justify this condition for the system we explore. As a consequence, the total membrane tension \( \Sigma \) reduces to the first term in eq 1, which represents the aspiration tension \( \Sigma_{\text{asp}} \). Furthermore, for negligible spontaneous curvature, the aspiration tension becomes equal to the mechanical tension which can now be deduced from the aspiration geometry and the aspiration pressure. In addition, the last term in eq 2 can be ignored because of the small mean curvature of the vesicle.

To perform tube pulling experiments, we switch between confocal fluorescence and bright-field imaging: the membrane nanotube is visualized in confocal mode and the force on the trapped object is obtained from bead position recorded in bright-field mode. At a fixed tube length, the force \( f_y \), acting on the trapped bead, can be estimated from the beam off-center displacement from the trap axis, \( \Delta x \), as \( f_y = k_b \Delta x \), where \( k_b \) is the trap constant determined independently, see Experimental Section. From the dependence \( f_y \) versus \( \Sigma_{\text{asp}} \), one can deduce the bending rigidity. Alternatively, this material property could be assessed from the membrane nanotube radius, if the latter could be measured: the radius of a cylindrical tube \( R \), depends on the aspiration tension through the relation

\[ R = \sqrt{\frac{\kappa}{2\Sigma_{\text{asp}}} \sum_{\text{asp}}} \]

In general, the tube radius is smaller than the resolution of the optical microscope (~200 nm), which makes it impossible to measure it directly. AFM imaging could be used when the tubes adhere to a substrate but, as a result of this adhesion, the tube morphology will be deformed into a noncylindrical shape which can no longer be described by the tube radius alone. In other studies, the tube radius is estimated indirectly from correlating the fluorescence intensity count. However, it is unclear how curvature influences the dye performance. Moreover, dye sorting taking place in membrane nanotubes necessarily affects the tube fluorescence intensity and thus the measurement of the nanotube diameter. The radii of spontaneously formed tubes (not pulled by tweezers) can be also roughly inferred from spontaneous curvature measurements (see pages 9–11 in ref 52 for a review of approaches to measure the membrane spontaneous curvature), but an assumption has to be made for the shape of the tube (cylindrical or necklace-like).

With the advent of super resolution microscopy, the optical microscope resolution has been improved to a few tens of nanometers and thus, in principle, can be used to measure nanotube diameters. Previously, STED microscopy has been used to study the membrane heterogeneity and to assess dimensions of endoplasmic reticulum structures using point spread function fitting. It has advantages over other super resolution microscopic techniques such as photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), which require the acquisition of a high number of images (typically a few thousand frames) and are thus slower imaging techniques. In this Letter, we report direct measurement of membrane nanotube diameters with unprecedented resolution using STED microscopy. For this purpose, we have integrated an optical tweezers setup in a microscope equipped for STED imaging.

**EXPERIMENTAL SECTION**

** Vesicle Preparation and Characterization.** GUVs were grown from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) doped with 0.1 mol % biotinyl cap phosphatidylethanolamine (PE) (both from Avanti Polar Lipids) and 0.5 mol % ATTO 647N dye (AttoTech) using electroformation in 100 mOsm/kg sucrose solution; for details see Section S1 in the Supporting Information (SI). Occasionally, we also explored vesicle membranes containing cholesterol (Chol), namely at POPC/Chol 9:1 molar ratio. An optically trapped streptavidin-coated bead of diameter ~2 μm adhered to the vesicles due to biotin–streptavidin bonding. The vesicles were diluted in isotonic medium of 40 mM glucose and 30 mM sodium chloride solution. This external solution was chosen (i) to enhance the optical contrast of the vesicle in phase-contrast observation but avoid vesicle deformation by gravity, (ii) to ensure strong biotin–streptavidin binding, which requires the presence of sodium chloride, and (iii) to establish conditions of low asymmetry across the membrane so that the spontaneous curvature is negligible (at these
conditions the spontaneous curvature is comparable to the mean curvature of the GUVs and the last two terms in eq 2 can be ignored). The bending rigidity of the membrane was measured from fluctuation analysis according to previously published protocol,62 see Section S2 in the Supporting Information. All experiments were performed at ∼22 °C. MATLAB (2014a) and Origin 2015 were used for the image and data analysis.

Experimental Setup. In our experiment, a membrane nanotube is extruded from an aspirated GUV using an optically trapped microsphere (Figure 1). The setup includes three parts

![Scheme of the experimental approach of pulling membrane nanotubes and example line scans acquired with confocal and STED microscopy.](image)

### RESULTS AND DISCUSSION

GUVs with diameters typically between ∼20 to 25 μm were aspirated via micropipettes with diameters of 3 to 5 μm. A floppy GUV was chosen (see Movie S1 for an example), aspirated by the micropipette at a low aspiration pressure and brought into the contact with streptavidin-coated bead trapped by the optical tweezers. The low vesicle tension allowed us to achieve a larger contact area of the bead with the membrane (occasionally the vesicle was displaced so that the position of the bead was well inside the GUV interior but still engulfed by the membrane). After waiting for few seconds, the aspirated GUV was moved away from the trapped bead and a membrane nanotube was extruded from the vesicle due to strong biotin–streptavidin noncovalent bonding. In all experiments, we kept the length of the enforced nanotube to be between 8 and 10 μm. By doing so, the hydrodynamic contribution arising from the vesicle wall is minimized.63 If the tube is shorter, not only the noncylindrical part of the vesicle and the noncylindrical part of the nanotube extrusion as seen in confocal fluorescence imaging (c) Confocal and (d) 3D STED image of a small portion of the extruded nanotube (scale bars correspond to 500 nm). (e, g) Schematic illustration and (f, h) experimentally acquired data from line scans (gray bands in panels e and g) across a membrane nanotube (red cylinder) when using confocal (e, f) and 3D STED (g, h) imaging; for lucidity, the rough dimensions of the scanning voxels are illustrated as gray ellipsoids in (e, g).
images with significant S/N ratio and higher effective resolution compared to 2D STED imaging. The STED resolution was measured using 20 nm beads and found to be <40 nm in both x- and y-axes (SI Section S3.3 and Figures S3 and S4). Therefore, in a STED line scan across the tube, the two wall-crossings of a membrane nanotube with radius larger than 20 nm should be, in theory, resolvable under these system settings. However, due to the inherent vibrations of the micropipette (~31 nm, over six measurements of the positional fluctuations of the micropipette tip), the thermal motion of trapped beads (~15 nm, obtained from the trap stiffness, which was measured to be 74 ± 2 pN/μm, see SI Section S3.1), and the GUV itself, the membrane nanotubes are found to laterally fluctuate with an amplitude of the order of few hundred nanometers in the y-direction (see Figure 2a), which is in the range of the expected tube diameter. As a result, in a major fraction of the line scans in a kymograph, instead of two clearly defined peaks (as sketched in Figure 1g), we detect several noisy maxima. The appearance of multiple peaks was reduced to some extent by adjusting the pixel size to 20 nm (at lower pixel size, the scans were significantly noisier, see SI Figure S6). Larger pixel sizes were not explored as the resolution of the STED microscope was found to be <40 nm in both x- and y-axes while the pixel size is typically kept about half of STED resolution as a rule of oversampling. To reduce contributions from nanotube fluctuations, the line scans were aligned (Figure 2c, see also Section S4 in the Supporting Information). Subsequent averaging allowed identifying two clearly resolved fluorescence maxima arising from the two tube wall-crossing of the line scan; see Figure 2d. STED line scans that did not show two clear peaks after applying all of the above-mentioned steps were discarded from the analysis; these discarded line scans represented approximately 56% of all scans collected and result from out-of-focus displacement, micropipette vibration, and membrane fluctuations. We denote the tube diameter determined from this interpeak distance as 2Rs,STED and measured it for different aspiration pressures ranging between 15–140 × 10⁻⁶ N/m; see Figure S7 in the Supporting Information.

To avoid excessive photobleaching and decrease in fluorescence signal of the pulled nanotubes, each measurement at a given tension was performed only once. To estimate the precision of the image processing, we performed three repeat measurements on a single tube and found the standard deviation to be 11 nm.

We then aimed at comparing the tube diameter measured from the STED images, 2Rs,STED, with the tube diameter, 2Rv, independently assessed from the applied aspiration pressure following eq 3. For this, a precise knowledge of the membrane bending rigidity is required. The bending rigidity was measured with two independent methods. Analysis of the thermal fluctuations of free GUVs using a previously established protocol yielded for the bending rigidity 23 ± 2 kBT as assessed on five different vesicles (see Section S2 in the Supporting Information). We also measured this membrane elastic property from tube pulling experiments using eq 2 which gave a bending rigidity value of 23 ± 5 kBT as assessed from measurements on different vesicles (see Section S5 in the Supporting Information). The results from the two approaches are in excellent agreement and are consistent with previous data.

Using the obtained value for the bending rigidity (κ = 23 kBT), we compared the diameters of tubes directly measured from the STED images, 2Rs,STED, with the respective tube diameters, 2Rv, independently assessed from the imposed membrane tension following eq 3. For the vesicles made of POPC/Chol we took κ = 32.5 kBT corresponding to the linearly proportional increase in the bending rigidity upon the incorporation of 10 mol % cholesterol in POPC membranes as reported in ref 68. The comparison shown in Figure 3 demonstrates that the experimental STED data and estimates from the elastic sheet model (with independently measured bending rigidity) are in excellent agreement. Presumably, for tube diameters approaching 50 nm and below, the accuracy of the STED measurements does not allow precise determination because the tube diameter reaches the size of a couple of pixels.

**CONCLUSIONS**

We have shown for the first time that super-resolution microscopy like STED can be used to directly measure the membrane nanotube diameter. For membrane tubes pulled in a controlled fashion from GUVs aspirated in micropipettes, the tube diameter measured microscopically is in excellent agreement with estimates inferred from knowledge of the membrane tension and membrane rigidity. Thus, we provide the first direct evidence for the validity of the widely used curvature elasticity model for nanotubes down to tube diameters of 50 nm. STED imaging of tubes pulled from vesicles of known bending rigidity offers a means of assessing the membrane tension without the need of operating micromanipulation setups such as micropipette aspiration.

In the current paper, we were able to measure the three quantities that enter eq 3 independently: the tube radius by STED, the aspiration tension Σmph from the aspiration pressure,
rigidity of cell membranes. Measurements of material characteristics such as tension and the bending rigidity by tube pulling. In this way, we were able to confirm eq 3 directly, see Figure 3. As it stands, eq 3 is based on the implicit assumption that the mechanical membrane tension is laterally uniform and that the mechanical tension Σ within the tube is equal to the aspiration tension. The latter assumption is, however, unnecessary. In fact, the mechanical balance within the nanotube leads to a slightly modified and more general form of eq 3 for which the aspiration tension is replaced by the tube tension Σ. As a consequence, the tube tension is given by Σ = k/(2R^2). The latter relation can be used to obtain the mechanical tube tension from the measured values of the bending rigidity and the tube radius. It will be interesting to use this more general form of eq 3 to estimate the tube tension of plasma membranes, combining the previously obtained bending rigidity of these membranes with the tube radius as measured by STED.

Furthermore, assessing the tube tension from super-resolution imaging as introduced here could be applied, for example, to study (i) the dynamics of migratory cells leaving behind membrane nanotubes from which migratosomes with signaling material will be released, (ii) curvature coupling of proteins to highly bent membranes, as well as (iii) flows and tension propagation in cells (it has already been shown that under the right conditions, STED microscopy can be applied to live-cell imaging without inducing substantial photodamage). The analysis described here was limited to symmetric membranes with zero spontaneous curvature. Comparing the minimal forces needed to pull out tubes and the second term in eq 2 suggests that our approach can be extended to asymmetric membranes for which the magnitude of the spontaneous curvature is comparable to or larger than 1/(240 nm). The imaging methodology developed here based on measuring tube diameters with STED offers access to direct measurements of material characteristics such as tension and rigidity of cell membranes.

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