Using patterned supported lipid membranes to investigate the role of receptor organization in intercellular signaling

Pradeep M Nair[1–3], Khalid Salaita[4,5], Rebecca S Petit[1–3] & Jay T Groves[1–3,6]

[1]Department of Chemistry, Howard Hughes Medical Institute, University of California, Berkeley, California, USA. [2]Lawrence Berkeley National Laboratory, Physical Biosciences Division, Berkeley, California, USA. [3]Lawrence Berkeley National Laboratory, Materials Sciences Division, Berkeley, California, USA. [4]Department of Chemistry, Emory University, Atlanta, Georgia, USA. [5]Winship Cancer Institute, Emory University, Atlanta, Georgia, USA. [6]Research Center of Excellence in Mechanobiology, National University of Singapore, Singapore, Singapore. Correspondence should be addressed to J.T.G. (jtgroves@lbl.gov).

Published online 31 March 2011; doi:10.1038/nprot.2011.302

Physical inputs, both internal and external to a cell, can directly alter the spatial organization of cell surface receptors and their associated functions. Here we describe a protocol that combines solid-state nanolithography and supported lipid membrane techniques to trigger and manipulate specific receptors on the surface of living cells and to develop an understanding of the interplay between spatial organization and receptor function. While existing protein-patterning techniques are capable of presenting cells with well-defined clusters of protein, this protocol uniquely allows for the control of the spatial organization of laterally fluid receptor-ligand complex at an intermembrane junction. A combination of immunofluorescence and single-cell microscopy methods and complementary biochemical analyses are used to characterize receptor signaling pathways and cell functions. The protocol requires 2–5 d to complete depending on the parameters to be studied. In principle, this protocol is widely applicable to eukaryotic cells and herein is specifically developed to study the role of physical organization and translocation of the EphA2 receptor tyrosine kinase across a library of model breast cancer cell lines.

INTRODUCTION

Juxtacrine signaling, in which receptors and their cognate ligands are expressed on apposed cell membranes and function within an intercellular junction, allows direct communication between neighboring cells[1]. One of the major challenges in studying such systems is the development of experimental tools to study the effects of confining ligand-receptor interactions within an intermembrane junction. This geometry is experimentally inaccessible using conventional biochemical techniques that generally require ligands to be immobilized on a surface or in solution. The following protocol describes a powerful experimental platform to reconstitute the juxtacrine signaling configuration between live cells.

One of the distinct advantages of this platform is that it recapitulates the laterally fluid context in which these systems natively function. By virtue of residing in a two-dimensional fluid, membrane receptors exhibit extraordinary sensitivity to physical characteristics of their environment that are not present in the context of soluble proteins, such as spatial organization[2,3], polyvalency[4], mechanical strain[5–7] and membrane curvature[8]. Thus, an understanding of any of these effects on cell signaling requires a platform that more accurately reflects the physiological context in which these proteins are presented. Additionally, the elucidation of molecular mechanisms in cell biology typically requires the use of mutational analysis. Correspondingly, this protocol describes powerful methods to introduce spatial mutations in the organization of membrane receptors, thus aiding in the elucidation of the physical mechanisms that are unique to cell surface signaling pathways.

The spatial mutations described above require lithographic techniques to pre-pattern the substrate onto which a supported membrane is deposited. For the application described here, electron-beam (E-beam) lithography was used to create metal lines that were on the order of 100 nm in line width and 10 nm in height. This is a technically demanding and time-consuming technique, and alternative lithographic techniques, which are simpler and quicker to perform but do not offer the same pattern resolution, are described below. Additionally, there are a number of strategies that can be used to attach chromophores to proteins of interest. Some of these alternatives, detailed below (see Experimental design), offer greater control of protein orientation and activity, but require more time to perform and must be tailored individually to each protein to be studied.

In principle, this protocol may be adapted to the manipulation and study of virtually any cell surface receptor, and has been previously used to investigate the immunological synapse[3,9–11]. Herein, we specifically describe its application for the study of the EphA2 receptor tyrosine kinase pathway in breast cancer cells[2]. EphA2 is implicated in a wide range of aggressive cancers and, in particular, 40% of human breast cancers are observed to overexpress the receptor[12]. Upon binding to natively membrane-anchored ephrin-A1, EphA2 undergoes dimerization, transphosphorylation of the cytoplasmic domains and subsequent activation[13]. Soluble ephrin-A1 fails to activate EphA2 and only if the ligand is chemically cross-linked or surface bound is its activity rescued[14]. Despite this observation, most biological and biochemical studies of EphA2 stimulation rely on soluble variants of the ligand[12]. To address this issue, we generated semisynthetic junctions between cells expressing the EphA2 receptor tyrosine kinase and a supported membrane displaying a membrane-tethered and laterally mobile ephrin-A1 ligand. Furthermore, physical barriers to lateral mobility within the supported membrane, prefabricated onto the underlying substrate, can be used to guide ephrin-A1 transport and thus EphA2 transport in the live cell. The cells that encounter diffusion barriers experience the spatial mutation, and are chemically identical to the other cells, differing only by the spatial organization of receptor-ligand complex. The observations using this platform recently

revealed a mechanism of mechanical regulation of the EphA2 receptor signaling pathway[2]. We anticipate that this protocol may be widely applicable for the study of the mechanoregulation of surface proteins in cellular systems.

Development of the protocol

In order to trigger and manipulate receptors expressed on the surface of living cells, it is necessary to couple the supported lipid membrane platform with patterning techniques, such as E-beam lithography that can produce surface features with nanometer scale line widths and heights. This ensures that cells interact with laterally mobile ligands within the lipid membrane, rather than the lithographically defined patterns themselves, and allows for the manipulation of the microscale organization of receptors in the live cell membrane[3,9]. Cell surface receptors are triggered by ligands that are biologically active and tethered to a lipid membrane using a variety of anchoring chemistries, as described below (see Experimental design).

The cellular response is then characterized using two main classes of biochemical analysis. The first consists of single-cell microscopy-based immunofluorescence techniques and real-time imaging of fluorescent protein tags. Microscopy-based characterization of individual cells is a valuable technique that is capable of detecting hidden subpopulations, but may suffer from reduced accuracy because a smaller number of cells is available for characterization[15]. Microscopy is typically complemented with a second class of analysis that consists of bulk biochemical assays, such as western blotting, used to measure the quantity of protein expressed by a population of cells. The two methods are used in parallel but may not be applied to the same sample, as western blotting requires a minimum of 1×10^5 cells to collect a sufficient amount of protein for detection, and these cells must be lysed, a process that destroys information about the spatial organization of proteins on the membrane surface. Typical E-beam lithography is a serial process that will cover an area of 200×200 μm, and the average footprint of a single mammalian cell is on the order of 10 μm × 10 μm. These constraints indicate that each patterned substrate will provide ~ 10^2–10^3 cells for analysis. Thus, nanopatterned substrates are not directly compatible with western blotting because of throughput constraints. Rather, western blotting is used to complement the nanopatterned spatial mutations analysis by measuring alterations to protein expression when molecular clustering is completely inhibited. This may be a limitation of the spatial mutation strategy in contexts where bulk, ensemble averaged biochemical assays are required rather than single-cell measurements. Another limitation of this protocol is that it can only be used to measure early response functions within the first few hours of cell-supported membrane engagement. Cells can remodel their environment and the fidelity of the supported membrane may be compromised after a few hours of cell-surface interactions. However, to the best of our knowledge this is the only technique available to selectively manipulate the spatial organization of receptors on the surface of living cells.

In the following sections, we describe the methods required to generate ligand-functionalized supported membranes on nanopatterned substrates. Cells are then engaged to these surfaces and methods to measure cell responses are described. In particular, microscopy-based analyses are highlighted and semiautomated data processing algorithms are included as supporting information because they were specifically developed for the protocol.

Experimental design

Supported proteolipid membrane design. Almost any peripheral membrane protein or peptide of interest can be incorporated into the supported lipid membrane through the use of a range of anchoring chemistries, such as biotin-streptavidin affinity binding[2,16], polyhistidine-Ni^{+2} coordination chemistry[17] or direct covalent coupling using thiol-reactive groups targeting cysteine residues[18]. Typically, proteins or peptides are targeted to a pre-formed lipid membrane and can be characterized using an array of microscopy techniques[19,20]. It is important to empirically optimize the concentrations of blocking and protein conjugation conditions to reduce aggregation and nonspecific interactions with the lipid membrane or the underlying substrate.

Protein labeling with fluorescent probes and biotin tags. The location of the membrane-bound proteins is measured using specifically tagged fluorescent reporters. The two main approaches to incorporating a fluorescent label on the protein consist of generating recombinant fluorescent fusion proteins[10,11,17] or direct covalent conjugation of synthetic organic dyes to target proteins[2,16]. The use of fluorescent fusion proteins allows for precise control of the orientation and labeling ratio of fluorophore per protein, but this technique can be time consuming, as it requires molecular biology techniques for generating the protein of interest. To see examples where such an approach was used, please see reference 11. An alternative method, which is used in this protocol, consists of using synthetic fluorophores that label accessible primary amine functional groups on the target protein using *N*-hydroxysuccinimide (NHS)-ester functionalized dyes. This approach is advantageous if you are labeling a large library of ligands is necessary, because it is less time consuming and can be performed in a high-throughput manner.

To tether the protein of interest to the supported lipid membrane, we use biotin-avidin affinity methods. NHS-ester functionalized biotin tags are coupled directly to the free amine groups in the ligand of interest. As both the biotin affinity tag and the organic dye are added to the protein through the reaction of NHS-esters with accessible amines on the target protein, we perform a one-pot coupling step, where both biotinylation and conjugation to the fluorescent probes are performed simultaneously. As both the biotin and fluorophore use the same nucleophilic addition chemistry, we assume similar kinetics and use the corrected fluorophore absorbance to quantify the degree of fluorophore labeling, which is assumed to be equal to the degree of biotinylation.

The target degree of labeling (DOL) for the reaction is 1 biotin and 1 fluorophore for each ligand molecule. We find that excess biotinylation can lead to cross-linking of the ligand to multiple streptavidin molecules, which may reduce the fluidity of the lipoprotein membrane (please see the section on fluorescence recovery after photobleaching and membrane characterization).

Ligand selection. Typically, it is recommended that a highly specific and high-affinity ligand is selected to bind to its cognate receptor. It is also possible to incorporate multiple ligands simultaneously, and this protocol describes the use of lipid membranes onto which multiple ligand biomolecules are anchored. The particular example highlighted here uses supported membranes that incorporate the cyclic peptide [Arg-Gly-Asp-d-Phe-Lys(Biotin-PEG-PEG)] (cRGD) that specifically targets integrin receptors, and ephrin-A1, which

targets the EphA2 receptor tyrosine kinase. Biotinylated conjugates of both ligands were mixed in varying amounts, yielding membranes that had different concentrations of each on the bilayer surface.

Cell selection. Any type of live cell can be used in this protocol. Over 30 different cell lines have been characterized using the protocol described here. However, if the receptor that is under investigation is not expressed by the cells then it will not interact with the membrane. Conventional cell culture conditions that include serum are compatible with the protocol[2].

E-beam lithography. The goal is to generate nanostructures that act as physical barriers to lateral lipid diffusion. Chromium[3], adsorbed protein[21], photoresist[22] and exposed areas of bare silica[23] have all been found to function in this capacity. In a typical experiment, E-beam lithography is used to generate chromium metal lines in various geometric configurations on a batch of 10–20 optically thin (2, although thinner glass may be necessary depending on the working distance of the microscope objective to be used) glass cover slips. Nanopatterned glass cover slips are subsequently cleaned using conventional piranha etching or UV oxidation procedures (see Procedure).

The specifications of this process will vary greatly depending on the dimensions of the patterns desired and the capabilities of the particular lithographic system used. Using the guidelines detailed in this protocol, a separation between functional features as small as 500 nm has been achieved. For smaller spacings, it will be necessary to adjust pattern parameters to control for proximity effects, where the exposure profile of one feature overlaps with that of an adjacent feature to expose an area substantially larger than that specified in the patterning software. Parallel lines such as those used to partition off a surface are particularly vulnerable to proximity-induced widening. Many lithography systems come equipped with software that attempts to correct these problems by, for example, automatically reducing dosage given to areas on the outside edges of features relative to their interiors. The effectiveness of this approach is limited in cases where the Gaussian range of the E-beam is on the same order as the separation between features. To narrow the effective profile of exposure, it may be helpful to reduce electron scattering through the depth of the resist by using the thinnest possible resist layer that is feasible for each process. Using a higher accelerating voltage will also aid in reducing the cone of scatter; low-voltage lithography systems are at an inherent disadvantage in this respect.

If appropriate E-beam writing capabilities are not available, researchers may choose to use more accessible and, in some cases, unconventional approaches to nanofabrication. Phase-shift photolithography[24], extreme UV lithography[25], microcontact printing and dip-pen nanolithography[26–28] may prove suitable substitutes for E-beam capability, given flexible enough resolution requirements. Importantly, some of these techniques offer certain advantages in terms of biomolecule compatibility, throughput and resolution that exceed those offered by E-beam lithography. The particular application will dictate the resolution, fidelity and throughput requirements of the desired patterns, all factors that should be considered carefully during experimental design.

Preparation of small unilamellar vesicles. A number of approaches have been adopted for generating small unilamellar vesicles (SUVs). These include sonication[29], extrusion[30] and dialysis[31]. Among these, we find that extrusion is facile and well tolerated by reactive or sensitive lipid conjugates. To generate SUVs using the extrusion approach, lipids are mixed in the correct proportions as detailed in the procedure section. In general, the major lipid constituent is 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and a small amount of fluorescent lipid or anchoring biotinylated lipid is introduced in organic solvent (typically methylene chloride or chloroform). The mixture is dried first by using a rotary evaporator and then under a gentle stream of N_2. Dried lipids are resuspended in ultrapure (Milli-Q) water as a milky suspension of multilamellar vesicles. The lipid solution is then extruded under high pressure N_2 using a temperature-controlled extrusion system fitted with a 100-nm nanopore membrane.

Forming supported lipid membranes. SUVs will self-assemble into planar lipid bilayers when exposed to clean silica surfaces at high ionic strength conditions[32]. The most stringent requirement for the formation of supported lipid bilayers (SLBs) is the use of inorganic silica supports (typically glass cover slips) that have been freshly etched clean. The etching procedure renders the surfaces hydrophilic, thus removing organic adsorbates and other contaminants. Note that recent reports suggest that polymeric or soft-matter interfaces can be used in place of solid silica supports[33]. In principle, these types of surfaces are compatible with the described protocol and may be adapted depending on the desired experiments.

Supported membranes should be blocked before streptavidin addition, otherwise immobile clusters of streptavidin are formed because of nonspecific binding of streptavidin to defects in the supported membrane surface (**Fig. 1a**). Blocking with 100 μg ml^{-1} BSA yields a fluid-supported membrane, but prevented nonspecific binding of streptavidin to the underlying support

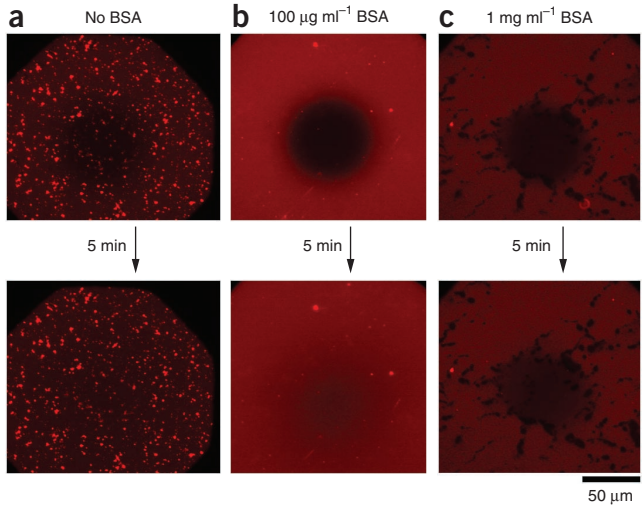

Figure 1 | Optimization of supported membrane blocking conditions. Sample fluorescence recovery after photobleach (FRAP) images of Alexa Fluor 647-labeled streptavidin on a supported membrane that was not blocked before streptavidin addition (**a**), on a supported membrane blocked with 100 μg ml^{-1} BSA (**b**) and on a supported membrane blocked with 1 mg ml^{-1} BSA (**c**). Each sample was imaged immediately after photobleaching and then once more after 5 min. These results show that incubation with 100 μg ml^{-1} BSA substantially reduces nonspecific binding, evidenced by the decreased number of bright immobile clusters of streptavidin, without enlarging defects in the bilayer, which appear as dark immobile patches present when too high a concentration of BSA is used.

(**Fig. 1b**). Blocking with 1 mg ml^{-1} BSA leads to patches in the bilayer, where BSA displaced the supported membrane (**Fig. 1c**). A similar blocking optimization can be carried out for different proteins or protein-anchoring strategies as needed.

To verify the formation of a laterally fluid-supported membrane, one can add a low concentration of fluorescent lipids (0.5 mol % 1-acyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl}-*sn*-glycero-3-phosphocholine or Texas Red-1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine (TR-DHPE)) to the lipid mixture during vesicle preparation. Then, after bilayer deposition, perform fluorescence recovery after photobleaching (FRAP) to determine lateral mobility. Briefly, close the field diaphragm of the microscope and photobleach a small area of the bilayer. Then immediately open the field diaphragm and acquire an image. After 1–5 min, acquire another image with the field diaphragm in the open position. If the bilayer is correctly deposited and fluid, the intensity of the photobleached area will recover. To ensure that nanopatterned metal lines serve as barriers to lateral diffusion of lipid dyes, a supported membrane containing Texas Red lipid dye can be deposited onto a nanopatterned surface and FRAP of the Texas Red should show that the metal patterns are effective diffusion barriers for lipid molecules in the supported membrane (**Fig. 2**, panel **b**).

Data analysis. A primary consideration in recapitulating intercellular signaling is the quantification of cell activation in the context of the receptor pathway under investigation. The spatial mutation strategy allows for the observation and characterization of single cells undergoing a signaling response to lithographically defined perturbations in lateral receptor organization on the cell membrane surface. Thus, single-cell microscopy-based analytical techniques have an important role in the described protocol. Furthermore, the intrinsic serial nature of E-beam lithography limits the utility of conventional biochemical analysis techniques such as western blotting that typically require large populations (~10^5) of cells. Herein, we detail a microscopy-based method for the measurement of ADAM10 recruitment to Eph-ephrin clusters upon dynamic ligand-induced reorganization of the EphA2 receptor.

It is important that data analysis is automated or semiautomated to reduce analysis times and to minimize human error and subjective distortion of data. Our algorithm is described in **Supplementary Methods 1–4** and **Supplementary Manual** and can be modified depending on researcher preference and data format. Briefly, our approach consists of normalizing nonhomogenous excitation intensities that are typically encountered in through-objective total internal reflection fluorescence (TIRF) microscopy, and then performing a series of measurements for each cell to quantify TIRF microscopy intensities and colocalization coefficients. The analysis was adapted to work with ImageJ, a freely available open source image analysis software package.

Controls. One of the most common problems encountered when preparing supported lipid membranes pertains to the homogeneity and fluidity of these materials. It is recommended that a control membrane containing a low concentration (< 3 mol %) of fluorescent lipids be generated as part of each experiment to confirm that the glass etching, lipid vesicle preparation and supported membrane deposition were all performed accurately. This routine control is not time consuming and can ultimately minimize wasted time because it is diagnostic of any issues with the lipid membrane.

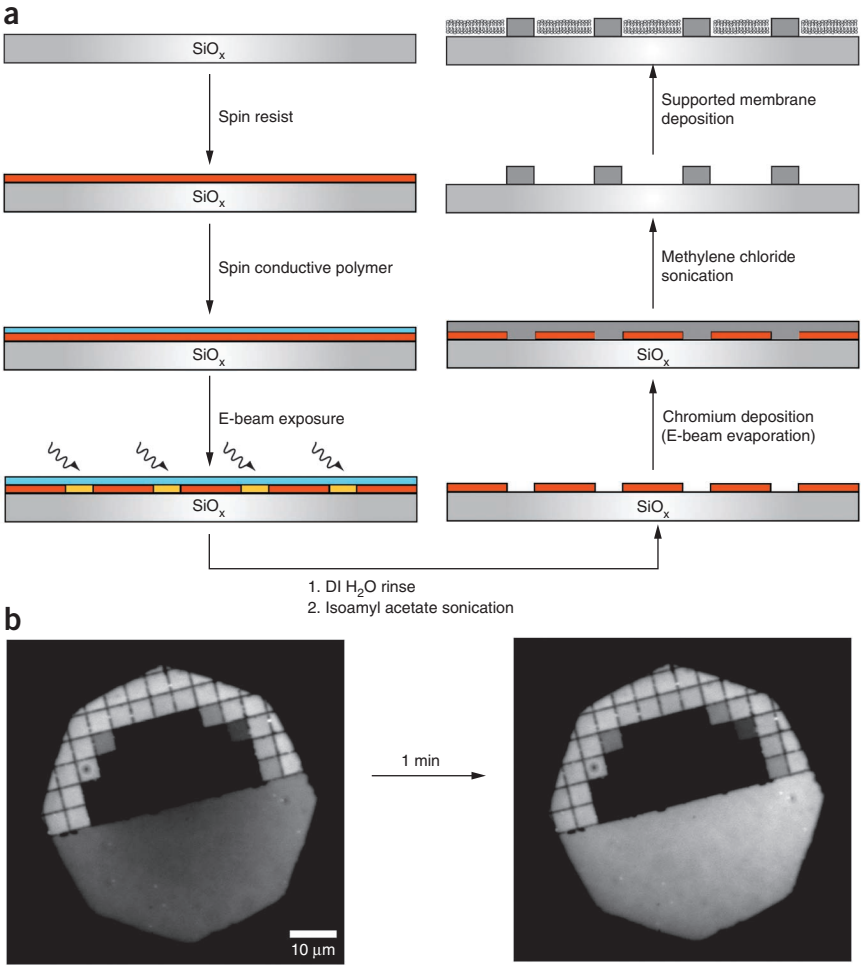

Figure 2 | Nanopatterned metal lines serve as barriers to lateral mobility of the supported membrane. (**a**) Metal lines were deposited onto the surface using E-beam lithography. (**b**) FRAP images of Texas Red lipid dye show that metal patterns serve as diffusion barriers for lipid molecules in the supported membrane. Texas Red fluorescence signal recovers completely in areas outside of grid lines, where nonphotobleached lipids can diffuse into the imaged area. In contrast, in grid squares that are entirely within the photobleached area, no active fluorophores can diffuse into the squares, and no fluorescence recovery is observed. Intermediate levels of recovery are observed in border grid squares, in which only a fraction of the fluorophores is photobleached. SiO_x, amorphous silica.

MATERIALS

REAGENTS

- 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; Avanti Polar Lipids, cat. no. 850375)
- 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl) (biotin-DOPE; Avanti Polar Lipids, cat. no. 870273)
- 1-Acyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl}-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, cat. no. 810129)
- Texas Red-1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine (TR-DHPE; Invitrogen, cat. no. T-1395MP)
- NaOH (1 M; EMD Chemicals, cat. no. SX0590)
- ACS grade chloroform (Acros Organics, cat. no. 423550010)
- Dry ice
- Distilled, deionized (DI) water
- PBS (10×; Invitrogen, cat. no. 70011-069)
- Dulbecco's PBS stock (10×; Mediatech, cat. no. 20-031-CV)
- H_2SO_4 (EMD Chemicals, cat. no. SX1244)
- H_2O_2 (Sigma-Aldrich, cat. no. 216763)
- Alexa Fluor 350, 488, 594 and 647 monoclonal antibody labeling kits (Invitrogen, cat. nos. A-20180, A-20181, A-20185 and A-20186, respectively)
- Biotin labeling kit (Pierce Biotechnology; EZ-Link Sulfo-NHS-biotin, cat. no. 21326)
- BSA (Sigma-Aldrich, cat. no. A3059)
- Cyclic peptide [Arg-Gly-Asp-d-Phe-Lys(Biotin-PEG-PEG)] where PEG = 8-Amino-3,6-dioxaoctanoic acid (Peptides International, cat. no. PCI-3697-PI)
- Recombinant Streptavidin (Sigma-Aldrich, cat. no. S4762)
- Ephrin-A1 (R&D Systems, cat. no. 602-A1-200)
- ZEP-520A E-beam resist (Zeon Chemicals L.P.)
- Extra pure anisole (99%; Acros Organics, cat. no. 100-66-3)
- Aqueous conductive polymer: Aquasave 53ZA (Mitsubishi Rayon America)
- CR-14 chrome etchant (Cyantek)
- Developer: isoamyl acetate (Acros Organics, cat. no. 123-92-2)
- Liftoff solvent: methylene chloride (Fisher Scientific, cat. no. 75-09-2)
- Isopropanol (Fisher Scientific, cat. no. 67-63-0)
- Chrome pieces (Alfa Aesar, cat. no. 7440-47-3)
- Trypsin (0.25%, vol/vol)/0.38 g per liter EDTA 4Na (Invitrogen, cat. no. 25200-114)
- Paraformaldehyde (Acros Organics, cat. no. 30525-89-4) **! CAUTION** Paraformaldehyde is a probable human carcinogen. Perform all work with paraformaldehyde in a chemical fume hood with adequate ventilation.
- Triton X-100 (EMD Chemicals, cat. no. TX1568)
- Hoechst 33342 (Invitrogen, cat. no. H3570)
- F-actin stains: Phalloidin Alexa Fluor 350, 488 and 647 conjugates (Invitrogen, cat. nos. A22281, A12379 and A22287, respectively)
- Laemmli buffer (Invitrogen, cat. no. NP0007)
- β-Mercaptoethanol (200 mM; Sigma-Aldrich, cat. no. M3148)
- Bis-Tris acrylamide gels (4–12%; Invitrogen, cat. no. NP0321BOX)
- Primary antibodies for immunostaining: anti-EphA2 antibody (Santa Cruz Biotechnology, cat. no. sc-924) and anti-ADAM10 (Santa Cruz Biotechnology, cat. no. sc-48400)
- Primary antibodies for western blotting: anti-EphA2 (Millipore, cat. no. 05-480), anti-phosphotyrosine (Millipore, cat. no. 05-321) and anti-actin (Santa Cruz Biotechnology, cat. no. sc-1616) **▲ CRITICAL** Primary antibodies for immunostaining recognize different epitopes on their target proteins than western blotting primary antibodies, due to differences in protein treatment during these two procedures. Use primary antibodies that are designed for whichever experiment is desired.
- Secondary antibodies for immunostaining: isotype-matched secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 647 (Invitrogen, cat. no. A-10680 or A-21244, respectively)
- Secondary antibodies for western blotting: isotype-matched secondary antibodies conjugated to Alexa Fluor 680 (Invitrogen, cat. nos. A-21058 and A-21084)
- NP-40 lysis buffer (Thermo Scientific, cat. no. 28324)
- Protease inhibitor cocktail set II (EMD Chemicals, cat. no. 539132)
- Phosphatase inhibitor cocktail set II (EMD Chemicals, cat. no. 524625)
- DMEM (Gibco/BRL, cat. no. 11965118)
- FBS (HyClone, cat. no. SH30070.03)
- L-glutamine (Invitrogen, cat. no. 25-005-CI)
- Penicillin/streptomycin (10,000 IU/10,000 μg; Invitrogen, cat. no. 30-002-CI)
- Milli-Q ultrapure water

EQUIPMENT

- Lipid Extruder (1.5 ml; Northern Lipid)
- Rotary Evaporator (Buchi) equipped with a vacuum pump
- Polycarbonate filters (100 nm pore size; Whatman)
- Cell scrapers (Fisher Scientific)
- Nikon Eclipse TE2000-E inverted fluorescence microscope
- Stabilite 2018 krypton/argon ion laser (Spectra-Physics)
- Model 177 argon ion laser (Spectra-Physics)
- Quantix charge-coupled device (CCD) camera (Roper Scientific)
- Cascade 512B electron-multiplying CCD camera (Roper Scientific)
- Metamorph image acquisition software (Molecular Devices)
- Cy5, TR, NBD/HPTS, DAPI/Hoechst/AMCA, RICM filter cubes (Chroma Technology)
- Physitemp TS-4 thermal microscope stage (Physitemp Instruments)
- Oil immersion objective heater (100×; Home-built)
- Teflon cover slip mini-rack (Molecular Probes, cat. no. C-14784)
- Model 75D Aquasonic bath sonicator (VWR Instruments)
- Model 100CB CEE spin coater (Brewer Science)
- Model PC-400D hot plate (Corning)
- Dektak 150+ surface profiler (Veeco Instruments)
- Bioscope atomic force microscope (AFM) system (Digital Instruments)
- Edwards EB3 electron-beam evaporator (Edwards BOC)
- CABL-9510CC high-resolution electron beam lithography system (Crestec)
- Gemini Ultra-55 analytical scanning electron microscope (SEM; Zeiss)
- 90Plus particle size analyzer (Brookhaven Instruments)
- Odyssey infrared scanning system (LI-COR Biotechnology)
- Attofluor cell chamber (Invitrogen)
- Glass microscope cover slips (round, number 2, 170 μm thick, 25 mm in diameter; Fisher Scientific, cat. no. 12-546-2)
- Cary 100 scan UV-visible spectrophotometer (UV-visible; Varian)
- ImageJ software (http://rsbweb.nih.gov/ij/)

REAGENT SETUP

PBS, 1× It is diluted using DI water from a 10× PBS stock. This can be stored at room temperature (25 °C) for periods of up to 1 month.

Piranha solution Piranha solution is H_2SO_4/H_2O_2 (3:1), freshly prepared. **! CAUTION** This is a strong oxidant that is explosive on contact with organics.

Membrane blocking solution BSA (100 μg ml^{-1}; 1.5 μM) in 1× PBS. This solution can be stored at 4 °C for up to 1 month.

Fixative Paraformaldehyde solution (4%, wt/vol) prepared in warm 1× PBS and stored at 4 °C for up to 1 month. **! CAUTION** Paraformaldehyde is a probable human carcinogen. Perform all work with paraformaldehyde in a chemical fume hood with adequate ventilation.

Immunostaining blocking solution BSA (10 mg ml^{-1}, 150 μM) in 1× PBS. This can be stored at 4 °C for up to 1 month.

Permeabilization solution Triton X-100 (0.1%, vol/vol) in 1× PBS. This can be stored at 4 °C for up to 1 month.

Nuclear stain Hoechst 33342 (1 μg ml^{-1}) in 1× PBS. This can be stored at 4 °C for up to 1 month.

E-beam resist solution Dilute ZEP520A 1:3 in 99% extra pure anisole. Excess solution may be stored at room temperature for later use; store according to the manufacturer's specifications.

Isopropanol/dry ice solution Add dry ice to isopropanol (enough that solid dry ice is visible in the solution) above the vacuum trap of the rotary evaporator immediately before evaporating chloroform from lipid solution. Use this solution to freeze lipid solution during lipid hydration (add more dry ice if no solid is visible in solution).

Cell medium Use DMEM supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine and 100 U penicillin/100 μg streptomycin for the MDA-MB-231 cell line. Medium solutions can be aliquotted and stored in a −20 °C freezer for up to 1 year. Other cell lines should be cultured according to the conditions provided by the Lawrence Berkeley National Laboratory

Integrative Cancer Biology Program database (http://icbp.lbl.gov/breastcancer/celllines.php).
Dulbecco's PBS buffer (1×) Dilute 10× Dulbecco's PBS stock 1:10 in H_2O and cool to 4 °C before rinsing. This can be stored at room temperature for 1 month.
Lysis buffer Supplement NP-40 lysis buffer with protease inhibitor cocktail set II and phosphatase inhibitor cocktail set II diluted to 1× .

EQUIPMENT SETUP
Stabilite 2018 krypton/argon ion laser Tune laser to 647 nm and align the laser to TIRF mode.
Model 177 argon laser Align laser to TIRF mode.
Physitemp TS-4 thermal microscope stage and home-built ×100 oil immersion objective heater For live cell experiments, preheat stage, ×100 objective, and Attofluor cell chamber to 37 °C before adding cells to substrates.

PROCEDURE

Preparation of E-beam fabricated substrates ● TIMING 12 h, depending on number of substrates

1| Bath-sonicate glass cover slips at room temperature in DI water for 5 min to remove gross particulate matter. Multiple cover slips may be placed in Teflon mini-rack to facilitate immersion and bath-sonication steps. Cover slips should be handled using forceps and powder-free gloves. Best results are achieved when substrate preparation is conducted under clean room conditions.

2| Clean cover slips by immersing them in piranha etching solution for 2 min.
! CAUTION Piranha solution is extremely dangerous and may spontaneously explode if exposed to organic solutions. Protective equipment and proper fume hood must be used. Piranha waste must be left to degas and must not be mixed with any organic solutions.

3| Rinse cover slips three times by 30-s bath sonication at room temperature in DI water, immerse briefly in isopropanol, and dry under a stream of clean air.

4| Bake cover slips on hot plate for 10 min at 140 °C to drive off any surface moisture that might interfere with resist adhesion.

5| Spin-coat the cover slips at room temperature for 45 s at 1,000 revolutions per minute (r.p.m.) with 1:3 ZEP-520A/anisole to achieve a film thickness of ~600 nm. The process of spin coating entails centered placement of the substrate on a rotating spin coater chuck, dropwise deposition of a generous amount of resist solution onto the center of the substrate surface and spinning the substrate—which is affixed to the chuck with a transient vacuum—at a predefined time and rotation speed. Ideally, the end result of this process is a resist film of a uniform height that varies depending on spin velocity and solution viscosity[34]. To measure film thickness, scratch the resist layer with a fine-pointed metal implement and run the stylus of a mechanical profilometric instrument over the score line to generate a surface height profile that can be measured using AFM or surface profilometry. Film heights generated in this way are generally quite reproducible, given the same spin parameters and resist solution composition, but spin layer height should be checked for each new batch of resist solution.
? TROUBLESHOOTING

6| Bake cover slips on hot plate for 5 min at 140 °C to remove solvent from resist coating.

7| Spin coat the cover slips at room temperature for 45 s at 1,000 r.p.m. with Aquasave conductive polymer. At the patterning dimensions used here, the process is relatively insensitive to the height of the conductive layer. Readers who desire to fabricate finer patterns than that we used may wish to use a thinner conductive layer to minimize exposure profile broadening because of lateral electron scattering, the effects of which increase in severity as a function of thickness height.
▲ CRITICAL STEP A conductive layer allows surface charges to dissipate during E-beam exposure. If conductive polymer is unavailable, a 150-nm-thick evaporated film of chrome may be substituted.

8| Bake cover slips on hot plate for 2 min at 140 °C to drive off solvent from polymer coating.
■ PAUSE POINT Spin-coated cover slips can be safely stored in a dry UV-free environment for at least a week before E-beam exposure.

9| Expose resist via E-beam lithography (line width 50 nm for 0.5-μm grids and 80 nm for all other spacings; acceleration voltage 50 kV, 100 pA; field size 600 × 600 μm, 3.6×10^9 dots per field, vector mode) at 100–150 μC cm^{-2}. Verify pattern dimensions and integrity by SEM and AFM[35].
▲ CRITICAL STEP Typical write times range from 0.5–2 h for a single cover slip, depending on the number, dosing and density of the patterns generated.
? TROUBLESHOOTING

10| Rinse off conductive polymer in DI water. If a chrome conductive layer has been substituted (Step 7), remove it with a 5-min bath sonication at room temperature in chrome etch.

11| Develop resist by bath sonication at room temperature in isoamyl acetate for 1 min to clear resist from exposed areas.

12| Immerse cover slips in isopropanol. Dry under a stream of clean air.

13| Deposit a 10-nm-thick chrome film on developed resist surface by E-beam evaporation at ~0.01 nm s^{-1} and pressure not exceeding 10^{-6} torr. Typical E-beam gun current used during chrome deposition ranges from 20–30 mA. Monitor deposition speed by quartz crystal microbalance[36]. The quartz microbalance is typically contained within the thin-film deposition apparatus. To receive accurate readings, users will be prompted to input parameters corresponding to the density of the metal (for chrome, 7.200 g cm^{-3}) and its acoustic impedance on quartz (the *Z*-ratio; for chrome, 28.950 10^5 g cm^{-2} s^{-1}) as well as the vertical distance between the crystal monitor and the deposition sample (the tooling factor; this varies according to the construction of the deposition apparatus).
? TROUBLESHOOTING

14| Remove resist mask from cover slip surfaces with a 10-min ice-cold bath sonication in methylene chloride. Verify gridline height by AFM or surface profiler measurements, and verify pattern integrity and gridline width by optical microscopy, AFM and SEM[35].
■ **PAUSE POINT** Nanopatterned substrates can be stored under a desiccator for extended periods of time (at least 2 years) and used at a later time point.
? TROUBLESHOOTING

Preparation of supported membranes ● TIMING 3 h
15| Combine desired phospholipids (99.9 mol % DOPC and 0.1 mol % biotin-DOPE) from chloroform stock solutions (<3 months old) in a round-bottom flask. To aid in calculation of the volumes of each phospholipid to be added, use **Supplementary Methods 5**, a Microsoft Excel spreadsheet that uses the desired molar ratios of each phospholipid, their initial concentrations in chloroform (in mg ml^{-1}) and their respective molecular weights to determine the volume of each phospholipid solution that must be added to create the desired lipid mixture.

16| Evaporate the chloroform solvent with a rotary evaporator to heat the lipid solution to 40 °C under vacuum.

17| Dry the lipids thoroughly under a stream of N_2 for 15 min.

18| Rehydrate lipids at room temperature by addition of ultrapure (e.g., Milli-Q) water such that the final lipid concentration is 1–2 mg ml^{-1}. To aid in hydration of the lipids, while swirling the lipid solution in a sealed round-bottom flask, quickly freeze the solution by immersing the flask in a dry ice/isopropanol solution. Then thaw the lipid solution by immersing the flask in a warm water bath (40 °C). Repeat this freeze-thaw cycle three times to release physically adsorbed lipids from the walls of the flask.

19| Extrude the milky suspension of hydrated lipids 11 times through 100-nm-sized polycarbonate pore filters, producing SUVs. Note that there are different types of extruders that are commercially available, but the main approach consists of forcing the solution through a nanometer-scale filter. This can be accomplished by using a manual syringe apparatus or using external pressure from a N_2 tank. The size and polydispersity of SUVs can be measured using dynamic light scattering[37].
■ **PAUSE POINT** Vesicles can be used without loss of function for at least 2 weeks if stored at 4 °C.

20| For supported membrane formation using nanofabricated substrates, follow option (A). For high-throughput experiments requiring several independent nonpatterned supported membranes, follow option (B).
(A) Supported membrane formation on nanofabricated microscopic cover glass ● TIMING 30 min
(i) Sonicate substrates (from Step 14) at room temperature in DI water for 5 min.
(ii) Clean substrates using piranha etching solution for 2 min.
! CAUTION Piranha solution is extremely dangerous and may spontaneously explode if exposed to organic solutions. Protective equipment and proper fume hood must be used. Piranha waste must be left to degas and must not be mixed with any organic solutions.
▲ CRITICAL STEP Piranha will etch chromium[38], so prolonged or repeated piranha exposure will degrade the quality of nanopatterned features. For repeated use of nanopatterned substrates, the surfaces can be made hydrophilic and amenable to supported membrane formation using alternative techniques, such as plasma oxidation[39] or exposure to UV light and ozone[40].

(iii) Rinse substrates with copious DI water.
(iv) Dry substrates under a stream of N_2.
(v) Spread lipid vesicle solutions (from Step 19) over these substrates by placing substrates on top of a 30 µl droplet of SUV-PBS solution (final lipid concentration 0.5–1 mg ml^{-1} in 1× PBS) for 1 min at room temperature in a standard Petri dish.
(vi) Submerge the substrate, atop the SUV-PBS solution in the Petri dish, in a 2-liter bath of 1× PBS at room temperature. Shake the lipid membrane functionalized substrate so as to remove excess SUVs.
▲ **CRITICAL STEP** Note that the substrate should never come in contact with air, as this will damage the supported membrane.
(vii) Seal the substrate in an Attofluor cell chamber in the water bath, again without allowing the substrate to come in contact with air.
(viii) Rinse excess vesicles off surface with three washes (5 ml each) of 1× PBS.

(B) Supported membrane formation in 96-well plates ● TIMING 1.5 h

(i) Pretreat each well with 1 M NaOH for 1 h and then thoroughly rinse with DI water.
(ii) Add 100 µl SUV (from Step 19)-PBS solution (final lipid concentration 0.5–1 mg ml^{-1} in 1× PBS) to each well and incubate for 5 min.
(iii) Rinse excess SUVs with two washes (5 ml each) of 1× PBS by aspirating the solution while adding fresh 1× PBS to remove excess vesicles.

Ligand labeling and biotinylation ● TIMING 3 h

21| Reconstitute $NaHCO_3$ (component B in desired Alexa Fluor labeling kit) in 1 ml of H_2O to generate 1 M $NaHCO_3$ solution.

22| Reconstitute 200 µg of ephrin-A1 in 200 µl of 1× PBS.

23| Add 20.5 µl of 1 M $NaHCO_3$ to the ephrin-A1 solution.

24| Reconstitute 1 aliquot of EZ-Link sulfo-NHS-biotin stock in 224 µl of H_2O to generate 10 mM solution of sulfo-NHS-biotin.
▲ **CRITICAL STEP** As both ligand labeling and biotinylation occur through the reaction of NHS-esters with accessible primary amines on the target protein, both reactions must be carried out simultaneously rather than in series to ensure the availability of amines for each chemical modification.

25| Add 180 µl of 1× PBS to 20 µl of 10 mM sulfo-NHS-biotin solution, generating 1 mM solution of sulfo-NHS-biotin in 1× PBS.
▲ **CRITICAL STEP** Sulfo-NHS-biotin will readily hydrolyze in aqueous solution and must be used immediately after preparation.

26| Add 4.49 µl of 1 mM sulfo-NHS-biotin to ephrin-A1 solution from Step 23.

27| Immediately add ephrin-A1 solution to one vial of reactive Alexa Fluor dye (component A in desired Alexa Fluor labeling kit)
▲ **CRITICAL STEP** Exposure to light will photobleach organic dyes. Immediately after adding the protein solution to the reactive dye, protect the solution from light exposure in all subsequent steps.

28| Allow reaction to proceed for 1 h at room temperature with continuous mixing.

29| Follow the manufacturer's protocols (Invitrogen) for purification and measurement of the absorbance of the labeled protein at the absorption peak of the fluorophore through the use of UV-visible spectroscopy. This information is used to determine the fluorophore DOL. The fluorophore DOL is taken as the degree of biotinylation, as both chemical modifications proceed through NHS-esters reacting with available primary amines on the protein. Typical DOL values range from 1 to 2 mol dye per mol protein. DOL values are approximately twice this value (4–5 mol dye per mol protein) when fluorophore labeling is performed in the absence of biotinylation, supporting the expectation that the same amino acid residues are biotinylated and fluorescently labeled.
▲ **CRITICAL STEP** Overbiotinyation increases the likelihood of cross-linking to multiple streptavidin molecules on the surface. Labeling and biotinylation should be optimized for each protein to be linked to the surface.
? TROUBLESHOOTING

Surface functionalization ● TIMING 2.5 h

30| Incubate substrates (from Step 20) for 45 min with a 100 μg ml^{-1} (1.5 μM) BSA in 1× PBS solution at room temperature to minimize nonspecific protein adsorption.

31| Rinse off BSA solution with copious amounts of 1× PBS.

32| Incubate the supported membranes in a 17 nM solution of streptavidin in 1× PBS for 45 min at room temperature. For experiments that quantify streptavidin surface density, substitute unlabeled proteins with streptavidin conjugated to Alexa Fluor 488.

33| Rinse streptavidin out with copious amounts of 1× PBS.

34| Incubate the substrates for 45 min at room temperature with biotinylated biomolecules of interest. For ephrin-functionalized substrates, incubate 50 nM ephrin-A1, previously biotinylated and labeled with Alexa Fluor 350, Alexa Fluor 488 or Alexa 647 in Steps 21–29. If cRGD is also desired on the surface, incubate streptavidin-functionalized membrane with cRGD peptide (in concentrations ranging from 1 nM to 1 μm in 1× PBS) during ephrin-A1 incubation. Then rinse out ligand with copious amounts of 1× PBS.

FRAP ● TIMING 30 min

35| FRAP can be used to check the formation of membranes and lateral diffusion of proteins. Mount the substrate containing a supported membrane on an inverted fluorescence microscope connected to a CCD camera.

36| Focus the specimen plane on the supported membrane surface.
▲ CRITICAL STEP Poorly focused FRAP images can easily be mistaken for evidence of fluidity because of blurred boundaries at the field diaphragm edges in the images. Care must be taken to focus the specimen plane initially, and we suggest keeping the diaphragm edges visible in all images as confirmation of proper focus during substrate excitation and image acquisition.

37| Close the field diaphragm.

38| Illuminate the fluorescent sample with high-intensity excitation until the fluorophore photobleaches to ~50% (or less) of its original intensity (typically 1–5 min, depending on the fluorophore, magnification and illumination conditions used).

39| Immediately open the field diaphragm such that the edges of the aperture are visible in the camera image.

40| Immediately acquire the "before recovery" image. A bleached region in the shape of the field diaphragm, possibly with blurry edges, should be visible in the center of the image.

41| Wait 1–5 min (lower magnifications requires longer times).

42| Acquire the 'after recovery' image. If the surface is fluid, the fluorescence intensity at the center of the image should have increased and the edges of the initially photobleached region should be blurry, or entirely invisible.
? TROUBLESHOOTING

Quantitative epifluorescence microscopy ● TIMING 7 h

43| As described in Steps 15–19, and with the aid of **Supplementary Methods 5**, prepare bulk lipid calibration standards made up of vesicles containing 0.1 mol % TR-DHPE, 99.9 mol % DOPC, and 1× PBS mixed in varying proportions, yielding solutions whose final concentrations range from 0 to 0.74 μM TR-DHPE.

44| Measure the fluorescence intensities of the bulk lipid calibration standards using a fluorescence microscope. Add an aliquot of the lipid vesicle solution of 50 μl to the well in a 96-well plate. Typically, a lower magnification objective, such as an ×20 or ×10 objective, is used to collect the bulk fluorescence intensity of the solution. Typical image settings for a 0.74 μM TR-DHPE solution include 100-ms exposure time with a TR filter cube, no neutral density filters and an ×20 microscope objective. Avoid imaging the bottom surface of the well plate, as there may be some physically adsorbed vesicles that could increase the measured intensity.

45| Use the bulk lipid calibration standards to establish a bulk lipid calibration plot, in which fluorescence intensities are plotted against the concentration of the TR-DHPE. Fit the data to a straight line with a *y* intercept of 0 and designate the slope as $I_{solu(lipid)}$.

46| Prepare bulk protein calibration standards by performing serial dilution of a solution containing Alexa Fluor 594–labeled ephrin-A1 (594-EA1) in 1× PBS to generate five solutions ranging from 0 to 0.305 μM 594-EA1.

47| Measure the fluorescence intensities of the bulk protein calibration standards using a fluorescence microscope with the same acquisition settings as those used for measurement of bulk lipid calibration standards. These settings may vary depending on the imaging system; they generally include the neutral density filters, aperture openings, exposure times and filter cubes used.

48| Generate a bulk protein calibration curve by plotting fluorescence intensity against 594-EA1 and fit to a straight line with a *y* intercept set to 0, and designate the calculated slope as $I_{solu(sample)}$.

49| From the two bulk calibration standards, calculate a scaling factor (F) to express the difference in fluorescence intensities between fluorescent lipids and proteins: $F = I_{solu(sample)}/I_{solu(lipid)}$.

■ **PAUSE POINT** Bulk calibrations can be completed separately from bilayer calibrations (below), as long as lipid and protein dyes remain consistent between the two types of calibration.

50| To prepare surface bilayer calibration standards, prepare two vesicle solutions as described earlier in Steps 15–19. The first should contain 0 mol % TR-DHPE and 100 mol % DOPC. The second should contain 0.5 mol % TR-DHPE and 99.5 mol % DOPC. Mix these solutions to obtain multiple (usually between 4 and 7) solutions with intermediate concentrations of TR-DHPE. Typical solution concentrations are 0, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mol % TR-DHPE. Use these vesicle solutions to prepare surface bilayer calibration standards by depositing supported membranes as described in Step 20B.

51| Measure the fluorescence intensities of the bilayer calibration standards using a fluorescence microscope. Use a high magnification and high-numerical-aperture objective to image the ligand-functionalized membrane. Note that the acquisition settings (neutral density filters, aperture openings, exposure times, filter cubes) must be consistent to permit accurate analysis. Typical illumination settings for a 0.5 mol % TR-DHPE bilayer include 100 ms exposure time with a TR filter cube, no neutral density filters and a ×100 oil-immersion microscope objective. Typically, ten different areas are imaged and the average signal is used for calculations.

52| Graph the fluorescence intensities of the surface bilayer calibration membranes against the number of TR-DHPE per μm^2, using 0.725 nm^2 as the average footprint of each DOPC lipid molecule[41], according to a recently developed method for quantitative fluorescence microscopy[42]. Fit these data to a straight line with a *y* intercept of 0, and label the calculated slope as $I_{bilayer(lipid)}$.

53| Multiply the scaling factor (from Step 49) by $I_{bilayer(lipid)}$ to obtain the slope of a line describing fluorescence intensity versus the number of 594-EA1 molecules per μm^2, $I_{bilayer(sample)} = F \times I_{bilayer(lipid)}$.

54| Measure the fluorescence intensity of sample bilayers (from Step 34) containing an unknown density of 594-EA1 using a fluorescence microscope with the same acquisition settings (neutral density filters, aperture openings, exposure times, filter cube) as those used for measurement of surface bilayer calibration membranes.

55| Plot the measured fluorescence intensities on a line with slope $I_{bilayer(sample)}$ and *y* intercept 0, allowing a determination of the corresponding molecular densities of 594-EA1 per μm^2.

Cell engagement ● **TIMING 1 h**

56| Release cells from the tissue culture dish with 0.25% trypsin/0.38 g per liter EDTA 4Na for ~5 min at room temperature. Use enough trypsin to cover the bottom surface of the tissue culture flask (typically ~2 ml for a 75 cm^2 flask). If cells remain attached to the tissue culture flask, perform trypsinization at 37 °C until cells are rounded and detached from the surface (typical trypsin treatment is a total of 3–13 min at 37 °C). Measure the cell density with a hemocytometer cell counter.

57| Exchange the solution in which the functionalized supported membrane (from Step 34) is immersed with appropriate cell media warmed to 37 °C.

58| Add 1 × 10^5 cells to functionalized lipid membrane substrates.

59| For radial transport analysis, follow option A. For colocalization analysis, follow option B. For western blotting experiments, follow option C.

(A) Radial transport analysis ● TIMING ~7 h, depending on number of cells

(i) Allow cells to engage the membrane by incubating them for 1 h at 5% CO_2 and 37 °C. The incubation time will depend on the specific signaling pathway that is under investigation, as well as the transport processes to be analyzed. Note that adherent cells deposit their own extracellular matrix and will degrade supported membranes at long incubation times.

(ii) Image cells on supported membrane using bright-field and epifluorescence microscopy to detect fluorescent ephrin-A1 underneath cells on the supported membrane surface. The fluorescence exposure times will depend on experimental parameters, including the fluorophore used, aperture openings, neutral density filters and alignment of the mercury arc lamp; these must be optimized for the each experimental setup. Typical illumination settings to image Alexa Fluor 647-labeled ephrin-A1 included 50–500-ms exposure times with a Cy5 filter cube, no neutral density filters and an ×100 oil-immersion microscope objective.

■ **PAUSE POINT** Data analysis can be performed at a later time.

(iii) Use the bright-field channel to select cells as regions of interest in ImageJ.

(iv) Use the Radial Profile Plot ImageJ plug-in available at http://rsbweb.nih.gov/ij/plugins/radial-profile.html to measure the radial distribution of ephrin-A1 for each cell. This plug-in measures the normalized radial distribution of fluorescent ligand on the surface.

(v) Perform linear regression to fit the radial distribution of each cell to a line, whose slope serves as a score for the propensity of the cell to radially transport receptor-ligand complex.

(vi) Average the normalized radial distributions of many cells for each cell line. Cell-to-cell variability can be denoted by calculating the standard error in radial distribution slope for many cells for each cell line.

(B) Colocalization analysis ● TIMING 15.5 h, depending on number of cells

(i) Allow cells to engage the membrane by incubating for 1 h at 5% CO_2 and 37 °C.

(ii) Rinse cells with Dulbecco's 1× PBS that is stored at 4 °C.

(iii) Fix cells with 1 ml of 4% (wt/vol) paraformaldehyde in 1× PBS for 12 min. When immunostaining of intracellular protein or protein domains is required, cells should be permeabilized with 1 ml of 0.1% (vol/vol) Triton X-100 in 1× PBS for 5 min.

! CAUTION Paraformaldehyde is a probable human carcinogen. Perform all work with paraformaldehyde in a chemical fume hood with adequate ventilation.

(iv) Incubate the cells overnight at 4 °C in 1× PBS containing 10 mg ml^{-1} BSA to block nonspecific antibody binding.

▲ **CRITICAL STEP** If time is limited, 4 h incubation is sufficient.

(v) Stain cells for 40 min with primary antibodies against EphA2 and ADAM10 at concentrations of 2 μg ml^{-1}.

(vi) Rinse away excess antibodies with 1× PBS containing 1% (wt/vol) BSA.

(vii) Incubate isotype-matched secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 647 for 20 min at concentrations of 2 μg ml^{-1}.

(viii) Rinse away excess secondary antibody with 1× PBS.

■ **PAUSE POINT** After immunostaining, samples can be stored at 4 °C for up to 3 d before imaging.

(ix) To prepare a TIRF calibration bilayer, prepare supported membranes as described before in Step 20A, using vesicles containing 99.9 mol % DOPC and 0.1 mol % biotin-modified DHPE.

(x) Incubate the supported membranes for 45 min with a 17 nM solution of fluorescently labeled streptavidin made up of a 1:1 mixture of Alexa Fluor 488 streptavidin and Alexa Fluor 647 streptavidin, each with a fluorophore/protein ratio of 2.

▲ **CRITICAL STEP** Excitation at 488 nm and 647 nm is typical, as there is minimal cross-talk when these two wavelengths are used to excite Alexa Fluor 488 and Alexa Fluor 647, respectively. Other fluorophores can be used instead, but care must be taken to minimize cross-talk between channels, and streptavidin conjugates with the appropriate fluorophore pairs should be used in Step 59B(x).

(xi) Rinse the bilayer with 1× PBS.

(xii) Image several (at least 10) distinct areas of the calibration bilayer in the 488-nm and 647-nm excitation channels by TIRF illumination. The fluorescence exposure settings will depend on experimental parameters, including the fluorophore used, laser power, TIRF alignment, aperture openings and neutral density filters, and they must be optimized for each experimental setup. Typical illumination settings to image Alexa Fluor 488 included an argon laser aligned to TIRF mode providing 50-μW power at 488 nm, a NBD/HPTS filter cube with 10–100 ms exposure times, and no neutral density filters. Alexa Fluor 647 illumination settings typically included a krypton/argon laser aligned to TIRF mode

providing ~50-μW power at 647 nm, using a Cy5 filter cube with 10–100-ms exposure times, and no neutral density filters. Laser excitation was further filtered using a 488/647-nm notch filter placed in the beam path, and a ×100 TIRF oil-immersion microscope objective was used for all TIRF image acquisition.

(xiii) Image the prepared samples (from Step 59B(viii)) using dual-color TIRF microscopy at ×100 magnification. Use the same exposure settings as those used in Step 59B(xii).

■ **PAUSE POINT** Data analysis can be performed at a later time.

? TROUBLESHOOTING

(xiv) Use the Calibration Calculator ImageJ macro (**Supplementary Methods 1**) for each channel. This macro creates an average image of the calibration bilayers, then selects an area that is not illuminated and takes the average intensity of that area as background. This value is subtracted from every pixel in the average image, yielding an average, background-subtracted image.

▲ **CRITICAL STEP** A collection of ImageJ macros (**Supplementary Methods 1–4**), as well as a detailed description of data formatting requirements to use this analysis package (**Supplementary Manual**), are included. This software aids in the automation of data analysis, and can be edited to suit specific user preferences and data formatting requirements that may differ from one experimental setup to another.

(xv) Designate cell areas (20 × 20 μm in size) as regions of interest in ImageJ.

(xvi) Use the Convert and Calibrate ImageJ macro (**Supplementary Methods 2**). This macro separates the three channels (bright field, TIRF Alexa Fluor 488 and TIRF Alexa Fluor 647). For the two TIRF channels, the same unilluminated area (as in Step 59B(xiv)) is measured and the average is used as background and subtracted from every pixel in the image. Background-subtracted sample images from each channel are then divided by the average background-subtracted calibration image for the same channel (obtained in Step 59B(xiv)), yielding sample images with normalized illumination intensities that can be quantitatively compared between the two channels for the entire field of view.

(xvii) Use the Cell Selector and Ratio Calculator ImageJ macro (**Supplementary Methods 3**). This macro crops areas occupied by cells as designated in Step 59B(xv), and calculates the ratio of signal intensities in the two TIRF channels within each cell.

(xviii) Use Pearson's Calculator ImageJ macro (**Supplementary Methods 4**). This macro calculates Pearson's correlation coefficient for the two TIRF channels for each cell.

(C) Western blotting ● TIMING 2 d, 4 h

(i) Allow cells to engage the membrane by incubating for 2 h at 5% CO_2 and 37 °C.

(ii) Place each sample on ice.

(iii) Collect supernatant from each substrate.

(iv) Rinse each substrate with 2 ml cold Dulbecco's PBS and add these rinses to the supernatant.

(v) For each substrate, centrifuge the combined rinses and supernatant at 250*g* for 5 min at room temperature.

(vi) Aspirate the supernatant (from Step 59C(v)), taking care not to disturb the cell pellet.

(vii) Resuspend the cell pellet in 2 ml cold Dulbecco's PBS.

(viii) Centrifuge the resuspended cell pellet at 250*g* for 5 min at room temperature.

(ix) Aspirate the supernatant (from Step 59C(viii)), taking care not to disturb the cell pellet.

(x) Resuspend each cell pellet (from Step 59C(ix)) in 50 μl NP-40 buffer.

(xi) Add 100 μl NP-40 buffer to each substrate (from Step 59C(iv)) and scrape off adhered cells.

(xii) Add these cells to the resuspended cell pellet (from Step 59C(x)), as well as the NP-40 remaining on the substrate surface.

(xiii) Centrifuge the solution (from Step 59C(xii)) at 15,000*g* for 15 min at 4 °C.

(xiv) Collect the supernatant from Step 59C(xiii)).

(xv) Store at −80 °C until the day of western blotting.

■ **PAUSE POINT** Cell lysates can be stored at −80 °C for at least 6 months without loss of western blot signal.

(xvi) Perform western blotting on cell lysates[43].

(xvii) Incubate western blots overnight at 4 °C with primary antibodies specific to EphA2, phosphotyrosine and actin.

(xviii) Incubate western blots at room temperature for 1 h with isotype-matched secondary antibodies conjugated to Alexa Fluor 680 or Alexa Fluor 780.

(xix) Image western blots with an Odyssey Infrared Scanning System (LI-COR Biotechnology).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
5	Poor resist adhesion	Cover slip surface not sufficiently cleaned before spin	Increase duration of pre-spin sonication and piranha cleaning
9	Incomplete pattern development	Particulate matter in resist	Use a 0.45-µm syringe filter to deposit resist before spin
		Resist is expired	Obtain a new lot of resist
		Cover slip surface not sufficiently cleaned before spin	See Troubleshooting for Step 5
	No pattern development	E-beam dosing too low	Create a dose matrix to ascertain the minimum effective shot time
		Resist and/or conductive polymer layer too thick	Ensure complete mixing of resist solution
			Thin resist solution with additional anisole
			Spin-coat resist and/or conductive polymer at a higher r.p.m.
		Cover slip was exposed resist-side down	Check cover slip orientation before exposure by scratching resist
		Poor resist adhesion	See Troubleshooting for Step 5
13	Breaks in chrome grid lines	Incomplete pattern development	See Troubleshooting for Step 9
		Cover slip surface not sufficiently cleaned pre-evaporation	Dust off cover slips with air gun before chrome deposition
	Chrome film is the wrong height	Quartz crystal microbalance (QCM) is not calibrated	Adjust the QCM tooling factor
		QCM adsorbate is above working limits	Obtain fresh QCM
	Chrome does not act as diffusion barrier	Chrome film is too thin	Evaporate chromium for longer time
		Film contains impurities	Vacuum-clean inside of evaporator before deposition
			Degas chrome target with E-beam gun for 5 min before beginning deposition
			After deposition, wait until samples have completely cooled to room temperature before venting the evaporation chamber and exposing the samples to air. A 15-min interval between the end of deposition and chamber venting should be sufficient
		Chrome filings have oxidized	Obtain fresh chrome filings
14	Chrome residue present on the surface after liftoff	Poor resist adhesion	See Troubleshooting for Step 5
		Stray chrome particles have adhered to the surface during liftoff sonication	Perform liftoff with two beakers of methylene chloride. Briefly sonicate in the first to remove the bulk of the chrome, then quickly transfer cover slip rack to the second, clean beaker for the remainder of the liftoff

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		Incomplete pattern development	See Troubleshooting for Step 9
		Cover slip surface not sufficiently cleaned before spin	See Troubleshooting for Step 5
29	Minimal fluorescence signal from labeled protein or no cell response to protein on supported membrane surface	Protein labeling needs to be optimized	Repeat fluorophore labeling with a range of fluorophore: protein molar ratios to optimize labeling conditions
42	Minimal FRAP	Oxidized phospholipids do not form fluid-supported membranes	Ensure vesicles are not more than 2 weeks old, and original lipid stocks are not more than 3 months old. Remake lipid stocks with lipid dyes (1 mol% NBD-PC, 98.9 mol% DOPC and 0.1 mol% biotin-DOPE) and examine FRAP of lipids as well as proteins to determine bilayer fluidity
		Poorly cleaned silica surface prevents supported membrane formation	See Troubleshooting for Step 5
59B(xiii)	Low immunostaining signal or high background	Primary or secondary antibody concentrations need to be optimized	Titrate samples with a range of antibody concentrations of primary and secondary antibody solutions
		Protein denatured	Relabel a fresh solution of protein
	No clustering in the SLB after cell incubation	Streptavidin solution is aggregated and aged or protein itself may be aggregated	Remake streptavidin solutions and protein solutions
	High degree of aggregation in SLB	Nonspecifically adhered vesicles are bound to the substrate	Use additional rinse of 1× PBS and DI water to remove SUVs

● **TIMING**

Steps 1–14, Preparation of E-beam fabricated substrates: 12 h
Steps 15–20, Preparation of supported membranes: Steps 15–19, 3 h; Step 20A, 30 min; Step 20B, 1.5 h
Steps 21–29, Ligand labeling and biotinylation: 3 h
Steps 30–34, Surface functionalization: 2.5 h
Steps 35–42, Fluorescence recovery after photobleaching: 30 min
Steps 43–55, Quantitative epifluorescence microscopy: Steps 43–49, 3 h; Steps 50–55, 4 h
Steps 56–58, Cell engagement: 1 h
Step 59A, Radial transport analysis: 7 h, depending on number of cells
Step 59B, Colocalization analysis: 15.5 h, depending on number of cells
Step 59C, Western blotting: 2 d, 4 h

ANTICIPATED RESULTS

Bioactivity of supported membrane-anchored ligands

We used the biotin-streptavidin linkage described in Steps 21–29 to anchor the ephrin-A1 ligand to supported membranes, and then engaged EphA2-expressing MDA-MB-231 cells with this ligand. Membrane-bound ephrin-A1 triggered the EphA2 RTK pathway and ultimately led to its degradation, as measured by western blotting (**Fig. 3**), confirming that the ligand was active on the supported membrane surface.

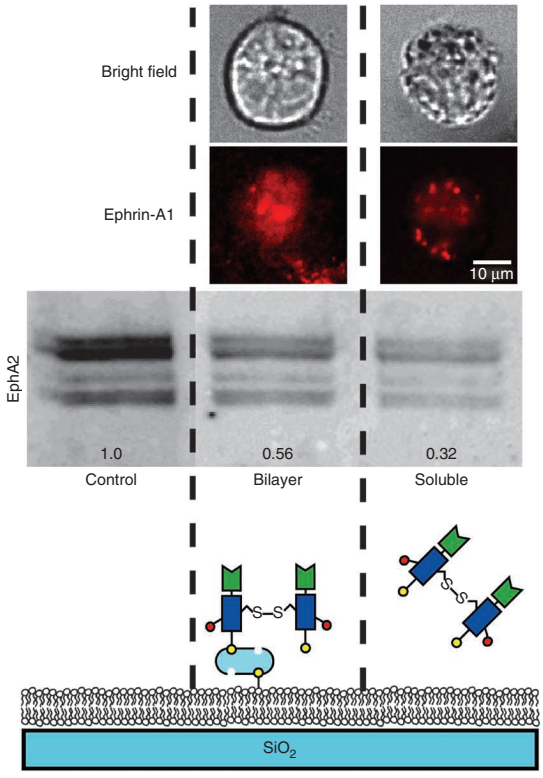

Figure 3 | Assessing membrane-anchored ligand activity. (Top) Epifluorescence images showing clustering of fluorescent ephrin-A1 by live MDA-MB-231 cells. Bright-field and epifluorescence images were taken 2 h after addition of cells. (Middle) Western blots showing that both soluble and supported membrane-bound ligands lead to increased EphA2 degradation[45], resulting in less EphA2 protein in MDA-MB-231 cells. Numbers denote amount of EphA2 signal relative to control. (Bottom) Schematic representation of ephrin-A1 presentation to live cells.

Evaluating ligand-induced receptor clustering, endocytosis and spatial mutations

Following initial ligand engagement, receptor-expressing cells may endocytose and degrade the receptor-ligand complex. After endocytosis of the ligand, the surface will offer no mechanical perturbations to transport of the receptor-ligand complex, nullifying the strengths of the spatial mutation strategy described herein. To confirm that the ligand remained on the supported membrane surface after cell engagement, the fluorescence intensity of each grid square on a nanopatterned surface was measured. Squares underneath cells, in which there were clusters of ligand, had the same fluorescence intensity as squares that were not underneath cells, in which ligand was homogeneously distributed throughout the grid square (**Fig. 4a**). This was taken as evidence that endocytosis of membrane-anchored

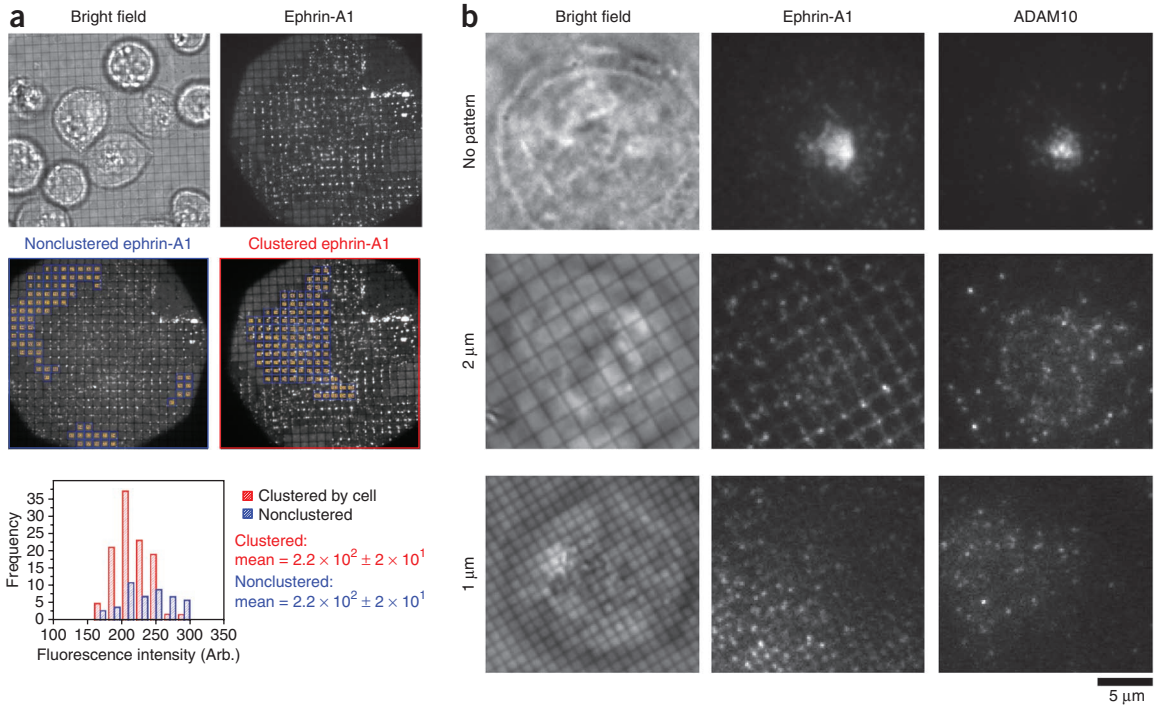

Figure 4 | EphA2 receptor lateral transport is hindered by nanopatterned diffusion barriers in the supported lipid membrane. (**a**) Bright-field and fluorescence images of MDA-MB-231 cells showing that cells are unable to move ephrin-A1 clusters across diffusion barriers on the surface. This is observed by the accumulation of ephrin-A1 at grid lines. Note that the ephrin-A1 clusters remain on the surface, as evidenced by equal fluorescence intensities in grid squares underneath cells compared with squares not underneath cells. (**b**) MDA-MB-231 cells were allowed to engage nanopatterned supported membranes showing laterally mobile ephrin-A1 for 1 h. Cells were then fixed, permeabilized, stained with a primary antibody specific for the metalloprotease ADAM10 (implicated in cleavage of ephrin ligands from cell surfaces, allowing endocytosis and degradation of Eph-ephrin complex[44]); this was followed by treatment with an isotype-matched fluorescently labeled secondary antibody and imaging with TIRF microscopy. Left panels are bright-field images showing cells and metal lines with 1- and 2-µm line spacings, as noted. Center panels show fluorescently labeled ephrin-A1 in corresponding areas. Right panels show antibody-labeled ADAM10 in corresponding areas. This supports the observation that ADAM10 is recruited to EphA2-ephrin-A1 clusters when lateral transport of receptor-ligand complex is allowed (no grid lines are present).

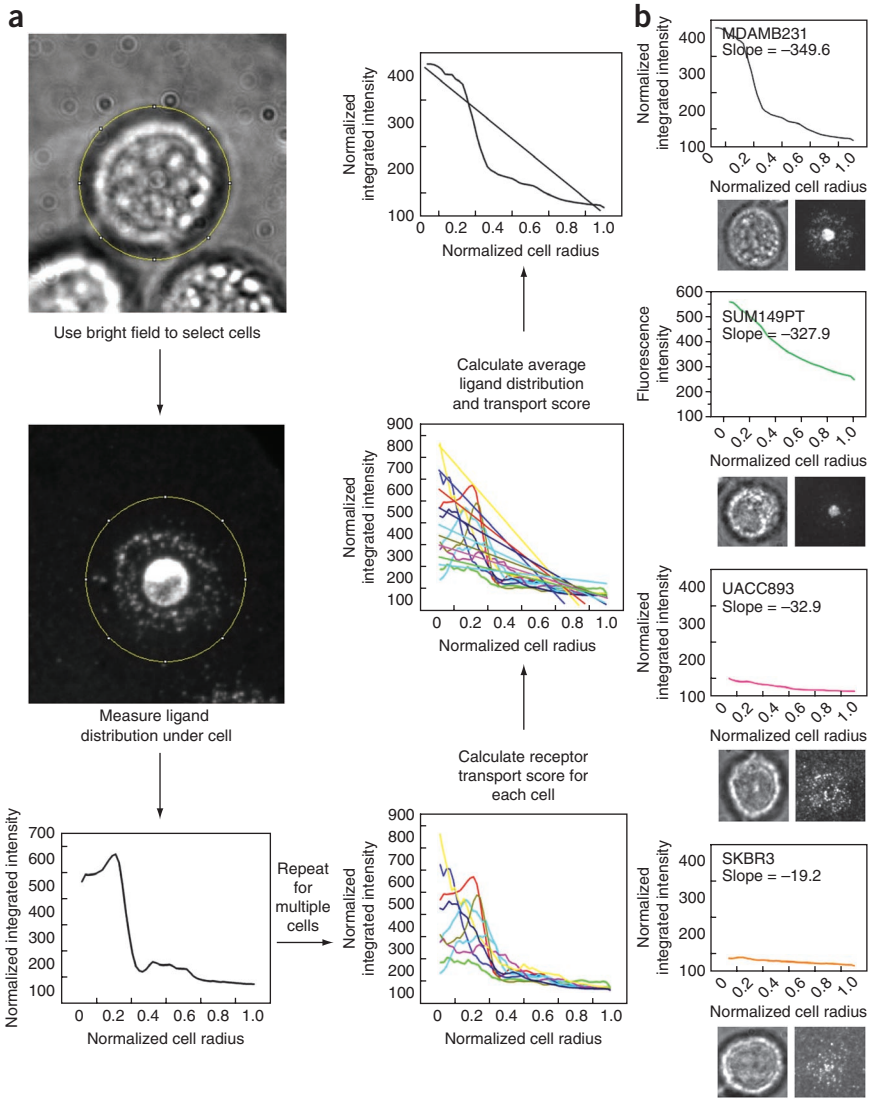

Figure 5 | Approach to quantifying EphA2-ephrin-A1 transport by different cell lines. (**a**) Radial distributions of ephrin-A1 underneath single cells were measured for many cells in each cell line. The radial distribution of the ligand underneath each cell was fit to a line whose slope served as a measure of radial transport. (**b**) Examples of average scores for tens to hundreds of cells in each cell type were then compared to determine the relative degrees of receptor transport across the cell line library.

ephrin-A1 does not occur to a significant extent within 1 h, and this time point was used for all experiments using nanopatterned supported membranes. Cells were triggered with fluid ephrin-A1 for 1 h and stained for ADAM10 (**Fig. 4b**), a metalloprotease implicated in Eph receptor degradation[44]. To quantitatively probe downstream signaling as a result of Eph-ephrin reorganization, the recruitment of ADAM10 to Eph-ephrin complex was measured as described in Step 59B(xiv–xviii), as a function of EphA2 spatial organization. This led to the observation that mechanical manipulation of EphA2 organization alters the cell response to EphA2 activation[2].

Quantifying transport across a library of cell lines

The radial distributions of ephrin-A1 were measured for single cells as described in Step 59A(i–vi). The radial distribution of each cell was fit to a line, whose slope served as a measure of radial transport (**Fig. 5a**). This technique was used to score tens to hundreds of cells for each cell line of a 26-cell line library. The average scored radial transport values for each cell line were compared to determine the relative degrees of receptor transport across the cell line library (**Fig. 5b**).

ACKNOWLEDGMENTS We thank J.W. Gray and R.M. Neve for discussions that led to the use of supported membranes to study EphA2-ephrin-A1 signaling, and for providing the cells used in this work. We also thank N. Bayani for assistance in performing western blotting, A. Smoligovets and C.-H. Yu for performing transfection and imaging with EGFP-actin-expressing MDA-MB-231 cells, and A. Bershadsky for helpful discussions. This work was supported by the Director, Office of Science, Office of Basic Energy Sciences, Chemical Sciences, Geosciences and Biosciences Division (K.S., P.M.N.; hybrid synthetic-live cell interfaces) and Materials Sciences and Engineering Division (R.S.P.; supported membrane substrates) of the U.S. Department of Energy (DOE) under contract no. DE-AC02-05CH11231. Patterned substrate fabrication was performed, in part, at the Molecular Foundry, Lawrence Berkeley National Laboratory (LBNL), and was supported by the Office of Science, Office of Basic Energy Sciences, Scientific User Facilities Division of the U.S. DOE under contract no. DE-AC02-05CH11231. This work was also supported by the Laboratory Directed Research and Development Program of LBNL under U.S. DOE contract no. DE-AC02-05CH11231. Seed support for biomedical aspects of this work was provided by the U.S. Department of Defense Breast Cancer Research Program Concept Award BC076701 under U.S. Army Medical Research Acquisition Activity no. W81XWH-08-1-0677 with follow-on support provided by Award U54 CA143836 from the National Cancer Institute (NCI) beginning in 2009. K.S. acknowledges Oak Ridge National Laboratory's Center for Nanophase Materials Sciences, Scientific User Facilities Division, Office of Basic Energy Sciences, U.S. Department of Energy (CNMS2009-269). K.S. is also grateful to the Georgia Cancer Coalition (GCC) for a Cancer Research Award. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NCI or the National Institutes of Health (NIH). The Regents of the University of California have filed a related patent application through LBNL.

AUTHOR CONTRIBUTIONS P.M.N. wrote the ImageJ software used to quantify metalloprotease recruitment to EphA2 clusters. P.M.N. and K.S. designed and performed all experiments described here. R.S.P. optimized and performed all E-beam lithography used to fabricate the patterned supported membranes. J.T.G. oversaw all aspects of the work.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

Published online at http://www.natureprotocols.com/.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

1. Singh, A.B. & Harris, R.C. Autocrine, paracrine and juxtacrine signaling by EGFR ligands. *Cell Signal.* **17**, 1183–1193 (2005).

2. Salaita, K. *et al.* Restriction of receptor movement alters cellular response: physical force sensing by EphA2. *Science* **327**, 1380–1385 (2010).
3. Mossman, K.D., Campi, G., Groves, J.T. & Dustin, M.L. Altered TCR signaling from geometrically repatterned immunological synapses. *Science* **310**, 1191–3 (2005).
4. Mammen, M., Choi, S. & Whitesides, G.M. Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors. *Angew. Chem. Int. Ed. Engl.* **37**, 2754–2794 (1998).
5. Discher, D.E., Janmey, P. & Wang, Y.L. Tissue cells feel and respond to the stiffness of their substrate. *Science* **310**, 1139–1143 (2005).
6. del Rio, A. *et al.* Stretching single talin rod molecules activates vinculin binding. *Science* **323**, 638–641 (2009).
7. Vogel, V. & Sheetz, M. Local force and geometry sensing regulate cell functions. *Nat. Rev. Mol. Cell Biol.* **7**, 265–275 (2006).
8. Hatzakis, N.S. *et al.* How curved membranes recruit amphipathic helices and protein anchoring motifs. *Nat. Chem. Biol.* **5**, 835–841 (2009).
9. Demond, A.L., Mossman, K.D., Starr, T., Dustin, M.L. & Groves, J.T. T cell receptor microcluster transport through molecular mazes reveals mechanism of translocation. *Biophys. J.* **94**, 3286–3292 (2008).
10. Manz, B.N. & Groves, J.T. Spatial organization and signal transduction at intercellular junctions. *Nat. Rev. Mol. Cell Biol.* **11**, 342–352 (2010).
11. Hartman, N.C., Nye, J.A. & Groves, J.T. Cluster size regulates protein sorting in the immunological synapse. *Proc. Natl. Acad. Sci. USA* **106**, 12729–12734 (2009).
12. Lackmann, M. & Boyd, A.W. Eph, a protein family coming of age: more confusion, insight, or complexity? *Sci. Signal.* **1**, re2 (2008).
13. Kullander, K. & Klein, R. Mechanisms and functions of Eph and ephrin signalling. *Nat. Rev. Mol. Cell Biol.* **3**, 475–86 (2002).
14. Davis, S. *et al.* Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. *Science* **266**, 816–9 (1994).
15. Muzzey, D. & van Oudenaarden, A. Quantitative time-lapse fluorescence microscopy in single cells. *Annu. Rev. Cell Dev. Biol.* **25**, 301–327 (2009).
16. Nam, J.M., Nair, P.M., Neve, R.M., Gray, J.W. & Groves, J.T. A fluid membrane-based soluble ligand-display system for live-cell assays. *Chembiochem* **7**, 436–40 (2006).
17. Nye, J.A. & Groves, J.T. Kinetic control of histidine-tagged protein surface density on supported lipid bilayers. *Langmuir* **24**, 4145–4149 (2008).
18. Gureasko, J. *et al.* Membrane-dependent signal integration by the Ras activator Son of sevenless. *Nat. Struct. Mol. Biol.* **15**, 452–461 (2008).
19. Axelrod, D., Koppel, D.E., Schlessinger, J., Elson, E. & Webb, W.W. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* **16**, 1055–1069 (1976).
20. Groves, J.T., Parthasarathy, R. & Forstner, M.B. Fluorescence imaging of membrane dynamics. *Annu. Rev. Biomed. Eng.* **10**, 311–338 (2008).
21. Kung, L.A., Kam, L., Hovis, J.S. & Boxer, S.G. Patterning hybrid surfaces of proteins and supported lipid bilayers. *Langmuir* **16**, 6773–6776 (2000).
22. Groves, J.T., Ulman, N. & Boxer, S.G. Micropatterning fluid lipid bilayers on solid supports. *Science* **275**, 651–653 (1997).
23. Cremer, P.S., Groves, J.T., Kung, L.A. & Boxer, S.G. Writing and erasing barriers to lateral mobility into fluid phospholipid bilayers. *Langmuir* **15**, 3893–3896 (1999).
24. Gates, B.D., Xu, Q., Love, J.C., Wolfe, D.B. & Whitesides, G.M. Unconventional nanofabrication. *Annu. Rev. Mater. Res.* **34**, 339–372 (2004).
25. Gonsalves, K.E., Thiyagarajan, M. & Dean, K.R. New resists for nanometer scale patterning by extreme ultraviolet lithography. *J. Micro/Nanolithography, MEMS, and MOEMS* **4**, 029701 (2005).
26. Salaita, K., Wang, Y. & Mirkin, C.A. Applications of dip-pen nanolithography. *Nat. Nano.* **2**, 145–155 (2007).
27. Salaita, K. *et al.* Massively parallel dip-pen nanolithography with 55 000-pen two-dimensional arrays. *Angew. Chem. Int. Ed. Engl.* **45**, 7220–7223 (2006).
28. Salaita, K. *et al.* Sub-100 nm, centimeter-scale, parallel dip-pen nanolithography. *Small* **1**, 940–945 (2005).
29. Tenchov, B.G., Yanev, T.K., Tihova, M.G. & Koynova, R.D. A probability concept about size distributions of sonicated lipid vesicles. *Biochim. Biophys. Acta* **816**, 122–130 (1985).
30. Lasic, D.D. *Liposomes in Gene Delivery* (CRC Press, 1997).
31. Ollivon, M., Lesieur, S., Grabielle-Madelmont, C. & Paternostre, M. Vesicle reconstitution from lipid-detergent mixed micelles. *Biochim. Biophys. Acta* **1508**, 34–50 (2000).
32. Richter, R.P., Him, J.L.K. & Brisson, A. Supported lipid membranes. *Mater. Today* **6**, 32–37 (2003).
33. Ochsner, M. *et al.* Micro-well arrays for 3D shape control and high resolution analysis of single cells. *Lab. Chip* **7**, 1074 (2007).
34. Schubert, D.W. & Dunkel, T. Spin coating from a molecular point of view: its concentration regimes, influence of molar mass and distribution. *Mater. Res. Innovations* **7**, 314–321 (2003).
35. Qin, D., Xia, Y. & Whitesides, G.M. Soft lithography for micro- and nanoscale patterning. *Nat. Protoc.* **5**, 491–502 (2010).
36. Cho, N., Frank, C.W., Kasemo, B. & Hook, F. Quartz crystal microbalance with dissipation monitoring of supported lipid bilayers on various substrates. *Nat. Protoc.* **5**, 1096–1106 (2010).
37. Hallett, F.R., Watton, J. & Krygsman, P. Vesicle sizing: number distributions by dynamic light scattering. *Biophys. J.* **59**, 357–362 (1991).
38. Gupta, K., Wasilik, M. & Williams, K.R. Etch rates for micromachining processing-part II. *J. Microelectromechanical Syst.* **12**, 761–778.
39. Hovis, J.S. & Boxer, S.G. Patterning and composition arrays of supported lipid bilayers by microcontact printing. *Langmuir* **17**, 3400–3405 (2001).
40. Yu, C., Parikh, A. & Groves, J. Direct patterning of membrane-derivatized colloids using in-situ uv-ozone photolithography. *Adv. Mater.* **17**, 1477–1480 (2005).
41. Nagle, J.F. & Tristram-Nagle, S. Structure of lipid bilayers. *Biochim. Biophys. Acta* **1469**, 159–195 (2000).
42. Galush, W.J., Nye, J.A. & Groves, J.T. Quantitative fluorescence microscopy using supported lipid bilayer standards. *Biophys. J.* **95**, 2512–2519 (2008).
43. Misawa, Y., Li, Y., Rekosh, D. & Hammarskjold, M.L. Western blot analysis of sub-cellular fractionated samples using the Odyssey Infrared Imaging System. *Nat. Protoc.* doi:10.1038/nprot.2006.219 (2006).
44. Janes, P.W. *et al.* Adam meets Eph: An ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans. *Cell* **123**, 291–304 (2005).
45. Walker-Daniels, J., Riese, D.J. & Kinch, M.S. c-Cbl-dependent EphA2 protein degradation is induced by ligand binding. *Mol. Cancer Res.* **1**, 79–87 (2002).