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Dynamic Light-Induced Protein Patterns at Model Membranes

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Abstract

The precise localization and activation of proteins at the cell membrane at a certain time gives rise to many cellular processes, including cell polarization, migration, and division. Thus, methods to recruit proteins to model membranes with subcellular resolution and high temporal control are essential when reproducing and controlling such processes in synthetic cells. Here, a method is described for fabricating light-regulated reversible protein patterns at lipid membranes with high spatiotemporal precision. For this purpose, we immobilize the photoswitchable protein iLID (improved light-inducible dimer) on supported lipid bilayers (SLBs) and on the outer membrane of giant unilamellar vesicles (GUVs). Upon local blue light illumination, iLID binds to its partner Nano (wild-type SspB) and allows the recruitment of any protein of interest (POI) fused to Nano from the solution to the illuminated area on the membrane. This binding is reversible in the dark, which provides dynamic binding and release of the POI. Overall, this is a flexible and versatile method for regulating the localization of proteins with high precision in space and time using blue light.

Introduction

The formation of protein patterns on cell membranes within subcellular regions gives rise to numerous biological processes, including migration, division, and localized cell-to-cell communication[1,2]. These protein patterns are regulated in space and time, and are highly dynamic. Replicating such protein patterns in synthetic cells is essential for mimicking cellular processes that arise from them and for gaining a better understanding of how such regulation works at a molecular level. Analogous to what is observed for membranes in living cells, methods for generating protein patterns on artificial membranes must capture their dynamics and provide precise spatiotemporal control.

Among various stimuli, light stands out for providing the highest spatiotemporal control and several additional advantages[3]. Through regulation with light, it is straightforward to illuminate a desired area at any desired time with unmatched precision. In addition, light provides high tunability as both the light intensity and the pulse durations can be adjusted. Furthermore, visible light is harmless to biomolecules, including proteins, and it is

possible to even address multiple functionalities with different wavelengths. Hence, light-responsive approaches based on visible light emerge as promising avenues for the controlled and biorthogonal regulation of protein patterns in space and time[4,5,6]. Utilizing photoswitchable protein pairs from optogenetics, acting as light-inducible dimerizers, provides a straightforward method for recruiting specific proteins to membranes. In particular, protein patterns have successfully been formed on artificial membranes using the blue light-triggered interaction between iLID (improved light-inducible dimer, based on the photoswitchable LOV2 domain from *Avena sativa*) and Nano (wild-type SspB)[7,8], the blue light-inducible SpyTag system (BLISS)[9], the green light-responsive protein tetramer CarH[10] and the red light-inducible interaction between PhyB and PIF6[11].

It has been demonstrated that the photoswitchable interaction between iLID and Nano[5] can be used to photo-pattern proteins on model membranes using blue light[7]. The iLID/Nano interaction is reversible in the dark, highly specific, and operates under physiological conditions. Anchoring iLID onto lipid membrane models, such as giant unilamellar vesicles (GUVs) or supported lipid bilayers (SLBs), enables light-regulated recruitment of Nano to these membranes, which is reversible in the dark. Notably, we observed that introducing a disordered domain to the N-terminus of iLID (resulting in a protein named disiLID) as a tether to a model lipid membrane enhances Nano recruitment efficiency and reversion dynamics[8].

By employing the disiLID/Nano interaction, we have developed a method for generating high contrast patterns of Nano-fused proteins of interest (POI) on SLBs and the external membranes of GUVs. This method allows for the creation of protein patterns with remarkable spatial and temporal resolution and high reversibility within minutes. The detailed protocol outlines the process for locally recruiting proteins onto artificial membranes. Specifically, this is achieved by immobilizing a biotinylated version of disiLID on SLBs and GUVs through the biotin-streptavidin (SAv) interaction. Subsequently, fluorescently labeled Nano (mOrange-Nano) is recruited to these disiLID functionalized membranes under blue light illumination. Our experimental protocol offers a straightforward and adaptable approach for achieving localized protein recruitment to membranes. Importantly, this methodology is not confined to the reported SLB and GUV interfaces or mOrange-Nano; it can be extended to other disiLID-functionalized materials and proteins fused to Nano.

Protocol

1. Experimental preparation

1. Express and purify biotinylated-disiLID (b-disiLID) and mOrange-Nano (see **Table of Materials**) by following previously reported procedures[7,8].
2. Prepare lipid mixtures in glass vials with the selected lipid composition and concentrations. First, dissolve lipids in chloroform to obtain a final lipid solution with 1 mg/mL concentration.
    1. Mix lipids in order to obtain a composition of 94.9 mol% 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 5 mol% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamineN-(cap biotinyl) sodium salt (DOPE-biotin) and 0.1 mol% 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine (DiD) (see **Table of Materials**).

NOTE: The lipid mixture can be adjusted with varying ratios and DOPE-biotin concentration and/or different membrane dyes. The recommended DOPE-biotin concentration (5 mol%) allows the formation of a high-density Streptavidin (SAv) layer in the following steps.

3. Prepare small unilamellar vesicles (SUVs) following previously reported methods[7,8,12]. For this step, it is recommended to prepare SUVs with a diameter ≤100 nm.

4. For this study, sonication method is employed. First, evaporate the chloroform solution in the glass vial with a nitrogen stream while rotating the vials in order to form a thin lipid film. Next, remove the residual chloroform for at least 1 h under vacuum.

    1. Rehydrate the dried film in ultrapure water with a final concentration of 1 mg/mL lipids by vortexing. Finally, sonicate the obtained solution for 10 min until the opaque solution turns clear.

        NOTE: Store the lipid mixture in a microcentrifuge tube in the refrigerator for a maximum of 2 weeks. Different SUV preparation methods (e.g., extrusion method) can also be used as long as the size of the final SUVs is ≤ 100 nm.

2. mOrange-Nano recruitment to disiLID functionalized SLBs

1. Add 150 µL of 2 M NaOH in each well of the µ-slide 18 well glass-bottom chamber (see **Table of Materials**) and incubate for 1 h at room temperature. Subsequently, remove the NaOH and wash the wells 3-5 times first with 150 µL ultrapure water, and then 3 times with 150 µL buffer (10 mM Tris pH 7.4, 100 mM NaCl) containing 10 mM $CaCl_2$.

2. Add 15 µL of freshly prepared SUVs (stock concentration 1 mg/mL in water) into the wells containing 150 µL buffer with 10 mM $CaCl_2$ in order to have approximately a factor 10 dilution of SUVs in the buffer. Let the SUVs incubate for 30 min at room temperature. After incubation time, biotinylated-SLBs will be formed.

3. Wash the SLBs at least 7 times with buffer (10 mM Tris pH 7.4, 100 mM NaCl) without $CaCl_2$ by first removing the solution and subsequently adding fresh buffer in each step. It is recommended to use 80 µL of buffer for each washing step.

    NOTE: An optimal washing of the SLBs is obtained by pipetting fresh solution up and down several times without touching the surface. Pipetting of the solution in the wells containing the newly formed SLBs should be gentle in order to reduce the formation of small air bubbles that would damage the formed SLBs. From this moment onwards, wells should contain a sufficient volume of buffer in order to prevent the SLBs from drying out.

4. For the further functionalization of the biotinylated SLBs with SAv, add a solution of SAv to a final concentration of 250 nM and incubate for 30 min at room temperature. Subsequently, remove the excess of SAv by washing it with a buffer (10 mM Tris pH 7.4, 100 mM NaCl) at least 5 times.

5. From this moment onwards, keep the samples under a protective red light in order to avoid unwanted photoactivation of photoswitchable proteins. Add b-disiLD (see **Table of Materials**) to a final concentration of 1 µM in the well. After 30 min incubation at room temperature, remove the excess protein by washing with buffer at least 5 times.

6. Add mOrange-Nano (see **Table of Materials**) to a final concentration of 200 nM and keep the sample in the dark by covering it with aluminum foil.
7. Place the µ-slide under the fluorescence microscope and adjust the imaging settings. Set the 552 nm laser for excitation of mOrange-Nano. Adjust the emission range to optimize the mOrange signal. The photoactivation of disiLID is achieved with a 488 nm laser, using light pulses with 2.58 s intervals.

3. Preparation of GUVs

1. Prepare a 5% (w/v) solution of polyvinyl alcohol (PVA, see **Table of Materials**) (MW: 145 000 g/ mol) with 100 mM sucrose in ultrapure water, mixing overnight at 80 °C at 400 rpm.
2. Prepare a lipid solution in chloroform with a desired composition (final concentration 10 mg/mL). For this method, it is recommended a composition consisting of 10 mg/mL POPC, 10 mol% 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG), 2 mol% DOPE-biotin and 1 mol% DiD (see **Table of Materials**).
3. Prepare GUVs with the hydration technique[7,8]. First, spread 40 µL of the prepared PVA solution as a homogeneous thin layer on top of a 60 mm x 24 mm glass slide, preferably with a pipette tip. Next, dry the thin layer at 50 °C for 30 min.
4. Spread 5 µL of the lipid solution with a needle on the PVA layer and let dry at 30 °C for 1 h.
5. Assemble a chamber on the functionalized glass slide using a spacer (~40 mm × 24 mm × 2 mm, see **Table of Materials**) and a second glass slide.
6. Add 1 mL of rehydration buffer (10 mM Tris pH 7.4, 100 mM NaCl) into the chamber for 1 h at room temperature to form GUVs. After 1 h, invert the chamber and gently tap on the glass surfaces with a pipette tip.
7. Carefully remove the glass slide on one side to open the built chamber and harvest the GUVs with a pipette.
8. Place the solution in a plastic tube and let the GUVs settle for 2 h.

4. mOrange-Nano Recruitment to disiLID functionalized GUVs

1. Add a solution of SAv to the freshly harvested GUVs and let sit for 30 min at room temperature.
   NOTE: The following steps should be performed with protective red light in order to avoid photoactivation of disiLID.
2. Add 1 µM of b-disiLID to the GUVs solution and place the sample for 30 min in the dark, covering it with aluminum foil.
3. Pretreat a µ-slide 18-well glass bottom chamber with 150 µL BSA solution (3% w/v in water) for 10 min. Next, remove the BSA solution and wash the wells with 150 µL ultrapure water 3 times.
4. Add 145 µL of 200 nM mOrange-Nano in buffer (10 mM Tris pH 7.4, 100 mM NaCl) to the well.
5. Next, add 5 µL of GUVs decorated with b-disiLID to the solution and wait ~15 min for the GUVs to settle.
6. Place the µ-slide under the confocal microscope. Excite the sample at 552 nm to visualize mOrange (λ_{ex} = 557 nm; λ_{em} = 576 nm) fluorescence and at 638 nm to visualize DiD (λ_{ex} = 644 nm; λ_{em} = 665 nm) in the GUV membranes. The mOrange-Nano recruitment

is triggered with blue light pulses (488 nm, intensity 1%) every 5.3 s in order to minimize undesired photobleaching effects.

NOTE: The excitation wavelengths can be adapted based on the type of microscope in use. Other common excitation wavelengths available for microscopes are also 532 nm or 561 nm and 633 nm, 647 nm, 639 nm, or 640 nm lasers.

Representative Results

The described procedures allow the formation of SLBs to recruit mOrange-Nano on the synthetic membranes. The formation of defined mOrange-Nano patterned on the SLBs functionalized with b-disiLID is shown in **Figure 1A**. As a squared (24 µm × 24 µm) region of interest (ROI) on the SLB is illuminated with 488 nm blue light, a rapid increase of fluorescence signal is observed in the mOrange channel (shown in red) in the ROI within 200 s. The pattern shows very defined and sharp edges (**Figure 1B**), indicating high spatial control over the photoactivated area. The interaction is fast and fully reversible as the illumination with blue light is interrupted. This method also allows the formation of patterns over several illumination cycles (**Figure 2**). Alternate cycles of ~200 s of blue light and 200 s of dark lead to reversible recruitment of mOrange-Nano in the selected area for multiple times with comparable values of Δ intensity of fluorescence in the patterns.

Figure 3 shows the schematic representation of the GUVs preparation. The recruitment of mOrange-Nano is also observed on GUVs. It is shown that GUVs placed in the dark do not exhibit mOrange fluorescence (**Figure 4A**). As the GUVs are globally illuminated with blue light, mOrange fluorescence is observed, colocalizing with the GUV membrane dye (DiD). The interaction is highly reversible as the illumination is terminated. The quantification of the mOrange intensity at the GUV membrane over time shows the fast and effective recruitment of proteins as well as the full reversibility (**Figure 4B**).

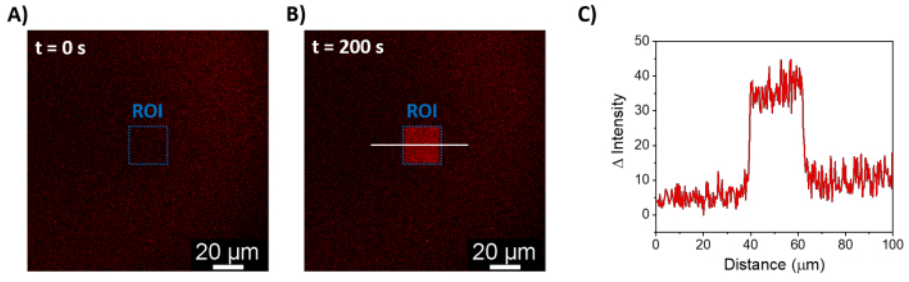

Figure 1: Fluorescence microscopy images of SLBs functionalized with b-disiLID. The fluorescence images in the presence of mOrange-Nano before (**A**) and during (**B**) local blue light (488 nm) illumination in the ROI. Scale bar = 20 µm. (**C**) Fluorescence intensity of mOrange measured in the ROI for SLBs functionalized with b-disiLID (at = 200 s). The figure is adapted from Di Iorio et al.[8]. Please click here to view a larger version of this figure.

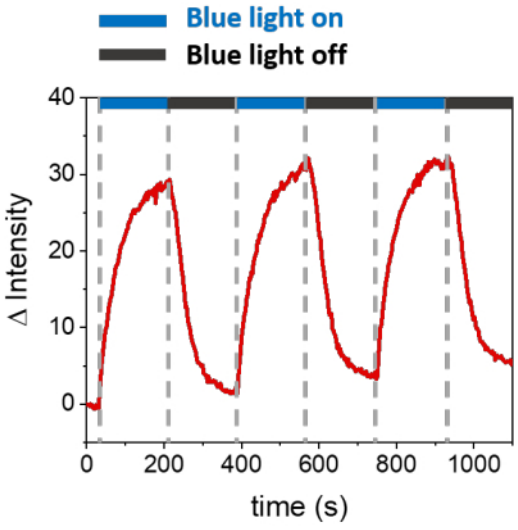

Figure 2: Fluorescence intensity of mOrange recruited in the ROI on SLBs decorated with b-disiLID during three recruitment cycles. After each photoactivation step, the mOrange-Nano fluorescence increased within the ROI. The pattern reaches saturation within 120 s, and the fluorescence decreases within 120 s, almost to background levels. No loss of pattern quality is observed over different blue light/dark cycles. The figure is adapted from Di Iorio et al.[8]. Please click here to view a larger version of this figure.

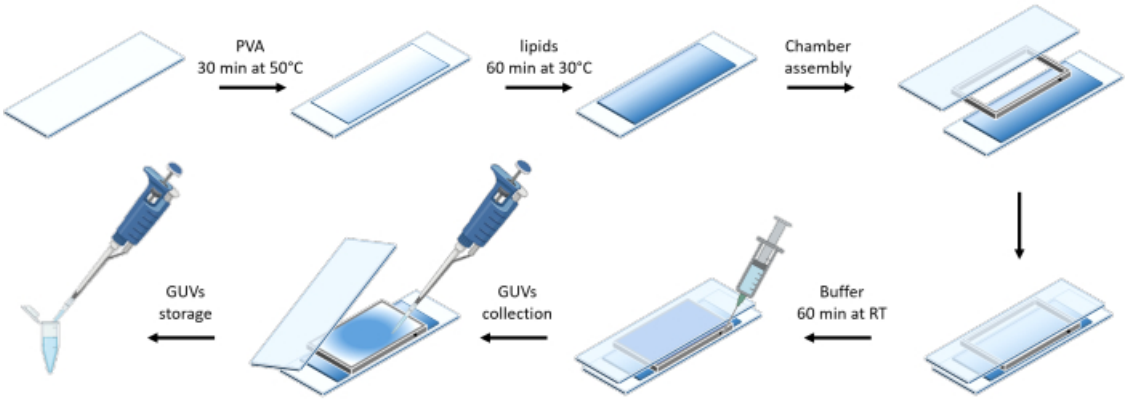

Figure 3: Schematic representation of the GUVs preparation using the gentle hydration method. The scheme offers a visual representation of the several steps and of the chamber built using two glass slides and a spacer. Please click here to view a larger version of this figure.

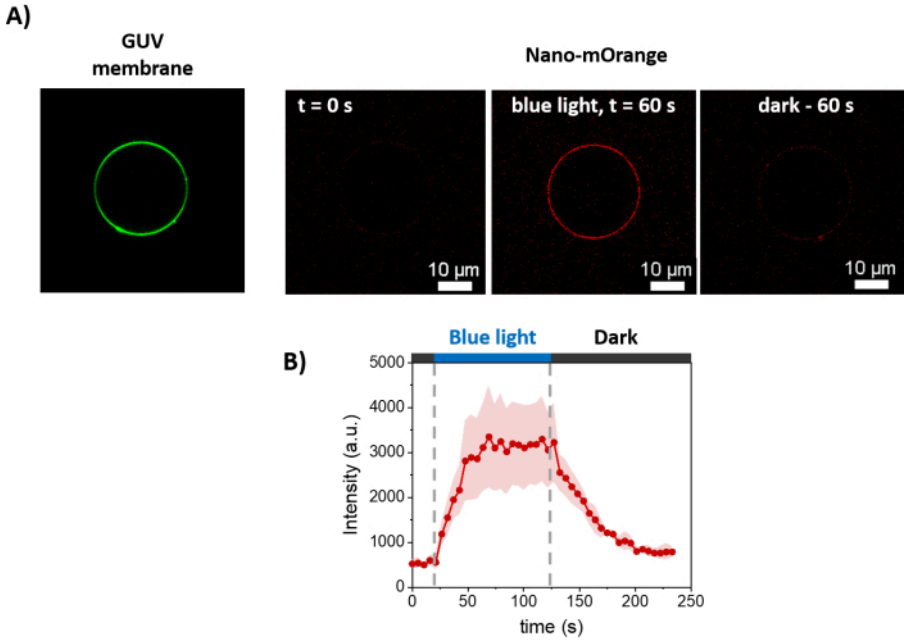

Figure 4: Fluorescence microscopy measurements of the light-dependent recruitment of mOrange-Nano on GUVs membranes. (**A**) Fluorescence images of a disiLID-functionalized GUV in the presence of mOrange-Nano. In green is the membrane dye of the GUVs, and in red is the mOrange fluorescence before, during, and after blue light illumination. Scale bars = 10 µm. (**B**) Fluorescence intensity of mOrange localized on GUV over time. Upon illumination, the mOrange fluorescence (shown in red) on the lipid membrane reaches maximum intensity within 60 s, with a 5.9-fold fluorescence intensity increase. As the illumination is stopped, the mOrange fluorescence decreases to almost pre-illumination values within 60 s (with a 90% recovery). The figure is adapted from Di Iorio et al.[8]. Please click here to view a larger version of this figure.

Discussion

We have described a method for the localized recruitment of mOrange-Nano proteins on model membranes, such as supported lipid bilayer and giant unilamellar vesicles by using the photoswitchable protein disiLID[8]. Aspects that contribute to the quality of the pattern include the quality of the proteins as well as the good quality of SLBs and GUVs.

To ensure a good protein quality after expression and purification, it is important to first evaluate the photoswitchable properties of disiLID. To this purpose, the absorption of the FMN cofactor has to be measured in the dark and after blue light illumination. UV-Vis spectra of disiLID are expected to show the characteristic triple peak of the cofactor FMN in the dark, which significantly decreases upon blue light illumination and recovers in the dark[13]. This photoswitchable behavior is crucial to obtaining reproducible and reversible recruitment in the following steps. Working with protective red light and exposing disiLID to minimum external

illumination during the preparation of the samples increases the performance of the experiments.

Another critical step, and perhaps the most crucial, is the formation of proper SLBs. Defects in the membranes and/or the formation of inhomogeneous SLBs (i.e., the presence of multilayers or patched SLBs) will affect the quality of the protein patterning. Therefore, for inexperienced users, it is recommended to reproduce the protocol by labeling the SUVs with some membrane dyes, such as DiD and DiO, in order to form fluorescently labeled SLBs. In this way, the properties and quality of SLBs can be well characterized with fluorescence microscopy. FRAP measurements represent a typical approach to assessing the quality of an SLB by evaluating the fluidity of the membranes. Alternatively, in the case of biotinylated SLBs such as the ones described in this protocol, fluorescently labeled SAv (e.g., Atto 488-SAv) can be used to visualize and assess the quality of SLBs.

The first part of the protocol describes the formation of patterns on SLBs. To ensure an optimal result, it is important to add mOrange-Nano onto the SLBs and let the sample incubate in the dark for 15 min. During the photoactivation, the selection of the ROI is not restricted to a specific size. However, laser intensity and exposure time need to be regulated in order to reduce undesired photobleaching of the fluorescent proteins.

This method is not restricted to biotinylated proteins, and other approaches can be used to anchor disiLID to SLBs. For example, His-tagged disiLID can be expressed and anchored onto Ni- NTA-containing SLBs. However, it is crucial to express Nano and disiLID with different tags in order to avoid replacement of the proteins on the SLBs. This method also allows the possibility to invert the order of the proteins, thus functionalizing SLBs with Nano and recruiting disiLID (or disiLID-fused proteins) upon blue light illumination.

For dynamic control of protein localization, the reversible localization of the protein to the selected region should be possible repeatedly. To achieve this, the concentration of Nano (200 nM) in the solution is a critical parameter to obtain high reversibility.

Another concern is the recruitment of Nano to the disiLID-functionalized GUV surface. As in the case of protein patterning on SLBs, this method can be extended to different membrane functionalization strategies. In this protocol, the entire GUV was illuminated with blue light to recruit mOrange-Nano on the entire GUV surface. However, the selection of small ROIs localized on the GUV membrane should lead to the precise localization of proteins in a more restricted area.

This method presents only a limitation related to the choice of fluorophore employed for imaging of the Nano recruitment at the SUVs' or GUVs' membrane. In particular, fluorophores with an excitation spectrum in the blue-light range must be avoided, as their usage will interfere with the photoactivation of (dis)iLID. Therefore, the choice of fluorophores in the green or red-light range (e.g., mOrange or Cy5) is recommended for this type of experiment.

The disiLID design offers a simple and adaptable way to improve local protein recruitment to membranes and broadens the dynamic range of iLID and Nano from optogenetics[4]. These methods focus on the recruitment of Nano onto mimic membranes such as lipid bilayers and GUVs. Nevertheless, this approach is extendable to the numerous optogenetic tools in cells where (dis)iLID or Nano are linked to a membrane.

Disclosures

The authors have nothing to disclose.

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