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Chapter 5

Reconstitution of TCR Signaling Using Supported Lipid Bilayers

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Abstract

Biochemical reconstitution has served as an important tool for understanding the mechanisms of many cellular processes including DNA replication, transcription, translation, vesicle trafficking, and ubiquitin-mediated proteolysis. Here, we demonstrate that biochemical reconstitution can be applied to studying a complex signaling pathway involving as many as 12 proteins or protein complexes acting at the surface of model membranes. We show that a temporal sequence of events in activated T cells beginning with phosphorylation of the T cell receptor and culminating in the activation of actin polymerization can be replicated in vitro. Our reconstitution demonstrates the sufficiency of these proteins in producing many of the complex behaviors observed during T cell activation. The ability to manipulate all of the components, measure reaction rates, and observe molecular behaviors, including at single molecule resolution, has enabled us to gain insight into some of the important biochemical features of this signaling pathway such as microcluster formation. The same system could be adapted to study other membrane-proximal signaling pathways, including growth factor receptors, death receptors, and Eph receptors.

Key words TCR, Microcluster, Reconstitution, Supported lipid bilayer, Multivalency, Phase separation, Actin, LAT

1 Introduction

T cell receptor (TCR) signaling mediates T cell activation during adaptive immune responses. A prominent feature of this signaling pathway is that multiple signaling components form submicron- to micron-sized clusters on the plasma membrane, though the mechanisms and functional consequences of forming these clusters remained poorly understood. It had been suspected that microclusters are hot spots for signaling because they are enriched in phosphorylated proteins, though direct evidence had been lacking [1, 2]. To understand the mechanisms of T cell microcluster formation, we developed methods for reconstituting microclusters using purified proteins assembled on supported lipid bilayers in vitro. We initially focused on LAT (Linker for activation of T cells) [3], a transmembrane adaptor protein that connects upstream TCR

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activation to multiple downstream signaling pathways including calcium mobilization, Ras activation, and actin polymerization [4, 5]. We recombinantly expressed a C-terminal fragment of LAT containing the four tyrosine residues that have been shown to be sufficient for mediating TCR signaling [6–8], phosphorylated the purified protein using the syk family kinase ZAP70, labeled it with maleimide-conjugated Alexa488, and attached it to planar lipid bilayers through the interaction of an N-terminal His_8 tag on LAT with Ni-NTA-functionalized lipids incorporated into the bilayer. pLAT-Alexa488 alone was uniformly distributed on membranes. However, when Grb2 and Sos1, two prominent, multivalent binding partners of LAT, were added, submicron-sized clusters formed and gradually grew in size and intensity (Fig. 1). Physical analyses

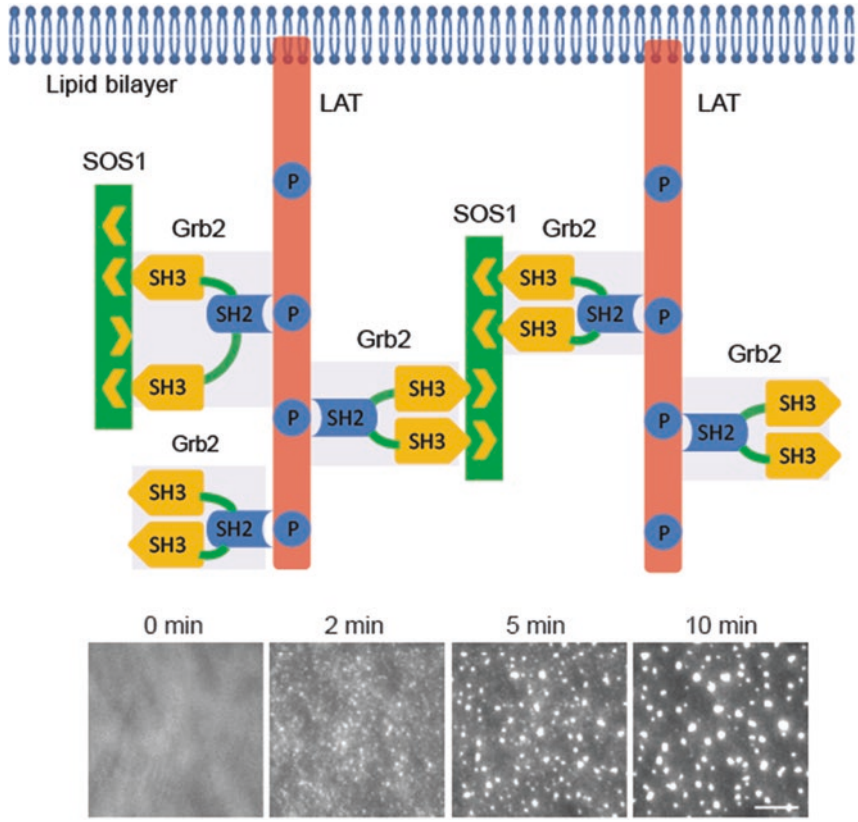


Fig. 1 Reconstitution of LAT microclusters on supported lipid bilayers. *Top*: Schematic of the clustering assay. Phosphorylated LAT (pLAT) was attached to Ni-NTA functionalized synthetic supported lipid bilayers through an N-terminal His_8 tag. The SH2 and SH3 domains of Grb2 bind the phospho-tyrosines in LAT and proline-rich motifs (*yellow pointy brackets*) of Sos1, respectively, thereby creating a network of multivalent interactions. *Bottom*: Total internal reflection fluorescence (TIRF) microscopy imaging of LAT clustering. Clusters were formed after Grb2 (0.5 μM) and Sos1 (0.25 μM) were added to membrane-bound pLAT-Alexa488 (300 molecules/μm^2) at 0 min. Scale bar: 5 μm

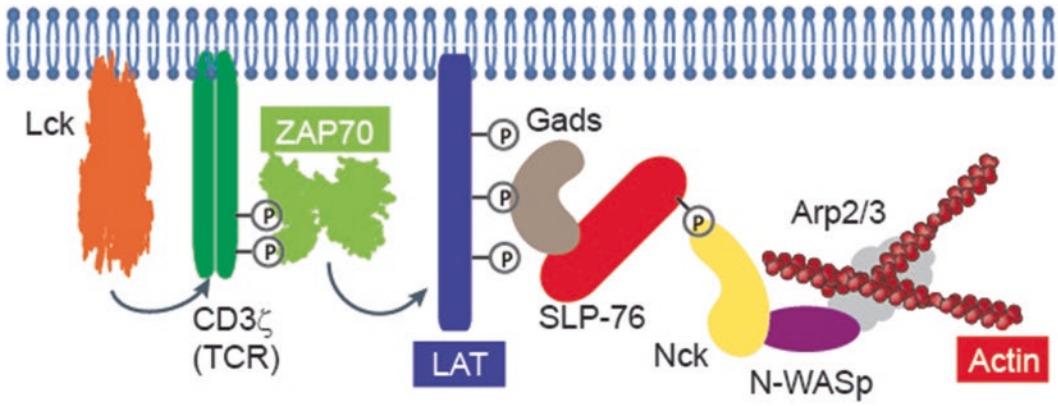
of these clusters indicated that they formed through a process of multivalent assembly and phase separation [9, 10].

Next we extended this reconstitution to a multi-step TCR signaling pathway. The first step in this pathway is phosphorylation of the TCR (CD3ζ subunit) by the src family kinase, Lck. Phosphorylated TCR recruits the kinase ZAP70 from solution and activates it on the membrane. ZAP70, in turn, phosphorylates LAT on the membrane [11, 12]. Phosphorylated LAT then recruits Grb2, SOS and the additional multivalent adaptor proteins Gads and SLP-76, which further recruit the actin regulators Nck, N-WASp, and the Arp2/3 complex to promote actin polymerization on the membrane [13, 14]. We fluorescently labeled ZAP70, LAT, and actin to serve as reporters for TCR phosphorylation, LAT clustering, and actin polymerization, respectively. We preincubated all the components in the experimental system. After adding ATP to the reaction to initiate CD3ζ phosphorylation by Lck, we observed a rapid recruitment of ZAP70 to the membrane, reflecting its binding to phosphorylated CD3ζ. This translocation of ZAP70 was then followed by the formation of LAT clusters. Actin then co-localized with the LAT clusters on the membrane and eventually polymerized and formed bundles (Fig. 2).

These data demonstrate that a multi-step signaling pathway can be reconstituted on supported lipid bilayers. The high spatial and temporal resolution of the tracking of this reconstituted system allows us to understand some critical aspects of the signal transduction process, including microcluster formation, exclusion of tyrosine phosphatases, and organization of actin regulators [15]. Moreover, the reconstitution itself demonstrates the sufficiency of the components to produce many of the complex behaviors observed in vivo during T cell activation.

2 Materials

1. Proteins: LAT, Grb2, Sos1, Gads, SLP-76, Nck, N-WASp, Arp2/3 complex, and actin (*see* ref. 15 for detailed protocols for producing these proteins).
2. Lipid components: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (for example (e.g.), Avanti, 850457C), 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (DOGS-NTA) (e.g., Avanti, 790404C), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (PEG-5000 PE) (e.g., Avanti, 880230C) (*see* **Note 1**).
3. Selected 96-well glass bottom plate (flat glass, upper plastic structure with low fluorescence background and resistant to detergent, acid, and base treatment, e.g., Matriplate MGB096-1-2-LG-L).



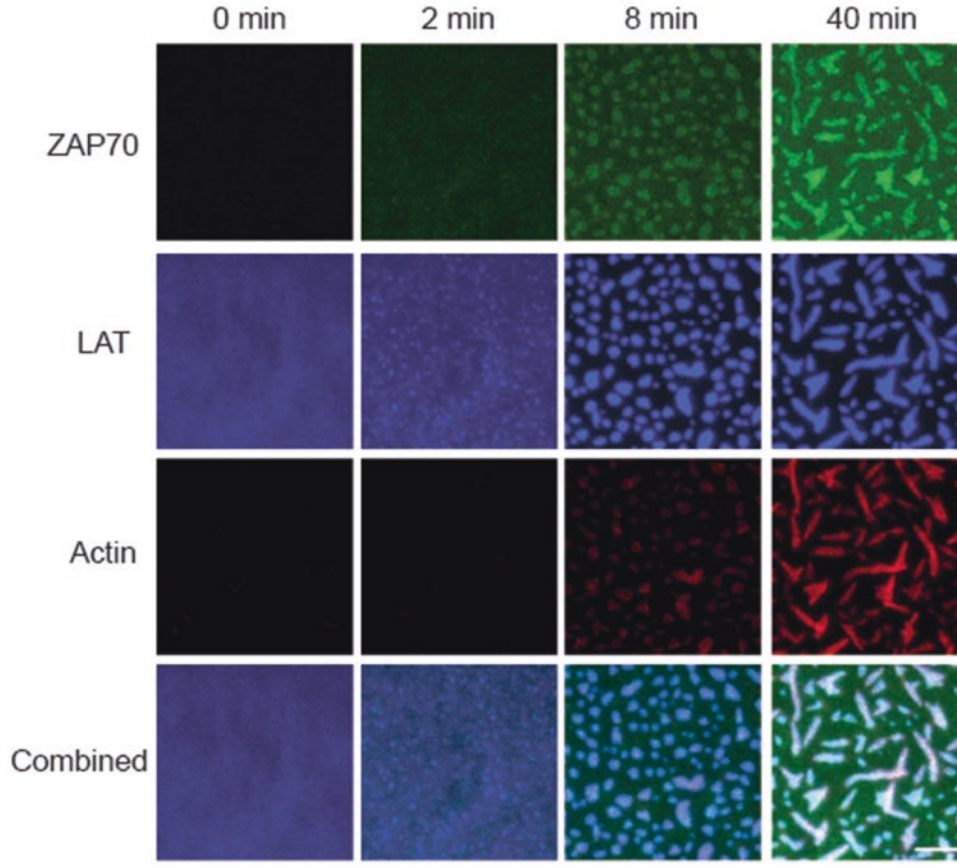


Fig. 2 Reconstituting a TCR-LAT-actin pathway. *Top*: Schematic of the components involved in a reaction designed to reconstitute a signaling pathway from CD3ζ/TCR phosphorylation to actin polymerization. ZAP70-505-Star, LAT-Alexa647, and actin-Rhodamine serve as reporters for TCR phosphorylation, LAT clustering, and actin assembly, respectively. Lck, CD3ζ, and LAT were attached to the membrane and incubated with other components in solution. *Bottom*: TIRF microscopy revealed ZAP70 membrane recruitment, LAT clustering, and actin polymerization in the reconstituted assay after the addition of ATP at time 0. Input: 2.5 nM His-tagged Lck, 5 nM His-tagged CD3ζ, 10 nM His-tagged pLAT-Alexa647, 10 nM ZAP70-505-Star, 250 nM Gads, 500 nM SLP-76, 1000 nM Nck, 500 nM N-WASp, 5 nM Arp2/3 complex, 1000 nM actin (5% Rhodamine labeled), and 0.5 mM ATP-Mg. Scale bar: 5 μm

4. Adhesive PCR Sealing Foil Sheets (e.g., Thermo Fisher AB-0626).
5. Glass vials (e.g., National Scientific B7800-2).
6. Hellmanex III (e.g., Sigma Z805939).
7. Gastight Syringes 25 μL (e.g., Hamilton 80,275), 250 μL (e.g., Hamilton 81,175).
8. Argon.
9. Chloroform (e.g., Electron Microscopy Sciences 12,550).
10. PBS buffer: 155 mM NaCl, 3.0 mM Na_2HPO_4, 1.1 mM KH_2PO_4, pH 7.4.
11. NaOH.
12. Basic buffer: 50 mM HEPES-Na^+, 150 mM NaCl, and 1 mM Tris(2-carboxyethyl)phosphine (TCEP), pH 7.4.
13. Clustering buffer: 50 mM HEPES-Na^+, 150 mM NaCl, 1 mM TCEP, and 1 mg/mL BSA (heat shock fraction, ≥98%), pH 7.4.
14. Signaling buffer: 35 mM HEPES, 60 mM NaCl, 30 mM KCl, 0.4 mM TCEP, 1 mM $MgCl_2$, and 0.4 mg/mL BSA, pH 7.2.
15. High speed centrifuge and polycarbonate centrifuge tubes.
16. Heat Block.
17. Glucose.
18. β-mercaptoethanol.
19. Glucose oxidase.
20. Catalase.

3 Methods

3.1 Preparation of Small Unilamellar Vesicles (SUV)

1. Clean the glass vial with 5% Hellmanex III, rinse thoroughly with ultrapure water (18.2 MΩ at 24 °C).
2. Warm up individual lipid stocks to room temperature (*see* **Note 2**).
3. Use glass syringes to prepare a DOGS-NTA lipid mix in the glass vial as follows: rinse the vial with chloroform, add ~1 mL of chloroform, and then individual lipids (*see* **Note 3**). The total lipids add up to 4 μmol.

   Lipid composition:
   98% POPC
   2% DOGS-NTA (*see* **Note 4**)
   0.1% PEG-5000 PE
4. Dry the lipid mix with a stable flow of argon. Use an ~45 °C water bath during drying (*see* **Note 5**). You will see multiple white layers adhered to the wall of the vial after the lipids are dried.

5. Continue to dry the lipids completely in a desiccator over 2 h.
6. Resuspend the dried lipids in 1.5 mL of PBS and vortex.
7. Transfer the resuspension into two 1.5 mL conical microcentrifuge tubes (750 μL each).
8. Freeze the resuspension in liquid nitrogen and thaw in a water bath at room temperature. Repeat the freeze-thaw for 30 cycles. The cloudy solution will become clear over the freeze-thaw cycles (*see* **Note 6**). The cleared resuspension can be stored at −80 °C for future use.
9. Centrifuge the resuspension at 33,500 × *g* for 45 min at 4 °C.
10. Transfer the SUV-containing supernatant to a clean tube. Avoid disturbing the white pellet at the bottom of the tube during the transfer. In the new tube, cover the SUV solution with argon atmosphere. Store it at 4 °C. Use the SUV within 2 weeks (*see* **Note 7**).

3.2 Preparation of Supported Lipid Bilayers

It is absolutely essential that clean glass is used when making supported lipid bilayers. Glass that is not extremely clean will cause defects in the bilayers and will impair lipid mobility and reproducibility of experiments. Here, we introduce the use of Hellmanex III and NaOH to clean the glass.

1. Immerse a 96-well imaging plate into 1 L of 5% Hellmanex III in a 1 L beaker. Microwave to 50 °C. Stir and incubate overnight at room temperature. The next morning, rinse each well with ultrapure water ten times. Blow-dry each well. Seal the plate with adhesive foil.
2. Use a blade to open the wells that will be used. Add 250 μL of freshly made 5 M NaOH to each well, incubate at 50 °C for 1 h on a heat block. Remove NaOH, repeat the cleaning twice (*see* **Note 8**).
3. Rinse each well with 500 μL of ultrapure water twice, and then 500 μL of basic buffer twice.
4. Add 200 μL of basic buffer to each well, and then 5 μL of SUV solution. Tap the tube containing the SUV solution gently to mix before transferring to the wells. Incubate the plate at 37 °C for 1–1.5 h to allow the bilayer to form. From here on, make sure the bilayers are always covered with buffer (a minimum volume of ~50 μL in the well).
5. Wash each well with 500 μL of basic buffer three times.
6. Block the bilayers with clustering buffer and incubate for 20 min at 37 °C.

3.3 Imaging LAT Microcluster Formation

1. Dilute His_8-tagged, pre-phosphorylated, and Alexa488 (or other dye)-labeled LAT in clustering buffer and add the mix to the well and incubate for 2 h at 30 °C (*see* **Note 9**).
2. Wash each well with 500 μL of clustering buffer three times.
3. Prepare an oxygen scavenger solution by mixing 0.2 mg/mL glucose oxidase, 0.035 mg/mL catalase, 25 mM glucose, and 70 mM β-mercaptoethanol in clustering buffer. Add the solution to the well. The total solution volume in well is 90 μL.
4. Start a time-lapse movie using TIRF (Total Internal Reflection Fluorescence) microscopy. At the beginning, LAT will appear evenly distributed on the supported lipid bilayer (*see* **Note 10**).
5. Add 0.5 μM Grb2 and 0.25 μM Sos1 in 10 μL of clustering buffer during the time-lapse acquisition. Pipette up and down gently several times to mix well. Avoid touching the bilayer with the pipette tips. Monitor the cluster formation process (Fig. 1).

3.4 Imaging a Signaling Pathway from TCR to Actin

1. Add His_{10}-tagged Lck, CD3ζ, and fluorescently labeled His_8-LAT in clustering buffer to the well and incubate for 3 h at 30 °C on a heat block or in an incubator.
2. Wash each well with 500 μL of clustering buffer two times, followed by 500 μL of signaling buffer once.
3. Prepare an oxygen scavenger mix by including 0.2 mg/mL glucose oxidase, 0.035 mg/mL catalase, 25 mM glucose, and 70 mM β-mercaptoethanol in signaling buffer. Add Alexa505-ZAP70, Gads, SLP-76, Nck, N-WASp, Arp2/3 complex, and Rhodamine-actin at desired concentrations to the scavenger mix (*see* Fig. 2 legend for recommended concentrations). Then add the entire mix to the well. The total solution volume in well is 90 μL.
4. Start a time-lapse movie using TIRF microscopy.
5. Add 0.5 mM ATP-Mg in 10 μL of signaling buffer to the reaction mix during the time-lapse acquisition. Pipette up and down gently several times to mix well. Avoid touching the bilayer with the pipette tips. Monitor the sequential signaling steps from TCR activation, to LAT clustering, finally resulting in actin polymerization (Fig. 2).

3.5 Analyzing Cluster Formation with FIJI (ImageJ)

1. Prior to image analysis, capture five images of an empty well (*see* **Note 11**). These will be used to determine the noise of the camera without fluorescence.
2. Also capture five images of an SLB coated with His_8 pLAT-Alexa488 (*see* **Note 12**) using microscope settings identical to those used for time-lapse acquisition. These will be used to create a flat-field image.

3. Prior to image analysis, set up the proper measurements in FIJI. The three essential measurements are the integrated fluorescent intensity of the image (the sum of all pixel intensities within the opened image), maximum intensity, and the standard deviation of the pixel intensities. To set these go to the Set Measurements menu (found in the Analysis menu) and select "Standard deviation," "Min & Max gray value," and "Integrated density."
4. To determine the camera noise, open the images from **step 1** using FIJI, create a stack using the "Images to Stacks" function (found in the Image menu under Stacks), Z-project the stack (also found in the Image menu under Stacks) using the "Average Intensity" projection type, and measure (Ctrl-M or found in the Analyze menu) the averaged image to determine the average pixel intensity. This value will be subtracted from each image used for flat-field correction as well as each frame of the time-lapse.
5. To flat-field correct the time-lapse, we first create a "ratio image" that is used to flatten the intensity within each frame of the time-lapse (*see* **Note 13**). Open the five images of His_8 pLAT-Alexa488-coated SLBs from **step 2** using FIJI, subtract the noise value determined in **step 3** from each image using the Subtract command (found in the Process menu under Math), create a stack using the "Images to Stacks" function (found in the Image menu under Stacks), Z-project the stack (also found in the Image menu under Stacks) using the "Average Intensity" projection type, and determine the maximum pixel intensity in the averaged image using the measure command (Ctrl-M or found in the Analyze menu). Divide the averaged image by the maximum measured intensity (the Divide command is found in the Process menu under Math) to obtain the "ratio" image. Keep the "ratio" image open and open the time-lapse that will be analyzed. Open the Calculator Plus (found in the Process menu) and select the "Divide" operation using the time-lapse as i1, the "ratio" image as i2, leaving k1 as 1.0, and k2 as 0.0. Upon completion of this process, the time-lapse will now be flat-field corrected.
6. We have used two approaches to analyze cluster formation in our assays. In the first approach, we measure the variance of clustering in the frame of interest from a time-lapse of cluster formation (*see* **Note 14**). The variance of the image is simply the standard deviation in pixel intensities from the image squared (Variance = Standard Deviation^2). To measure variance in FIJI, open the time-lapse to the desired frame, measure the standard deviation (Ctrl-M or found in the Analysis menu using the Measure command), and square the standard deviation. We use variance measurements to determine whether

clusters have formed under different sets of experimental conditions. If the variance of an image containing clustering agents is significantly different than the variance of an image of a bilayer coated with an unclustered His_8 pLAT-Alexa488 (*t*-tests or ANOVA can be used depending on the number of conditions being compared), we consider the critical concentration for phase separation has been reached. This is important for determining whether the conditions being used result in cluster formation that can lead to downstream signaling.

7. In the second approach, we measure the fractional intensity of fluorescent molecules found in clusters (*see* **Note 14**). This requires two independent measurements. First, measure the total intensity of the image with the measure command (Ctrl-M or found in the Analysis menu) and record the integrated density of the image. Next, use the triangle algorithm to set a threshold for the image (Ctrl-shift-T or found in the Adjust tab in the Image menu) (*see* **Note 15**) and measure the total intensity of the thresholded region (to measure the integrated density within only the thresholded region, go the to Set Measurements menu in the Analysis menu and select "limit to threshold"). Divide the thresholded integrated density by the total integrated density to obtain the fractional intensity. We use fractional intensity as a metric for how many molecules are located within clusters versus outside clusters. This measurement provides a metric for comparing the level of clustering between different experimental conditions (*see* refs. 9, 10, 15) as examples of how clustering can be compared between different experiments).

4 Notes

1. New users of lipids should read FAQ and tips for storage and handling of lipids from Avanti Polar Lipids, Inc.

   http://avantilipids.com/tech-support/faqs/
   http://avantilipids.com/tech-support/storage-handling-of-lipids/

2. Once a lipid vial is opened, store the remaining unused lipids in a clean capped glass vial. Fill the space above lipids with argon to prevent potential oxidation of lipids. Store the vial at −20 °C. Alternatively, for a modest price one can request bottling service from Avanti Polar lipids, which provides small aliquots of lipids of a specific volume (chosen based on your experimental needs).
3. When transferring small amount of lipids, rinse syringe with the lipids first to avoid any dilution of lipids.

4. We have found that supported lipid bilayers containing 2% DOGS-NTA lipids provide more stable and reproducible attachment of His-tagged proteins than bilayers containing 1% DOGS-NTA. We have also found that bilayers containing 5% DOGS-NTA or more tend to become less fluid (longer recovery time when tested by FRAP).
5. Adjust the argon flow to allow only small ripples during the drying process. Usually, it takes ~5 min to dry 2 mL of lipids in chloroform. It is highly recommended to immerse the lower half of the vial in a warm water bath (~40 °C) because evaporation of chloroform will take heat away and cool the bottle down, which could potentially reduce the solubility of lipids or cause phase separation.
6. Using bath sonication can greatly reduce the cycle numbers.
7. The duration that SUVs can be stored at 4 °C depends on the stability of the specific lipids used in an experiment. It is worthwhile testing the quality of bilayers by FRAP (fluorescence recovery after photobleaching) to determine the maximal storage time for a specific lipid mixture.
8. Keep the temperature of the heat block below 50 °C. Overheating will cause the glue of the plate to melt and solutions will leak into neighboring wells.
9. In our experience, an input of 3 nM phosphorylated LAT with an incubation of 2 h will result in a membrane density around 300 molecules/μm^2. The final membrane density scales roughly linearly with the input concentration in a range of 0.1–10 nM. For any new proteins, we recommend measuring the membrane density [15] at the range needed for the assay.
10. When using a new membrane-bound protein, it will be necessary to determine the mobility of that protein by FRAP to ensure that the protein behaves well on the membrane. Sometimes, black holes appear on the bilayer when imaging fluorescently labeled protein, indicating membrane defects. Membrane defects are usually caused by insufficient cleaning of the glass.
11. It is important to know (and subtract) the baseline noise for the camera used to capture time-lapse images of cluster formation when quantifying fluorescent intensities within the time-lapse. The easiest way to determine the noise of the camera is to capture images in an empty well.
12. There are multiple methods for creating a flat-field image. Alternative methods can be found at http://imagej.net/Image_Intensity_Processing.
13. TIRF microscopy often results in an uneven illumination field. To accurately quantify clustering, we need to "flatten" the

uneven field so that the intensity of both clustered and non-clustered regions of the image can be accurately measured.

14. We use both variance and fractional intensity to compare the degree of clustering in any given set of experimental conditions (*see* refs. 9, 10, 15 as examples of how clustering can be compared between different experiments). Variance is a good measure of how clustering changes the distribution of molecules, from a uniform density to small, intense regions upon the clustering of molecules. Larger, more intense clusters within images will result in an increased variance. Likewise, an increased number of clusters within an image will also result in an increased variance. Fractional intensity is a good measure of the percentage of molecules that are clustered within any given image. As the number of clusters within an image or the size and intensity of clusters within an image increases, the fractional intensity of clusters will also increase.
15. The triangle thresholding algorithm yielded similar results to an iterative manual procedure that was used to identify pixels with intensities greater than three standard deviations above the mean of regions outside of clusters [10].

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