A protocol for the systematic and quantitative measurement of protein–lipid interactions using the liposome-microarray-based assay

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Published online 5 May 2016; doi:10.1038/nprot.2016.059

Lipids organize the activity of the cell's proteome through a complex network of interactions. The assembly of comprehensive atlases embracing all protein–lipid interactions is an important challenge that requires innovative methods. We recently developed a liposome-microarray-based assay (LiMA) that integrates liposomes, microfluidics and fluorescence microscopy and which is capable of measuring protein recruitment to membranes in a quantitative and high-throughput manner. Compared with previous assays that are labor-intensive and difficult to scale up, LiMA improves the protein–lipid interaction assay throughput by at least three orders of magnitude. Here we provide a step-by-step LiMA protocol that includes the following: (i) the serial and generic production of the liposome microarray; (ii) its integration into a microfluidic format; (iii) the measurement of fluorescently labeled protein (either purified proteins or from cell lysate) recruitment to liposomal membranes using high-throughput microscopy; (iv) automated image analysis pipelines to quantify protein–lipid interactions; and (v) data quality analysis. In addition, we discuss the experimental design, including the relevant quality controls. Overall, the protocol—including device preparation, assay and data analysis—takes 6–8 d. This protocol paves the way for protein–lipid interaction screens to be performed on the proteome and lipidome scales.

INTRODUCTION

Plasma and organelle membranes contain important signaling lipids such as phosphoinositides and sphingolipids that organize myriad intracellular reactions in space and time[1–3]. It is estimated that the lipidome comprises more than 40,000 lipid species (LIPIDmap database: http://www.lipidmaps.org)[4] and that integral and peripheral-membrane proteins account for one-third of the entire proteome[5]. Despite the importance of protein–lipid interactions in biology[6] and medicine[7], the full repertoire is still unknown. Protein–lipid interactions are diverse and may take place in indifferent cellular environments[8]. For example, integral membrane proteins interact with lipids within the plane of the membrane, whereas peripheral-membrane proteins are recruited to the membrane surfaces, where they recognize specific lipid head groups or membrane properties. Each type of interaction has a companion set of dedicated analysis techniques[8]. The classical assays (e.g., isothermal titration calorimetry[9], lipid flotation assay[10] and surface plasmon resonance[11]) have provided great insights into the mechanisms of protein–lipid interactions, but they are labor-intensive and difficult to scale up to the proteome and lipidome scales. Innovative methodologies (e.g., protein array[12], chemically engineered lipids[13] and affinity-purification lipidomics[14,15]) are emerging to probe protein–lipid interaction on the systems level[8]. Extensive comparison between classic and emerging methods has been published elsewhere[8]. This review can be used as a guide to choose which method fits best to the scientific question at hand. The new protocol that we report here is particularly well suited for measuring the recruitment of soluble, peripheral-membrane proteins to biological membranes, and compared with other approaches (e.g., surface plasmon resonance, lipid flotation assay and isothermal titration calorimetry) it offers high throughput.

Molecular signaling events arising in the vicinity of the membrane are notoriously problematic to study *in vivo*. Therefore, *in vitro* approaches are used to reconstitute the precise and often complex sequence of molecular events that lead to lipid targeting by a protein, and to study how a protein can influence the molecular composition of the membrane and how a lipid can shape protein function[16]. Additional complexity arises from the fact that proteins can be promiscuous in their lipid-binding specificity[17,18]; the interaction can involve multiple lipids simultaneously[18–20], and the biophysical properties of the membrane (e.g., charge, curvature) are sensed by protein modules[21,22]. The systematic study of direct protein recruitment to biological membranes *in vitro* therefore requires the reconstitution of thousands of surrogate membranes and the quantitative measurement of the various interactions, two technical challenges that to date have prevented the study of a system-wide protein–lipid interactome[8].

We have recently developed LiMA[23], which is a miniaturized assay that measures the membrane recruitment of proteins in an automated, multiplexed, quantitative and high-throughput manner. The core technology of LiMA is the parallel self-assembly of giant unilamellar liposomes (also called giant unilamellar vesicles[24], which have a diameter of >5 μm) onto a microfluidic platform (**Fig. 1a**). In the current version of the assay, one liposome microarray can accommodate 120 different liposome types distributed (at a density of 100 types per cm^2) in four independent microfluidic chambers so that multiple proteins can be studied in parallel (**Fig. 1a**). LiMA relies on fast prototyping and robust

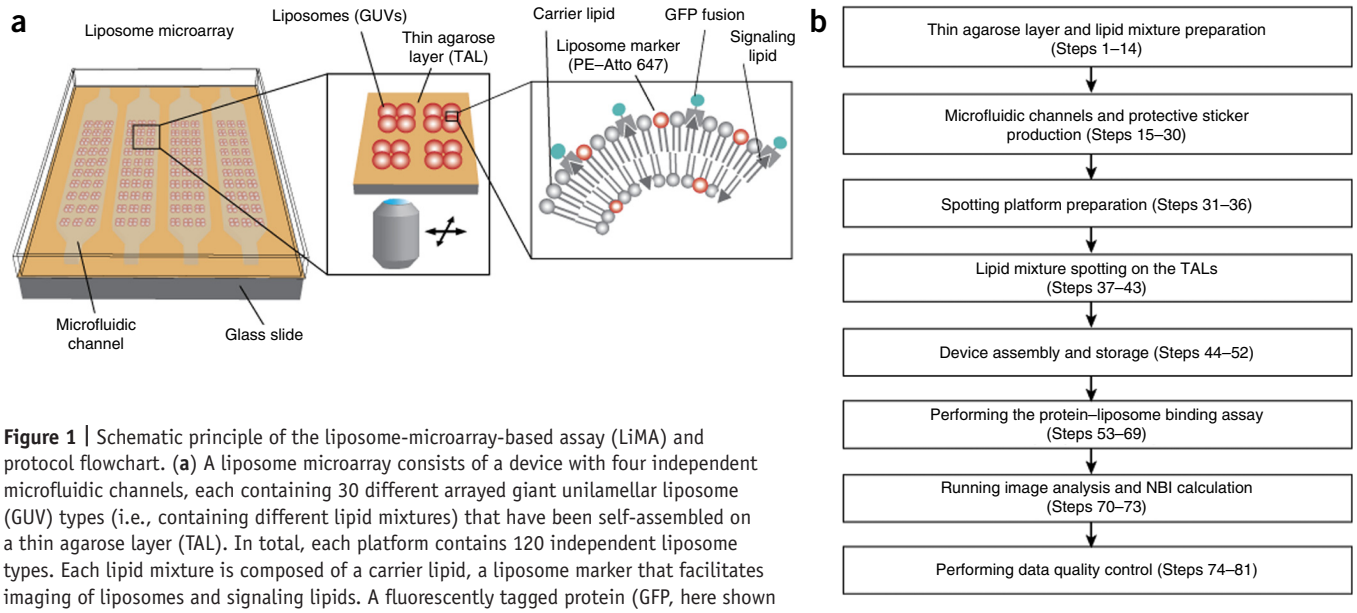


Figure 1 | Schematic principle of the liposome-microarray-based assay (LiMA) and protocol flowchart. (**a**) A liposome microarray consists of a device with four independent microfluidic channels, each containing 30 different arrayed giant unilamellar liposome (GUV) types (i.e., containing different lipid mixtures) that have been self-assembled on a thin agarose layer (TAL). In total, each platform contains 120 independent liposome types. Each lipid mixture is composed of a carrier lipid, a liposome marker that facilitates imaging of liposomes and signaling lipids. A fluorescently tagged protein (GFP, here shown in turquoise) is injected into the device and interacts with its cognate lipids, and the interaction is imaged by an automated high-throughput microscope. (**b**) Flowchart of the protocol including references to the steps in the PROCEDURE section.

methods that can be adapted for the serial production of up to 20 devices in 1 d. The use of microfluidics reduces the amount of protein or cell lysate required to as low as a few picomoles, so the assay can be performed in parallel in a highly reproducible manner. A glass slide is covered with a thin agarose layer (TAL), and lipid mixtures (i.e., lipids dissolved in an organic solvent) are sprayed and dried onto predefined places[23]. All liposomes form spontaneously and simultaneously within a few minutes after the hydration of the spotted TAL in a physiological buffer[23,25]. Microfluidic channels that are bonded on the platform (**Fig. 1a**) allow fluorescently labeled protein(s)—either purified or in a cell lysate[23]—to be incubated with the liposomes. The recruitment of proteins to liposomes is measured via an automated high-throughput microscope (**Fig. 1a**). Each interaction is quantified after image analysis through the measurement of a normalized binding intensity (NBI), which reflects the quantity of proteins bound to the liposome membrane[23].

We have previously shown that the liposomes efficiently integrate all classes of signaling lipids such as glycerophospholipids, phosphatidylinositol phosphates, sphingolipids and sterols, and the sensitivity of the assay ranges from nanomolar to micromolar interactions[23]. As proof of principle, LiMA has been used to quantify more than 32,000 protein–lipid interactions to study the role of lipid cooperativity in the recruitment of Pleckstrin homology domains to membranes[20].

Here we describe an accessible end-to-end protocol (**Fig. 1b**) for LiMA, including the serial production and storage of the array, its integration into a microfluidic format, the setup required to perform parallelized experiments and the automatic high-content screening microscopy, as well as automated image and data quality control.

Applications of the protocol

As a result of its high throughput, LiMA opens new perspectives in the field of protein–lipid interactions. The first application that we envision for LiMA is the systematic screening of peripheral-membrane protein–lipid interactions, which is currently an important challenge, as lipids are notoriously absent from published interactome projects[19,26–28]. Many diseases have been linked to a misregulation of protein–lipid interactions, and point mutations can abolish or change the lipid-binding specificity of a protein[29]. With increasing sequencing data, more and more genetic variants are being described, but their functional implication requires high-throughput and quantitative profiling methods, both of which are provided by LiMA[20,23]. LiMA could also be used to identify novel and specific probes for lipids to comprehensively map the subcellular localization of lipids. Some progress in this area has been made[30], but many lipids remain poorly characterized, particularly with respect to the dynamics of their intracellular localization. Finally, lipid-binding domains are increasingly being recognized as potential drug targets[31], and LiMA could be used to screen for inhibitors of protein–lipid interactions.

Current limitations and perspectives

From a technical point of view, the spotter used in this protocol inherently restricts the spot density to 100 spots per cm^2, which limits the current throughput of LiMA. Ideally, it would be desirable to achieve a density of 1,000 spots per cm^2, which means a spot size of $100 \times 100\ \mu m^2$. To date, we have restricted the use of LiMA to tracking the interaction between soluble peripheral-membrane proteins and membranes[20,23]; however, because LiMA has a readout based on imaging, many other features could be tracked. For example, many proteins have been shown to modify membrane properties by inducing the formation of micro or nano domains[32], and LiMA can be an ideal platform to systematically analyze such proteins using classical confocal microscopy, or it could be coupled with more advanced microscopy techniques such as fluorescence correlation microscopy or Förster resonance energy transfer to probe for subdiffraction-sized lipid domains[33].

In the current format, LiMA builds on the existence of genome-wide collections of cell lines expressing GFP protein fusions.

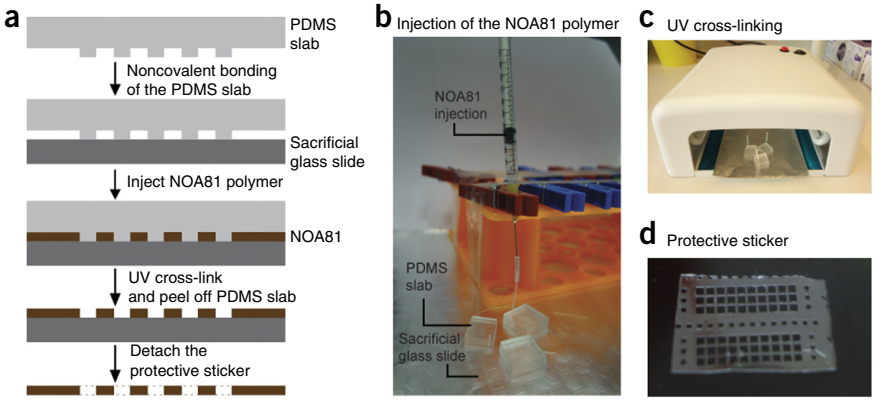


Figure 2 | Protective sticker preparation (Steps 21–30). (**a**) Schematic representation of the preparation of the protective stickers and photos illustrating the key steps of the protocol. (**b**,**c**) A polydimethylsiloxane (PDMS) slab is bonded noncovalently to a sacrificial glass slide. (**b**) A liquid UV-sensitive polymer is injected between the slab and the glass slide before (**c**) being subjected to UV illumination and polymerization. (**d**) The PDMS slab is removed and the protective stickers are detached from the glass slide.

However, the assay should be readily adaptable to other readouts that would allow researchers to avoid fluorescent labeling of proteins, such as mass spectrometry.

Protein–lipid interactions go beyond the study of soluble peripheral proteins with lipid membrane[8]. Furthermore, it has been shown that membrane proteins can be incorporated during the formation of giant vesicles using the agarose-based method[34,35]. Thus, combining LiMA and proteoliposomes[34,35] would be a straightforward approach to the formation of a proteoliposome array. In the future, the assay could be extended to study the interaction between integral membrane proteins and their neighboring lipids using confocal microscopy[34] or Förster resonance energy transfer–based approaches[36]. Finally, LiMA may become a platform with which to study the transport capabilities of solute carrier proteins[37].

Experimental design

A flowchart of the protocol is summarized in **Figure 1b**. It involves eight major stages that are described in detail below:

(i) Preparation of the TAL on glass slides and the lipid mixtures (Steps 1–14)
(ii) Production of the microfluidic channels and the protective stickers (**Fig. 2**; Steps 15–30)
(iii) Preparation of the spotting platform (**Fig. 3**; Steps 31–36)
(iv) Spotting of the lipid mixtures on the TALs (Steps 37–43)
(v) Device assembly and storage (**Fig. 4**; Steps 44–52)
(vi) Performing the protein–liposome binding assay (Steps 53–69)
(vii) Running image analysis and NBI calculation (Steps 70–73)
(viii) Performing data quality control (Steps 74–81)

Preparation of the TAL on glass slides and the lipid mixtures: formation of the TAL. Glass slides are coated with a TAL by dip-coating in a 1% (wt/vol) solution of low-melting-temperature agarose solubilized in deionized water that will support the formation of the liposomes once soaked with lipids[23,25]. After drying, a 300-nm agarose layer is typically generated[23] that does not impair the transparency of the glass slide. Increasing the concentration of agarose leads to an increase in the height of the TALs and the formation of liposomes with a larger diameter[23]. A homogeneous agarose coating is essential for an even formation of liposomes over the whole surface of the array. We found that a cleaning protocol using Hellma detergent renders the glass surface hydrophilic, and it results in a uniform agarose coating over the entire glass surface and reproducible results without requiring treatments with harsh chemicals such as hydrofluoric acid and NaOH. Coated glass slides can be arranged on a glass-slide holder (**Supplementary Fig. 1a** and **Supplementary Data 1**) and stored in a clean space (not necessarily a dedicated clean room) covered with Parafilm for at least 1 month.

Preparation of the TAL on glass slides and the lipid mixtures: lipid mixture preparation. Lipids are usually shipped by suppliers in solid form, and they must be solubilized in a proper solvent in glass vials. They can then be incorporated into lipid mixture solutions (with all the mixtures having an equal molar concentration) in glass vials closed with a septum and stored on spotting racks designed to fit in the spotter (**Supplementary Fig. 1b**). In every lipid mixture, signaling lipids are mixed with a carrier lipid that has a neutral charge to minimize the nonspecific binding to charged lipids. We opted for 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), which is considered to be a major constituent of lipid membranes[38]. A small amount (<1 mol% (mole fraction)) of a fluorescently labeled lipid (phosphatidylethanolamine (PE)–Atto 647) called a 'liposome marker' (**Fig. 1a**) that has a fluorescence spectrum that does not overlap with the signal from the protein (i.e., GFP) is added to visualize and further isolate the liposomes during the image analysis procedure (see 'Formation of liposomes' and 'Image acquisition' sections). To maximize liposome yield, lipid mixtures should be prepared with the following points in mind: the concentration of the lipid mixtures must be carefully chosen, because too few or too many lipids on the TALs prevents the formation of liposomes (from our experience, lipid mixtures having a total lipid concentration of 0.26 mM are optimal); the fatty acid chains of the signaling lipid must include at least one unsaturation, without which the signaling lipids will integrate into the liposomes with very low efficiency at room temperature (RT, 25 °C; i.e., lipids with fatty acid chains composed of palmitoyl–oleoyl or oleoyl–oleoyl combinations are better incorporated than lipids composed of palmitoyl–palmitoyl fatty acid chain combinations); and a small amount of PEGylated lipids should be added to every lipid mixture, as they are known to favor the formation of liposomes[39] (the more highly charged the signaling lipid, the higher the amount of PEGylated lipid needed; for example, in our experience for lipids with charges down to −4.97, corresponding to double-phosphorylated PIPs such as $PI(3,4)P_2$ and $PI(4,5)P_2$, adding 0.5 mol% (phosphatidylinositol phosphates) of PE-PEG350 is sufficient, whereas for lipids with charges as low as −6.95, corresponding to triple-phosphorylated PIP $PI(3,4,5)P_3$, 5 mol% of PE-PEG2000 should be added).

Box 1 | Protocols for microfabrication

Several parts of the protocol require the use of microfabrication techniques based on soft lithography approaches, and we refer readers to protocols that are already described in the literature[40–42,54]. In summary, soft lithography is a set of techniques and tools based on the printing, molding and embossing of soft material—often PDMS—and always requires the following steps: (i) a mask is designed using computer-assisted design (CAD) software; (ii) the mask is printed on a plastic film or a quartz wafer; (iii) the master is fabricated in a clean room; and (iv) the master is serially replicated using polymeric replica. Several soft lithography tools are used thorough the protocol, namely, microcontact printing, micromolding in capillaries and microfluidics[40–42,54]. They have the advantage of being easy to establish and to handle even in laboratories that have minimal access to microfabrication facilities and are amenable to rapid, low-cost and large-scale production[40,41].

An automatic spotter that is compatible with organic solvents is required, as the lipids are solubilized in a chloroform-based solution. Chloroform is corrosive, and most spotters (e.g., for nucleic acid spotting) are not suitable for such harsh conditions. Furthermore, most of the DNA and protein microarray robots use spotting technologies that are similar to ink-jet printing and that are extremely sensitive to the physico-chemical properties of the solutions (e.g., wetting properties), which vary markedly between different lipid mixtures. For this protocol, we recommend the use of the Camag TLC4 liquid-handling robot, which was initially designed to perform thin-layer chromatography (TLC) and can mechanically dispense liquid solutions (down to 10 nl) via an automated syringe pump; we used only this robot for our research, but a MALDI spotting robot compatible with chloroform might be an alternative. The liquid is nebulized at the syringe tip, which allows the lipid to be dispensed without physical contact between the needle and the TAL. The smallest spot achievable with this machine is 1 mm^2. A customized platform termed the 'spotting platform' and 'protective stickers' are used to perform efficient automatic spotting and to prevent cross-spot contamination, as described below.

Production of the microfluidic channels and the protective stickers: microfabrication protocols. Many steps in the protocol require the handling of microfabricated components, but these are all feasible on a laboratory bench with minimal costs to allow mass production. **Box 1** contains useful information to familiarize the reader with soft lithography technologies[40]. We also provide the lithography mask design ready for drawing (**Supplementary Data 2**).

Production of the microfluidic channels and the protective stickers: microfluidic channel production. Polydimethylsiloxane (PDMS) microchannels are prepared by classical molding techniques[41,42], and they have a height of >600 μm. The procedure is easy to implement and relatively inexpensive.

Production of the microfluidic channels and the protective stickers: protective stickers. The lipid solutions are dispensed by nebulization (see 'Lipid mixture preparation' section above), and each one must be constrained on a 1-mm^2 spot to avoid cross-contamination. In addition, an even distribution of the lipid on the TAL must be ensured during the spotting to minimize any inhomogeneity in liposome formation within the area of spotting. For this, TALs are covered during spotting with a microfabricated polymeric mask called a protective sticker, which restricts the spotting of the lipid within a dedicated area and is removed after the spotting. The protective sticker must be easy to produce on the bench with minimal material and be suitable for handling with tweezers; it must be made of a soft material that is capable of bending; it must be adherent to the TAL via electrostatic interactions, and this adhesion must be stable throughout the period of the spotting; it must be able to be detached from TALs without peeling off the surface; and it must be able to be reused without introducing cross-contamination between the lipid-spotting cycles. To fulfill these criteria, we chose a UV-cross-linked membrane made of a thiolene-based resin (NOA81)[43]. The protective stickers are produced via a molding method called 'micromolding in capillaries'[40,42,44]. Briefly, a PDMS slab is bonded non-covalently with a sacrificial glass slide, and the NOA81 is injected between the two. The polymer is cross-linked (i.e., exposed to UV light), after which the PDMS slab is removed (**Fig. 2**). Finally, the protective stickers, which are about 80–100 μm in height, are removed from the glass slide and stored at RT between two sheets of clean-room-grade paper to prevent them from sticking to each other. The UV-cross-linking intensity alters the physical properties of the protective stickers: an underillumination will lead to stickers that are too soft and extremely sticky and can detach the TAL; conversely, overilluminated stickers will be hard and will lack sufficient adhesive force to the TAL. Fine-tuning the cross-linking conditions of the protective stickers will be necessary, as UV illumination sources differ between laboratories. It is not necessary to create stickers before each spotting, as they can be stored without losing their adhesive properties and can be attached and removed at least five times.

Preparation of the spotting platform. Because of the need for the protective stickers to eliminate cross-contamination, the spotter needs to be calibrated so that it dispenses lipid solutions and mixtures at precise positions delimited by the holes in the protective sticker. We introduced a spotting platform (**Fig. 3a**,**b**; **Supplementary Figs. 1c** and **2a**) on which 20 glass slide positions are printed, and for each of them 120 lipid spots (size 800 × 800 μm^2, 200 μm apart) are defined. First, the agarose-coated glass slides are mounted on the spotting platform using a drop of deionized water, which creates adhesion by capillary action. The precise position of each glass slide can be easily adjusted with tweezers under a binocular microscope. The capillary force ensures that the glass slides remain attached to the spotting platform and at the same time can be easily detached with a scalpel after spotting. Subsequently, the protective sticker is placed on each glass slide under a binocular microscope (**Fig. 3b**).

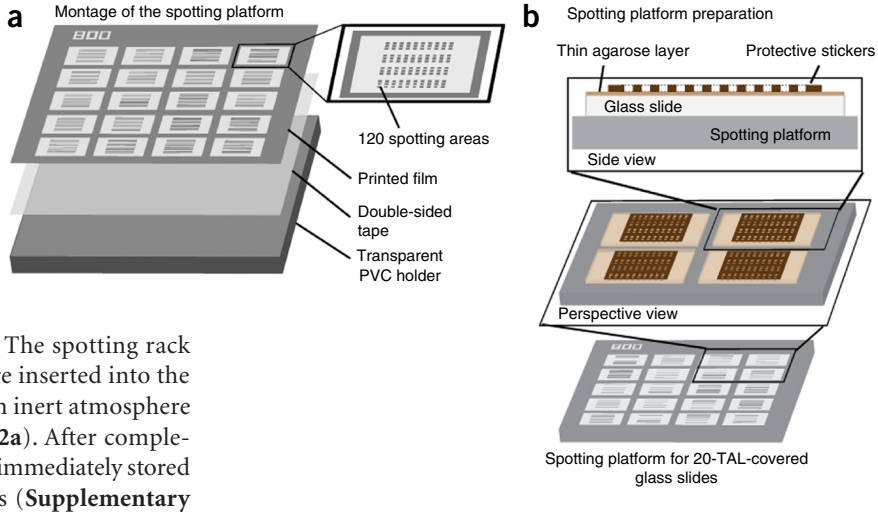
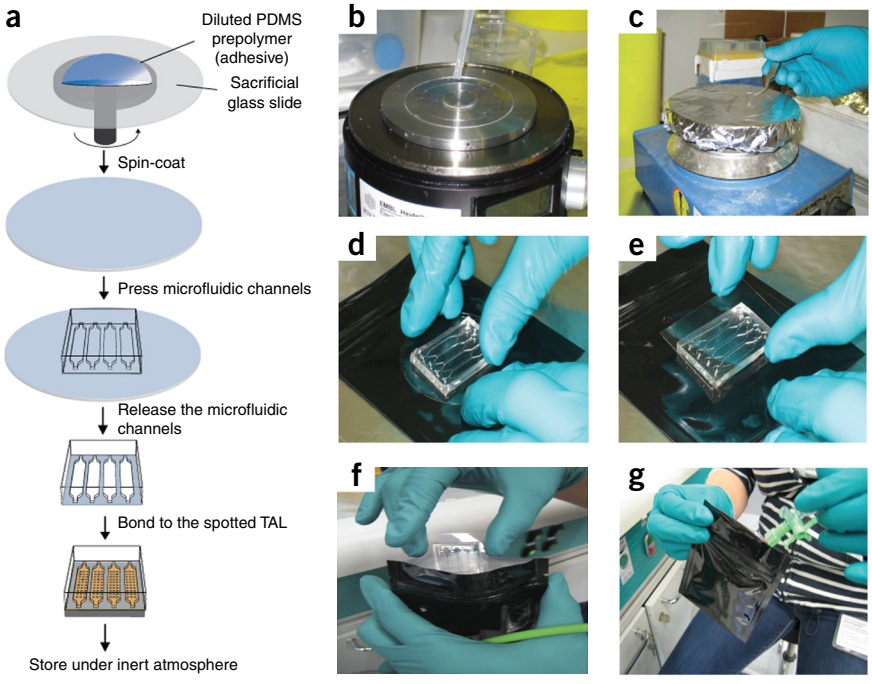


Figure 3 | Spotting platform. (**a**) Spotting platform montage. The spotting platform is formed by a PVC holder on top of which double-sided tape and a printed film are layered. The spotting platform can accommodate 20 glass slides. For every glass slide slot, 120 squares (spotting areas) are drawn (inset), representing the areas where lipids will be spotted. (**b**) Scheme of the spotting platform final assembly before spotting.

Spotting of the lipid mixtures on the TALs. The spotting rack (with the lipid mixture vials) and platform are inserted into the robot, and lipid spotting commences under an inert atmosphere (nitrogen or argon gas; **Supplementary Fig. 2a**). After completion, the spotting racks with lipid mixtures are immediately stored at −20 °C in a Mylar bag filled with inert gas (**Supplementary Fig. 1b**), and the spotted TALs are detached from the platform (**Supplementary Fig. 2b,c**), at which point they are ready for the addition of microfluidic channels (see below).

Microfluidic device assembly. To complete the liposome array, a PDMS device containing four separate microfluidic channels is bonded on top of the TAL-covered glass slide spotted with lipids (**Figs. 1a** and **4**). Classical methods to bond PDMS devices to glass slides use plasma treatment[41,42], but this would damage the lipids; thus, an alternative method had to be established. We adapted a bonding method[45] using diluted liquid PDMS as an adhesive (**Fig. 4**). The adhesive is spin-coated on a coverslip, and the PDMS microchannels are inked with the adhesive by being dipped onto the coverslip and peeled off after a few seconds (**Fig. 4**). The coated PDMS devices are then placed in contact with the lipid-spotted TAL-covered glass slide. No further treatments are necessary. After the bonding, it is advisable to wait for 24 h before using the microfluidic devices to allow the adhesive to dry.



This procedure ensures no cross-contamination of injected liquids between adjacent channels.

Microfluidic device storage. Immediately after microfluidic channel assembly, the devices are stored at 4 °C in opaque Mylar bags filled with argon that constitute a protective barrier against oxygen (**Fig. 4**). The devices can be stored for up to 2 months with no noticeable effects on the assay.

Performing the protein–liposome binding assay: protein extract. We have previously shown[20,23] that LiMA is compatible not only with purified (fluorescently labeled) proteins but also with cell extracts of *Escherichia coli* and human cell lines expressing recombinant proteins (GFP-tagged). The buffer used for the lysis should not contain detergent, as it may interfere with the assay.

Performing the protein–liposome binding assay: formation of liposomes. The microfluidic devices are first connected to a short piece of tubing so that liquids can be injected into the chambers. Giant liposomes (>5 μm) form within 5 min of the manual injection of assay buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) into the microfluidic chambers with a Hamilton syringe. The liposomes are fully swollen and remain stable and attached to the surface for at least 6 h without rupturing. The successful formation of liposomes can be confirmed by fluorescence microscopy in the fluorescence channel dedicated to the lipid marker (Atto 647). In some cases (~1% of experiments,

Figure 4 | Microfluidic channel bonding protocol. (**a**) Schematic representation of the bonding protocol and photos illustrating the key steps. (**b**) A diluted polydimethylsiloxane (PDMS) prepolymer (called adhesive) is spin-coated onto a sacrificial glass slide and briefly heated (**c**). (**d**) The microfluidic channel is inked with the adhesive and bonded with the lipid-spotted TAL (**e**). (**f**) Finally, the device is stored in a Mylar bag under an inert atmosphere (**g**).

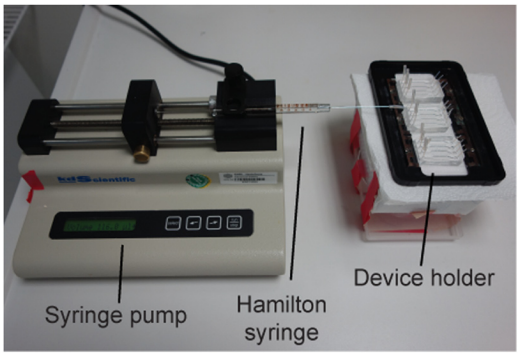


Figure 5 | Setup for the injection of sample into the microfluidic device. Three microfluidic chips are plugged onto a device holder that is ready to be assembled on a microscopic stage for automatic imaging of the liposomes. Sample is injected via a Hamilton syringe attached to an automatic syringe pump (optionally, a dual- or triple-channel syringe pump can be used for the simultaneous injection of the sample).

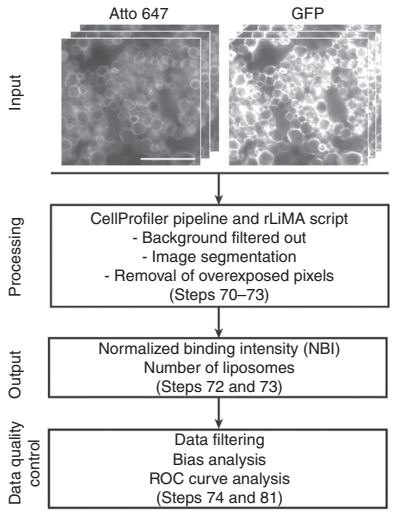


Figure 6 | Workflow of automated image analysis and data quality control. The set of acquired images (in both Atto 647 and GFP channels) serve as input. The images are subsequently processed with the CellProfiler pipeline and rLiMA script, which outputs the two main readouts: normalized binding intensity (NBI) and estimated number of liposomes. Series of statistical methods are then applied to assess the data quality. Scale bar, 50 µm.

in our experience), we noticed that the liposomes themselves can autofluoresce in the GFP channel. Therefore, before the sample injection, it is advisable to automatically image each liposome spot in both Atto 647 and GFP fluorescence channels; we call these images 'blank images'.

Performing the protein–liposome binding assay: incubation with protein extract. After acquisition of the blank images (see above), 40 µl (the volume of one chamber (20 µl) plus the dead volume of the tubing) of cell extract is injected into each chamber using a syringe pump (**Fig. 5**). The flow rate is adjusted so that it does not detach the liposomes from the surface, and these settings are kept constant throughout the experiments. After 20 min of incubation, unbound proteins are washed with 120 µl (four chamber volumes (80 µl) plus twice the dead volume of the tubing) of assay buffer. Subsequently, the microfluidic device is inserted into the holder, which allows automatic image acquisition from three such devices in parallel (**Fig. 5**).

Running image analysis and NBI calculation: image acquisition. The device holder (**Fig. 5**) is transferred to the automated epifluorescence microscope (IX-81; Olympus; 20×/0.7-NA (numerical aperture) objective), and images are acquired with a CCD camera (Hamamatsu Orca-R2)[46,47]. The field of view offered by this objective is ~400 × 400 µm^2 (which equates to one-quarter of a liposome spot). The user can program the microscope to take several images per spot to cover the whole liposome spot or take only one image (of a selected area) per liposome spot. For illumination, we use a xenon lamp (MT-20, Olympus) that exhibits exceptional stability over time. The microscope autofocuses automatically on every liposome spot. With bright field or with phase contrast, we found that the liposomes on the TAL do not provide sufficient contrast for automatic focusing. Therefore, it is important to supply each lipid mixture with a small amount of a fluorescently labeled lipid (PE–Atto 647, 0.1 mol%). The fluorescence from PE–Atto 647 not only provides a signal for automatic focusing, but also is exploited further during the image analysis and processing. After the focusing step, two sets of images are acquired in all experiments (**Fig. 6**): one set for Atto 647, which represents the position of liposomes, and a second set for GFP, which represents protein–lipid binding events. Overall, the imaging time for one array of 120 spots is about 50 min.

Running image analysis and NBI calculation: image processing. Acquired images are processed with the open-source image analysis software CellProfiler, the scripts for which are provided (**Supplementary Data 3**). **Figure 6** summarizes the image analysis pipeline.

The image processing starts with background filtering using the top-hat morphological filter. The filtered Atto 647 image is segmented in two different ways: the first identifies the centers of the liposomes, which are used later for counting the number of analyzed liposomes in one image (**Fig. 7a**); the second generates a mask representing the position of liposomal membranes. This mask defines a surface area that is further quantified (**Fig. 7a**).

Subsequently, all overexposed pixels are identified and removed in both Atto 647– and GFP-filtered images. This ensures that the number of non-overexposed pixels is exactly the same in both images at the end of the image-processing step. Both of the processed images are then overlaid with the mask, and the following information is extracted from both images: the number of non-overexposed pixels that overlap with the mask and the corresponding mean intensities of these pixels in both GFP and Atto 647 images. Finally, the ratio between the mean intensities of the GFP and Atto 647 images is calculated. This ratio defines an NBI that represents the amount of proteins bound per membrane surface area (**Fig. 6**).

The output from the CellProfiler pipeline is a comma-separated value (csv) file summarizing the key values extracted during the image-processing step (i.e., the estimated number of liposomes on the image, the mean fluorescence intensity in the Atto 647 image, the mean fluorescence intensity in the GFP image, the number of pixels analyzed and the NBI).

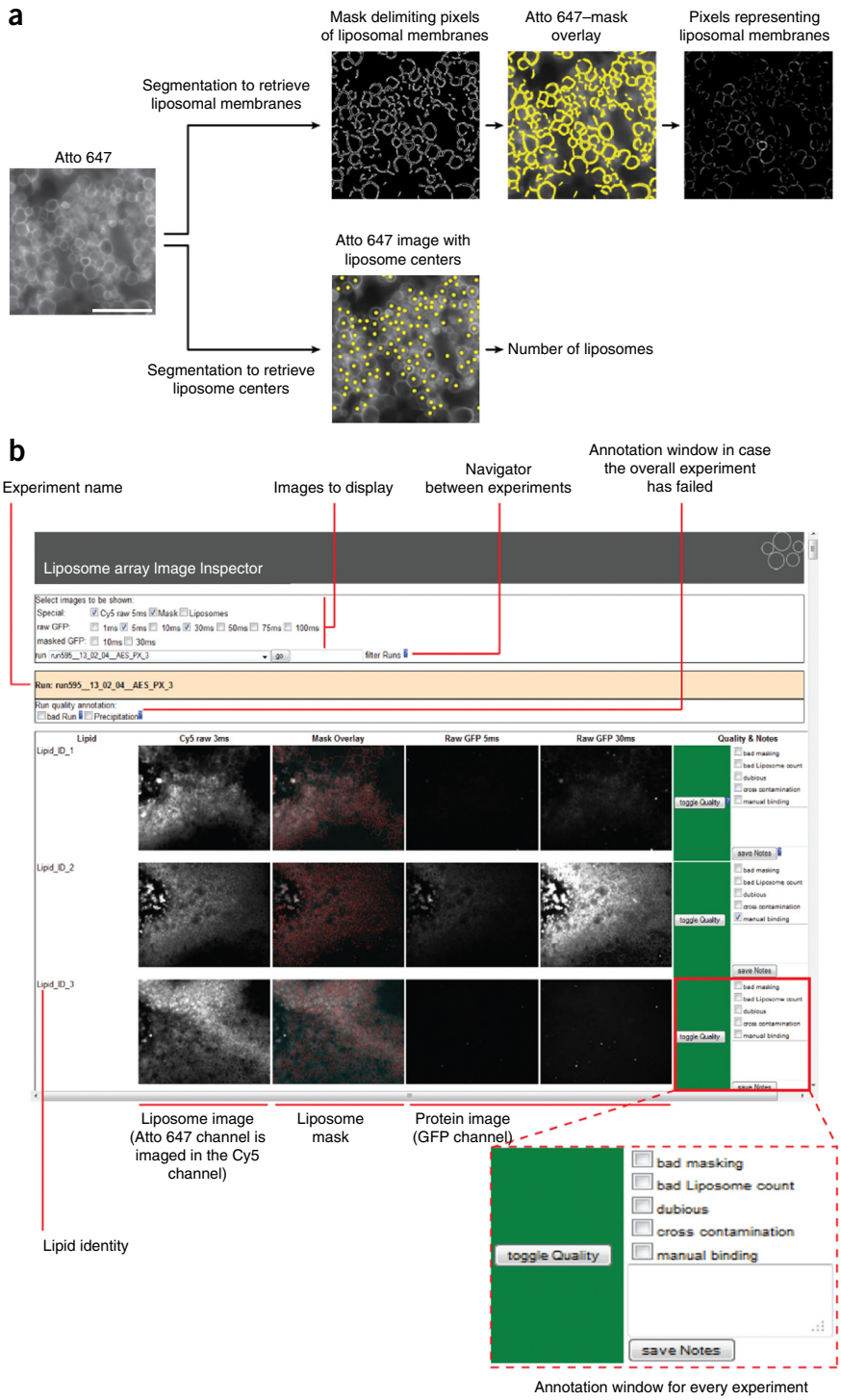


Figure 7 | Anticipated results. (**a**) Image segmentation. The Atto 647 image (left center image) is used for image segmentation. The Atto 647 image serves as a template for retrieving the mask delimiting the pixels representing liposomal membranes in the image (top images). It also allows one to estimate the number of liposomes on each image by counting their centers (bottom image). All processes illustrated in this panel are part of the CellProfiler pipeline (**Supplementary Data 3**). (**b**) Display of images on an interactive web page interface is ideal for quick data browsing and annotation. The experiment and series of images to be displayed are both selected in the top part. The name of the selected experiment and the images are then displayed below. The annotation line on the top of the displayed images serves for labeling experiments that are overall of bad quality; the annotation window right from each image series allows detailed and individual annotation of images.

Capturing protein–lipid interactions with different affinities. The affinity of individual protein–lipid interactions can vary markedly from nanomolar to micromolar[23], and one protein can interact strongly with one lipid mixture (reflected by a high NBI) but weakly with another (reflected by a low NBI). We found it necessary to acquire GFP images with increasing exposure times and then use them to calculate a mean NBI value[23] (**Supplementary Data 3**). The 'Atto-647-to-GFP' ratio must be normalized for each exposure time (i.e., divided by the exposure time) before calculation of the mean. In addition, the mean NBI must be calculated only from images with a low number of overexposed pixels. We determined that if the number of analyzed pixels between two subsequent (increasing) exposure times decreases by more than 5%, the images acquired at the higher exposure time should not be considered for further analysis. All the calculations are facilitated by use of the specialized R package (**Supplementary Data 3**).

Performing data quality control: data set filtering. After the acquisition of a screen, some images cannot be considered for downstream analysis because, for example, the liposomes failed to form, or because a protein precipitate or the liposomes are incorrectly focused. In these cases, the failed experiments must be filtered out of the data set. We have built a web interface that allows browsing and annotation of the images (**Fig. 7b**).

Performing data quality control: reproducibility and bias analysis. As for siRNA screens[48], LiMA output must be carefully analyzed, and we provide a tutorial for performing the first steps of data analysis (http://www.bork.embl.de/Docu/LiMA_protocol/).

First, the researcher must assess the reproducibility of the data by performing several replicates and plotting their NBI against each other and then extracting a Pearson's correlation coefficient. We also recommend shuffling the position of the lipid spots between the replicates to test for potential spatial effects. Second, several sources of bias must be specifically tested: any spatial bias—i.e., if some parts of the array show systematic abnormal increases or decreases in binding; the effect of protein

concentration; and the effect of the number of liposomes on the NBI values. Finally, the sensitivity and the specificity of the assay are computed using receiver operating characteristic (ROC) and precision recall curves analysis in order to extract an optimal NBI threshold to automatically distinguish between binding and no binding.

MATERIALS

REAGENTS

- dH_2O
- Hellmanex III (Hellma Analytics)
- Agarose type IX-A low melting point (Sigma-Aldrich, cat. no. A2576)
- Antibodies to GFP (Miltenyi Biotec, cat. no. 130-091-833)
- cOmplete, EDTA-free, protease inhibitor cocktail tablets (Roche, cat. no. 11873580001)
- Benzonase nuclease (Sigma-Aldrich, cat. no. E1014)
- NOA81 (Norland Products)
- Polydimethylsiloxane (PDMS) and curing agent (Dow Corning, Sylgard 184)
- Hexane (Sigma-Aldrich, cat. no. 296090) ! **CAUTION** Hexane is an inhalation risk. Manipulate it under a fume hood.
- Acetone (Merck, cat. no. 1000141000)
- 2-Propanol (Merck, cat. no. 1096341000)
- Chloroform (Merck, cat. no. 1024451000) ! **CAUTION** Chloroform is toxic and highly volatile. Handle it only under a fume hood. Do not handle it with plasticwares. Use proper gloves.
- Methanol (Merck, cat. no. 1060091000) ! **CAUTION** Methanol is toxic. Handle it under a fume hood.
- Silicon wafer (2-inch (50.8 mm) or 4-inch (101.6 mm))
- SU-8 photoresist (MicroChem, SU-8 2075 and 2150) ! **CAUTION** Manipulate it in the clean room only.
- SU-8 developer (MicroChem) ! **CAUTION** Manipulate it in the clean room under a fume hood only.
- Trimethylchlorosilane (TMCS; Sigma-Aldrich, cat. no. 92360) ! **CAUTION** TMCS is highly toxic, and it should be manipulated under a fume hood only.
- Streptavidin AF-488 (Life Technologies, cat. no. S-11223)
- One Shot BL21 Star (DE3) chemically competent cells, *E. coli* (Thermo Fisher Scientific, cat. no. C6010-03)
- LB medium (Thermo Fisher Scientific, cat. no. 10855)
- IPTG solution (Thermo Fisher Scientific, cat. no. R1171)
- HEPES (Sigma-Aldrich, cat. no. H3375)
- Sodium chloride (Merck, cat. no. 1.06404. 1000)
- DTT (Biomol, cat. no. 04010.10) ! **CAUTION** DTT is toxic. Handle it with gloves.
- ZYP-5052 autoinduction media (Amresco, cat. no. N990)

Lipids

▲ **CRITICAL** All lipids were supplied by Avanti Polar Lipids, Sigma-Aldrich, Echelon Biosciences.

- Phosphatidylcholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, Cas no. 26853-31-6 (Avanti, cat. no. 850457)
- Pegylated lipid PE-PEG350, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(methoxy(PEG)-350), Cas no. 474922-90-2 (Avanti, cat. no. 880430)
- Pegylated lipid PE-PEG2000, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(PEG)-2000], Cas no. 474922-90-2 (Avanti, cat. no. 880130)
- PE-Atto 647 (Atto-Tec, cat. no. AD 647N-161)
- For positive control spots: biotinylated lipid, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(biotinyl), Cas no. 384835-53-4 (Avanti, cat. no. 870282)
- Protein (purified or from cell lysate; see Reagent Setup)

EQUIPMENT

- Transparent polyvinyl chloride (PVC), 210 × 205 × 2 mm (to be requested from a mechanical workshop)
- Glass-slide holder (**Supplementary Data 1**; to be requested from a mechanical workshop)
- Transparency mask for photolithography (**Supplementary Data 2**; Selba)
- Glass slides (Menzel, 30 × 45; thickness #1). Available from Fisher Bioblock, cat. no. 11727055 and upon request from VWR (cat. no. BC030045A1)
- Glass slides (Menzel, 40-mm diameter, thickness #1, cat. no. CB00400RA1). Available from Fisher Bioblock (cat. no. 11757065)
- Kimtech tissue (Kimtech, cat. no. 05511)
- Clean-room-grade tissue (e.g., Ted Pella, cat. no. 81228)
- Tweezers (7-SA, Rubis)
- Syringe, 27-gauge (Becton Dickinson and Company (BD), cat. no. 302200)
- Syringe, 1 ml (BD, cat. no. 300013)
- Millex GV filter unit, 0.22 µm (Merck Millipore, cat. no. SLGU033RS)
- Silicon tubes, diameter 0.3 × 0.76 mm (Fisher Scientific, Novodirect, cat. no. 11919445)
- Silicon tubes, diameter 0.5 × 2.1 mm (Fisher Scientific, Novodirect cat. no. 11988199)
- Harris Uni-core 1-mm hole puncher (Ted Pella, cat. no. 15110-10)
- Harris cutting mat (Ted Pella, cat. no. 15085)
- Magnetic stirrer (VWR, cat. no. 442-0664)
- Double-sided tape film (3M, cat. no. 9828)
- Parafilm (Sigma-Aldrich, cat. no. P7793)
- Cylindrical magnetic stirring bar, 25 mm × 8 mm encapsulated in PTFE (polytetrafluoroethylene) (VWR, cat. no. 74950-288)
- Syringe pump dual way (common distributors: KD scientific, Harvard Apparatus, Cetoni, http://www.syringepump.com)
- Sonicator bath (Bandelin Sonorex RK156)
- Digital sonifier W-250D (Branson, cat. no. 101-063-588)
- Binocular microscope (Leica MZ8)
- BioTek microplate reader (BioTek)
- Profilometer (Dektak 8, Veeco Instruments)
- UV illuminator (36 watts, Proxima Direct)
- Hamilton syringes, 10, 50, 100, 250, 500 µl and 1 ml (Hamilton, cat. nos. 80000, 80900, 81000, 81100, 81217 and 81317) ▲ **CRITICAL** The plunger needs to have an organic-solvent-resistant tip (made of PTFE in the Hamilton syringe), and we recommend using beveled needles.
- Camag TLC spotter 4 (Camag) ! **CAUTION** This spotter has the unique ability to handle organic solvents such as chloroform.
- Spotter racks for lipid vial (Camag, cat. no. 022.7430)
- Spotter syringe (Camag, cat. no. 695.0061)
- Spotter needle for spray-on application (Camag, cat. no. 695.0046)
- Camag plunger (Camag, cat. no. 695.0061)
- Scanning microscope (ScanR, Olympus Biosystems)
- Objective, 20×, 0.7 NA (Olympus)
- Camera (Hamamatsu, Orca-R2)
- Xenon lamp (MT-20, Olympus) ▲ **CRITICAL** The lamp intensity must be checked regularly to make sure that the intensity is stable over time.
- Filter set (emission/excitation) appropriate for GFP and Atto 647 channels
- Spin-coater benchtop (SCI-10, LOT Quantum Design)
- Crystallizing dish, 140 mm (VWR, cat. no. 216-0074)
- Crystallizing dish, 70 mm (VWR, cat. no. 216-0066)
- Scalpel (B. Braun, cat. no. 5518040)
- Vials and caps kit, 1.5 ml (Sigma-Aldrich, cat. no. 29307-U)
- Caps (Sigma-Aldrich, cat. no. 29319-U)
- Rack for vials (Sigma-Aldrich, cat. no. 23207)
- Plastic bag sealer for 200-mm bags
- Mylar bag, foil on one side and clear on the other, 15.0-inch × 16.25-inch outside dimensions (Sorbent Systems, cat. no. VST152516560)
- Mylar Ziploc bags, black, outside dimensions of 3.4 × 4.0 inches (Sorbent Systems, cat. no. 034MFB04ZTE)

REAGENT SETUP

Lipids Lipids should be ordered in powder form for long-term storage, and then they should be dissolved in an appropriate solvent. The final lipid mixture that is ready for spotting should be dissolved in chloroform:methanol:water at a 20:9:1 vol:vol:vol ratio and stored at −20 °C. Under these storage conditions, lipid mixtures can be stored for more than 2 months without noticeable degradation of lipids. Hamilton syringes should be used to manipulate the lipids. All lipid mixtures are composed of a mixture of POPC

as the carrier lipid (up to 99.4 mol%), a fluorescent lipid (PE–Atto 647, 0.1 mol%), a PEGylated lipid (PE-PEG350) and additional signaling lipid(s) (up to 10 mol% each)—e.g., phosphatidylinositol phosphates or sphingolipids (**Supplementary Table 1**). Complete lipid mixtures should be prepared to a final total molar concentration of 0.26 mM in a glass vial. Each lipid mixture should be labeled with an identification number (ID number) and placed in the spotting rack (**Supplementary Figs. 1b** and **2a**). For every device, you can include a positive control that is a lipid mixture containing a biotinylated lipid (instead of the signaling lipid) recognized by a fluorescently labeled streptavidin. As a negative control, a lipid mixture composed of POPC, the fluorescent lipid and PEGylated lipid only (i.e., without any signaling lipid) is used. ▲ **CRITICAL** Preparation of the lipid mixture should be carried out under a fume hood. All plastic tips should be avoided when lipids are manipulated. Lipids are prone to degradation when they are stored for a long time. The quality of the dissolved lipids must be checked regularly: the concentration of stock solution can be assayed using the phosphorus assays, and the integrity of lipids can be checked by TLC[49].

Proteins LiMA works with cell lysates as well as with purified proteins. Recombinant proteins of interest fused to GFP can be expressed in mammalian cell lines or in bacterial cells (*E. coli*; see Vonkova *et al.*[20] and Saliba *et al.*[23]). For bacterial cells, it is preferable to use a superfolder GFP (sfGFP) that folds faster and exhibits higher brightness[50]. Detergents should be avoided. The protein also can be detected in the assay through the use of fluorescently labeled antibody. ! **CAUTION** The mammalian cell lines used in your research should be regularly checked to ensure that they are authentic and that they are not infected with mycoplasma.

HEPES, 100 mM (pH 7.4) Dissolve 23.8 g of HEPES in 800 ml of deionized water, adjust the pH to 7.4 and add water up to 1 l. Store it at RT for up to 2 months.

NaCl, 5 M Dissolve 292 g of NaCl in deionized water up to 1 l. Store it at RT for up to 2 months.

DTT, 1 M Dissolve 1.5 g of DTT in deionized water up to 10 ml. Store it in 100-µl aliquots at −20 °C for up to 6 months.

Solution of protease inhibitor cocktail, 25× Dissolve 1 tablet of cOmplete, EDTA-free, protease inhibitor cocktail in 2 ml of deionized water. Store the solution in 200-µl aliquots at −20 °C for up to 6 months.

Lysis buffer Lysis buffer is 10 mM HEPES (pH 7.4), 150 mM NaCl, 0.5 mM DTT, protease inhibitor cocktail and Benzonase nuclease. Prepare 5 ml of lysis buffer as follows: 500 µl of 100 mM HEPES (pH 7.4), 150 µl of 5 M NaCl, 2.5 µl of 1 M DTT, 200 µl of 25× protease inhibitor cocktail, 1 µl of nuclease and water up to 5 ml. After mixing the components, keep the buffer on ice and use it immediately. Do not store the buffer; freshly prepare it before each experiment.

Assay buffer Assay buffer is 10 mM HEPES (pH 7.4), and 150 mM NaCl. Prepare 10 ml as follows: 1 ml of 100 mM HEPES (pH 7.4), 300 µl of 5 M NaCl and deionized water up to 10 ml. Filter the buffer using a 0.22-µm syringe filter. Do not store the buffer; freshly prepare it before each experiment.

EQUIPMENT SETUP

Glass-slide holder Glass slides need to be cleaned upright in a crystallizing dish, and thus a specific holder is required. To our knowledge, such products are not available on the market, and they need to be produced in a mechanical workshop. Two glass slide holders should be prepared (**Supplementary Data 1**): one to accommodate glass slides of 30 × 45 mm^2 (**Supplementary Fig. 1a**) and another for round glass slides 40 mm in diameter. The holder should also allow a magnetic stirrer to be fitted underneath.

Spotting platform This platform is designed to layer the glass slides for spotting, and it has dimensions such that it fits in the Camag spotter (**Supplementary Fig. 2a**). First, take some transparent polyvinyl chloride (PVC; 210 × 205 × 2 mm) and overlay it with double-sided tape without forming bubbles (**Fig. 3a**). Print the spotting layout on a transparency film (photolithography-grade ready for printing from Selba (http://www.selba.ch); **Supplementary Data 2**) and stick the printed plastic film on top of the double-sided tape (**Fig. 3a**). ▲ **CRITICAL** The spotting layout should be stuck perfectly horizontal to the PVC.

Microfluidic device holder for microscopy Assembled microfluidic devices can be mounted on a 'device holder' that allows three devices to be fitted into the microscope stage (**Fig. 5**). They should be specifically fabricated in a mechanical workshop, with dimensions specific for the microscope used (**Supplementary Fig. 1d**), and at least three of these holders are required.

Microfabrication The photomask can be drawn using CAD software (e.g., Qcad, http://www.qcad.org) or AutoCAD (Autodesk) and further printed on a transparency film at a resolution of 12,000 dpi. Samples of the photomask design are provided in **Supplementary Data 2**. Photolithography should be carried out in a clean room if available. If not, a company specializing in microfluidics such as Micronit Microfluidics or Dolomite Microfluidics can provide a microfabrication service.

Calibrating the lipid spotter The lipid mixtures need to be dispensed precisely on the allocated space in the spotting platform. An initial spatial calibration is required: insert the spotting platform in the spotter (**Supplementary Fig. 2a**), spot a fluorescent lipid at precise locations over the entire spotting platform and observe the glass slides under a fluorescent microscope. Adjust the positions until every lipid spot is properly localized. Chloroform can damage the inner fluidics (pumps, tubes) inside the spotter, so regular checks by a specialized technician are required.

Software requirements The image analysis is performed using open-source image analysis software CellProfiler 2.1.1. or later (http://www.cellprofiler.org/). We provide an example pipeline suitable for the analysis of experiments using one ('example_pipeline_one exp time.cpproj' in **Supplementary Data 3**) or multiple ('example_pipeline_two exp times.cpproj' in **Supplementary Data 3**) exposure times in the GFP channel. Each step in the pipeline is introduced by a short description to help orient the user in case some adjustments are needed. The output in both cases is a .csv file, which can be viewed in Microsoft Office Excel, for example, together with images that can be viewed in open-source software such as ImageJ (http://imagej.nih.gov/ij/) or Fiji (http://www.fiji.sc/Fiji). For the analysis of experiments with multiple GFP exposure times, the final calculations to obtain the mean NBI values are facilitated by a script in the statistical program R that we have provided (**Supplementary Data 3**). R and its integrated development environment RStudio (http://www.rstudio.com/products/rstudio/download/) are required for quality control and bioinformatics analysis. ▲ **CRITICAL** To enable automatized image processing, all acquired images should be named according to a standardized scheme that indicates the protein and lipid under analysis (see the example images in **Supplementary Data 3**).

PROCEDURE

Preparation of the TALs ● TIMING overnight

1| Wipe 20 glass slides of size 30 × 45 mm^2 with acetone and isopropanol using Kimtech tissue until all visible dust is removed, and place the glass slides on a glass-slide holder (**Supplementary Fig. 1a**).

2| Prepare 500 ml of a solution of Hellmanex III 1% (vol/vol) in dH_2O.

3| Submerge the holder with the glass slides in the prepared Hellmanex III solution, and clean them for at least 3 h under constant magnetic stirring.

4| Meanwhile, prepare a solution of agarose 1% (wt/vol) in dH_2O. Briefly heat the solution in the microwave until all of the agarose is dissolved. As advised by Horger *et al.*[25], the agarose should be gelified once before use. Store it at RT for a maximum of 2 weeks.

5| Remove the glass slides from the Hellmanex III solution and clean them twice in dH_2O. Put the glass slides in a new dH_2O-filled crystallizing dish and sonicate in a sonication bath for 10 min at RT.
▲ **CRITICAL STEP** After cleaning, take the glass slide in your hands, pour dH_2O on the glass slide and observe whether the water covers the entire surface of the glass slide without forming droplets. This quality check ensures that the glass slides are highly hydrophilic and that they are ready to be coated with agarose.

6| Heat the prepared agarose solution from Step 4 in a microwave, and transfer the solution to a 70-mm crystallizing dish.

7| Using tweezers, briefly dip each glass slide in the agarose solution. Excess agarose is removed by tilting the glass slide by 90°. Lay the glass slide on a clean-room-grade tissue to let the agarose dry out.
▲ **CRITICAL STEP** Agarose should cover the entire glass slide uniformly without forming droplets.
? TROUBLESHOOTING

8| Let the agarose dry overnight. Once the glass slides are dry, label each on the left bottom corner with a sign to show which side is coated and to orient the glass slide in Steps 31 and 51. Put the coated glass slides on the glass-slide holder and cover them with Parafilm.
■ **PAUSE POINT** The TALs can be stored at RT for 3 weeks in a dust-free, dry and dark environment.

9| (Optional) To measure the height of the TAL, remove a small piece of the agarose layer gently with a scalpel without scratching the glass slide (for example, slide the scalpel gently on the agarose layer). Blow away the agarose dust with a stream of nitrogen. Measure the agarose height with a profilometer (e.g., Dektak 8, Veeco Instruments). Repeat the measurement at several locations on the glass slide to ensure that the TAL is homogeneously covered.

Lipid mixture preparation ● TIMING 6 h for the preparation of 30 different lipid mixtures
▲ **CRITICAL** The entire process of lipid mixture preparation should be performed exclusively in a fume hood. The lipid solutions must be handled only with Hamilton syringes.
10| Solubilize the lipid powders in the appropriate solvent to create stock solutions, and store them at –20 °C in 1.5-ml glass vials under an argon atmosphere and sealed with Parafilm, as described in Reagent Setup.
▲ **CRITICAL STEP** If the lipid powder does not dissolve easily, the solution can be heated for a few minutes at 60 °C. If lipids are heated, it is critical to perform a quality check of the lipid stocks using phosphorus assays or TLC (see 'Lipids' in the Reagent Setup section).
? TROUBLESHOOTING

11| Reserve fresh 1.5-ml glass vials, one for each lipid mixture to be prepared, and label them with a lipid identification number and/or the lipid composition. Wash each vial by injecting and removing 1 ml of chloroform, and let the residual liquid evaporate under a fume hood.

12| Use the stock solutions from Step 10 to prepare the desired lipid mixtures, all with an equal molar concentration of 0.26 mM in chloroform:methanol:water at a 20:9:1 vol:vol:vol ratio (**Supplementary Table 1**).
▲ **CRITICAL STEP** Use several Hamilton syringes (from 10 to 500 µl) to optimize the pipetting of different volumes.

13| Replace the air in the vials with argon, close them with caps with a septum and place a strip of Parafilm around the side of the caps.

14| Place the vials into a spotting rack (**Supplementary Fig. 1b**).
▲ **CRITICAL STEP** For longer storage, enclose the rack in a Mylar bag (foil on one side, clear on the other), fill it with argon, seal the bag and keep it at –20 °C; the lipid mixtures can be stored for 2 months under these storage conditions.

Preparation of microfluidic channels ● TIMING 1 d
15| Print the microfluidic channel pattern on a photolithography mask. In **Supplementary Data 2**, we provide a designed pattern for the microfluidic channels in the standard .dxf format that is ready for printing on a transparency film. A printing resolution of 12,000 dpi is sufficient.

16| Perform photolithography to fabricate a master using SU8-2150 photoresist on a 4-inch silicon wafer to reach a channel height of ~600 µm. A detailed protocol for the lithography and a troubleshooting guide are available from Microchem (SU8-2100 and SU8-2150 data sheet). We advise that three masters be prepared. Two masters should be used to prepare the microfluidic channels, and one should remain stored as a back-up.
▲ **CRITICAL STEP** The height of the channels should not be <500 µm, because this increases the risk that the formed liposomes enclosed in these channels will be washed away during the washing step (Step 64).

17| After photolithography, coat both newly prepared masters with the antiadhesive silane trimethylchlorosilane (TMCS). Under a dedicated desiccator, place each master on a glass Petri dish and add one drop of TMCS on the border of the Petri dish without letting the TMCS contact the master. Allow 15 min for coating.
▲ **CRITICAL STEP** This step should be carried out under a fume hood.
▲ **CRITICAL STEP** After Step 17, the masters can be taken out of the clean room.

18| Prepare PDMS elastomer prepolymer with 10:1 PDMS:curing agent. Detailed descriptions of this step can be found in **Box 1** and elsewhere[41,51].

19| Place the masters in a plastic Petri dish, pour the PDMS prepolymer over them and allow it to cure at 70 °C for 4 h. Detach the PDMS with a scalpel.
▲ **CRITICAL STEP** Heating above 70 °C will damage the Petri dish by deforming the plastic, and it may damage the masters.

20| Using the cutting mat as a support, punch each end of the channels with a histological puncher to make a 1-mm hole.
▲ **CRITICAL STEP** Change the puncher regularly; we recommend using a new one after punching 400 holes (i.e., 50 PDMS devices).
■ **PAUSE POINT** Steps 19 and 20 can be repeated multiple times to prepare sufficient stock of the PDMS channels, which can be stored (for a few weeks at RT) in a dish or a box for further use.

Protective sticker preparation ● **TIMING 2 d**
▲ **CRITICAL** A schematic description of the protocol is shown in **Figure 2**.
▲ **CRITICAL** If the protective membranes are already prepared, you can jump to Step 31.
21| Print the pattern of the holes required in the protective sticker on a photolithography mask. In **Supplementary Data 2**, we provide a designed pattern in the standard .dxf format that is ready for printing on a transparency film. A printing resolution of 12,000 dpi is sufficient.
▲ **CRITICAL STEP** After curing, PDMS has the tendency to shrink when released from the master. To take this into account, the photolithography mask is slightly increased in size; we provide the pattern with an increase of 2%, 3% and 4%. During the optimization of the protocol, we advise testing all three increasing ratios and choosing the one that fits best.

22| Perform photolithography to fabricate a master, as described in Steps 15–17. Use the SU8-2150 photoresist, 4-inch silicon wafer and a channel height of ~100 µm.

23| Prepare PDMS prepolymer as described in Step 18, and then place the masters in a plastic Petri dish. Pour the PDMS prepolymer on the master and allow it to cure at 70 °C for 4 h. Detach the PDMS with a scalpel.

24| Using the cutting mat and a histological puncher, make one 1-mm hole in a corner of the PDMS slab.
▲ **CRITICAL STEP** Repeat Steps 23 and 24 to reach the required number of copies of the PDMS slabs (we recommend preparing at least 50 replicates).

25| Place the PDMS slab on a round glass slide 40 mm in diameter, prewashed as described in Steps 1–4, with the pattern side in contact with the glass slide.

26| Prepare the connecting tubing by combining two pieces of silicon tube: one with a diameter of 0.3 × 0.76 mm (length ~4 cm) and one with a diameter of 0.5 × 2.1 mm (length ~1 cm).

27| Insert the connecting tubing with its thinner end into the hole in the PDMS slab on a glass slide. The thicker end of the tubing should connect with a needle (G30) on a 1-ml syringe filled with a thiolene-based resin (NOA81; **Fig. 2b**).

28| Inject the resin between the PDMS slab and the glass slide by slowly pressing the plunger.
? TROUBLESHOOTING

29| Remove the syringe with the needle and place the PDMS slab on a glass slide into the UV lamp (**Fig. 2c**). Cross-link the resin by exposing it to UV light for a few seconds.
▲ **CRITICAL STEP** Fine-tuning the cross-linking conditions of the protective stickers is necessary, because UV illumination sources differ from one to another. The UV cross-linking intensity alters the physical properties of the protective stickers: underillumination will lead to stickers that are too soft and extremely sticky and can detach the TAL; conversely, overilluminated stickers will be hard and will lack sufficient adhesive force to the TAL.
? TROUBLESHOOTING

30| Remove the PDMS slab and detach the protective sticker from the glass slide (**Fig. 2d**). Store the stickers between two sheets of clean-room-grade paper at RT for a couple of weeks.
▲ **CRITICAL STEP** To prevent tearing the protective sticker during detachment from the glass slide, drop a few drops of isopropanol on the sticker and use a scalpel or a razor to slowly peel it off. Perform this step under a fume hood.

Spotting platform preparation ● TIMING 2 h hands-on time + overnight adhesion
31| Place the spotting platform (see Equipment Setup) into a binocular microscope, and position it in such a way that the area for a glass slide in the top left corner is in the light.

32| Take up 5 µl of deionized water into a pipette and dispense it in two equal drops on the area for the glass slide.

33| Align the TAL-covered glass slide from Step 8—with the sign identifying the TAL uppermost—on the area and gently press with tweezers to create adhesion by capillary action. Adjust the position of the glass slide so that it precisely matches the mask on the spotting platform.

34| With tweezers, take the protective sticker from Step 30 and place it on the glass slide so that it precisely matches the pattern on the spotting platform, and gently press to ensure adhesion.
? TROUBLESHOOTING

35| Repeat Steps 32–34 to completely fill the spotting platform (20 glass slides in total).

36| Gently cover the filled spotting platform with a sheet of clean-room-grade paper and then with a thin PVC board the same size as the platform. Place a 2-l bottle filled with water on top and leave it to stand overnight to facilitate the adhesion between the glass slides and the protective stickers.

Spotting ● TIMING 10 h (for 20 arrays of 120 spots each)
37| Place a 10-µl glass syringe for the spotter with a needle into the Camag TLC4 automatic spotter. Fill up the reservoir for a washing solution with chloroform:methanol at a 2:1 vol:vol ratio.
▲ **CRITICAL STEP** The needle and the plunger should be changed regularly; we recommend changing both after spotting of 500 lipid arrays (i.e., 60,000 spots).

38| Place the spotting rack with the lipid mixtures (prepared in Steps 10–14) into a dedicated space in the spotter (**Supplementary Fig. 2a**).

39| Insert the spotting platform with the glass slides (prepared in Steps 31–36) onto a spotting area (**Supplementary Fig. 2a**), and use pieces of double-sided tape to attach it properly.

40| Fill the spotter with argon gas and then seal the doors with tape.

41| Set up the spotting program to dispense 200 nl of lipid mixture per each coordinate that corresponds with the positions of the pattern on the spotting platform. Start the spotting.

42| When the spotting is complete, take out the rack with the lipid mixtures and place it, closed in a Mylar bag filled with argon, at –20 °C.

43| Remove the spotting platform from the spotter and carefully peel off the protective stickers from the glass slides (**Supplementary Fig. 2b,c**). Store the protective stickers on a clean-room-grade tissue. Then, use a scalpel to carefully detach the lipid-spotted glass slides (**Supplementary Fig. 2b,c**).
▲ **CRITICAL STEP** The protective stickers can be reused multiple times until they lose their stickiness (i.e., about ten times, provided they maintain sufficient adhesive properties). To prevent lipid contamination between individual uses, place the stickers into a glass beaker, cover them with isopropanol and sonicate in a sonication bath for 10 min. Use tweezers to remove individual protective stickers, and allow them to dry on a sheet of clean-room-grade paper.

Device assembly and storage ● TIMING 3 h for 20 devices
▲ **CRITICAL** A schematic depiction of the protocol is provided in **Figure 4**.

44| Prepare a set of 20 sacrificial glass slides (round, 40 mm in diameter) prewashed as described in Steps 1–4.

45| Prepare the adhesive by diluting the PDMS prepolymer—silicone elastomer:curing agent in a 2:1 ratio—3× (vol:vol) in hexane.

46| Prepare 20 Mylar Ziploc bags labeled with a date and a note identifying the spotted array.

47| Place 20 PDMS channels (prepared in Steps 15–20) in a beaker, cover them with isopropanol and sonicate them in a sonication bath for 10 min. Remove the isopropanol and let the PDMS channels dry thoroughly.
▲ **CRITICAL STEP** The PDMS channels must be completely dry, as any residual isopropanol will prevent bonding. To speed up the drying process, the PDMS channels can be placed in an exicator for 30 min.

48| Place one of the glass slides from Step 44 in the table-top spin-coater (**Fig. 4b**). Pour some adhesive (~0.5 ml) in the middle of the glass slide and spin briefly to create a thin equal layer of the adhesive over the entire surface of the glass slide.

49| Lift the coated glass slide with tweezers and place it for 1 min on a heating block heated to 65 °C (**Fig. 4c**).
▲ **CRITICAL STEP** The adhesive on the glass slide must remain liquid after the heating phase. If it dries out, it cannot be used for the subsequent steps and a new one must be prepared.
? TROUBLESHOOTING

50| Take one piece of the PDMS channels and press it briefly, with the pattern side down, on the coated glass slide, and then peel it off (**Fig. 4d**). The pattern side should then be covered by a thin layer of the adhesive.

51| Place the coated PDMS channels in contact with the spotted glass slide from Step 43 so that the spots are enclosed in the channels (**Fig. 4e**). Press gently using tweezers to support the adhesion.
▲ **CRITICAL STEP** The PDMS channels must be aligned on the spotted glass slide so that all the spots are inside the channels and not accidentally covered. We advise that the PDMS channels be placed on a desk with the coated side uppermost and the spotted glass slide positioned carefully (with the spotted side down) on the top. The spots on the glass slide may be difficult to see, so it is advisable to increase the contrast by working on a dark background (the black surfaces of the Mylar Ziploc bags are ideal for this purpose).

52| Place the final microfluidic device into the Mylar Ziploc bag from Step 46 (**Fig. 4f**), fill it with argon and close it completely using a plastic bag sealer (**Fig. 4g**). Store it at 4 °C for up to 2 months. Repeat Steps 48–52 to complete all 20 microfluidic devices.
▲ **CRITICAL STEP** It is preferable to use the final microfluidic device 24 h after the bonding to provide sufficient time for the PDMS channels and the spotted glass slide to adhere. Earlier use increases the risk of leakage of the microfluidic device.

Low-volume cell extract preparation ● TIMING 1 h (excluding time needed for protein expression)
▲ **CRITICAL** If you are using purified proteins, you can jump to Step 57.
53| Express the protein of interest as an sfGFP fusion in *E. coli* (use bacterial strain BL21 Star (DE3) One Shot; follow the manufacturer's instructions for transformation and expression protocols). For one sample, a 5-ml culture yielding an ~100-µl pellet is sufficient.
▲ **CRITICAL STEP** If a high number of proteins are to be tested, use autoinducing ZY medium[52] for the culture instead of LB medium with IPTG solution. This medium gives higher yields of cells and eliminates the need to induce each culture independently.

54| Resuspend the pellet in 5× pellet volume of the lysis buffer (see Reagent Setup) and lyse the cells by mild sonication in a microtube. For the sonicator Digital Sonifier W-250D (Branson), we optimized the settings as follows: pulsing time, 0.5 s; pause, 10 s; total pulsing time, 1 min; and amplitude, 15%.
▲ **CRITICAL STEP** A sufficient pause must be left between pulses to avoid heating the sample and damaging the protein.
▲ **CRITICAL STEP** The use of detergent in the lysis buffer is not recommended, as it can lead to the disruption of liposomes.

55| Clear the lysate by centrifugation in a table-top centrifuge (16,000*g*, 20 min, 4 °C), and then transfer the supernatant to a fresh microtube (the final cell extract).

56| To estimate the amount of the protein of interest in the final cell extract, run an SDS–PAGE gel and western blot and detect the protein with an antibody to GFP. Another possibility is to measure the fluorescence intensity of the sfGFP-tagged protein in the final cell extract with a microplate reader (BioTek) and compare it with values of an sfGFP-tagged protein standard of known concentration.
▲ **CRITICAL STEP** Ideally, both methods—western blot and microplate reader—should be performed, as the western blot also provides an indication of the protein quality (i.e., level of degradation), and the fluorescence intensity gives a quantitative readout that can be easily compared.
■ **PAUSE POINT** Provided that the protein is stable and functional after freezing and thawing, the cell extract can be snap-frozen in liquid nitrogen and stored at –80 °C for up to 3 months and reused one more time.

Protein–lipid interaction assay ● TIMING 1 h
57| Prepare four inlet tubings per chip (one for each chamber) by combining two silicon tubes—one with a diameter of 0.3 × 0.76 mm (length ~4 cm) and one with a diameter of 0.5 × 2.1 mm (length ~1 cm). You will also need four pieces of 0.3 × 0.76 mm silicon tubing (length ~1 cm) as outlets (one for each chamber).

58| Open the Mylar bag from Step 52 and take out the microfluidic device. Insert the tubing into the holes with the inlet tubings on the side of the longer connecting channels and the outlet tubings on the opposite side.

59| Using a 100-µl Hamilton syringe, manually inject the assay buffer (see Reagent Setup) into the first channel until a small drop comes out from the corresponding outlet tubing. Fill the remaining three channels in the same way. The liposomes will form within 5 min.
▲ **CRITICAL STEP** Inject the assay buffer into the channels slowly to avoid creating air bubbles inside. Assay buffer should be filtered (0.22-µm pore size) to avoid the introduction of dust in the channels.
▲ **CRITICAL STEP** To check the quality of the formed liposomes, you can briefly browse the spots on the array in the fluorescent microscope. If the liposomes are nicely formed, you can continue with the next steps. You can also acquire images of liposomes at this point and use them later as blank images.
▲ **CRITICAL STEP** In the case of particular lipids that systematically do not integrate in liposomes, it is possible to add a very little amount (<5% mol/mol) of a PEGylated lipid, as advised by previous protocols[39,53]. However, the introduction of PEGylated lipids might introduce bias, so their use must be restricted to the minimum.
? TROUBLESHOOTING

60| Connect the inlet tubing of the first channels to the 250-µl Hamilton syringe, filled with the protein sample (from Step 55 if using cell lysate, or purified protein otherwise) and mounted in the automatic syringe pump (**Fig. 5**).

61| Inject 40 µl of the protein sample into the channel (injection speed, 10 µl/min).

62| Repeat Steps 60 and 61 to fill the remaining three channels.

63| Incubate the liposomes with the protein for 20 min.

64| Wash each channel with 120 µl of the assay buffer.

Image acquisition ● TIMING 50 min per array (120 spots)
65| Mount the microfluidic device into the microfluidic device holder for microscopy (see Equipment Setup), and then place the holder into the microscope.

66| Enter the spot positions into the microscope settings (i.e., define the array diameters, the size of the spots and the distance between spots). Set the exposure times for both channels, Atto 647 and GFP.

67| Set the parameters for automatic focusing.
▲ **CRITICAL STEP** In our experience, the microscope focuses better on the agarose layer on the glass slide than on the liposomes. Therefore, it is important to set an offset in the *z*-dimension that roughly corresponds to half of the diameter of liposomes (in our experience, 4–6 µm works the best). The difference between the wavelengths during the image acquisition in the GFP and Atto 647 channels leads to an imperfect alignment of the corresponding fluorescent signals. You can easily correct this discrepancy by increasing the offset in the GFP channel by 2 µm.
▲ **CRITICAL STEP** As binding affinities vary by several orders of magnitude between different proteins and even between different liposome spots, several exposure times should be acquired in the GFP channel. Overexposed images are not considered in the image analysis pipeline (**Supplementary Data 3**).

68| Assign the first position (top left corner).
▲ **CRITICAL STEP** The holder can accommodate three microfluidic devices. The software allows multiple starting positions to be set; therefore, it is possible to set the first position for each device at the start. The microscope will then acquire images from all three devices one after another.

69| Start automated image acquisition.
▲ **CRITICAL STEP** The microscope assigns a name/label to each image within one experiment that identifies the position of the imaged spot. However, this naming is always the same, and it does not vary between different experiments. Therefore, the images have to be renamed in order to be assigned a unique identifier. We suggest that image names contain an experiment identifier (e.g., date, type of liposome array or series number), a protein identifier and a lipid identifier (see the names of the example images we provide in **Supplementary Data 3**). For advice regarding automated renaming, please contact the authors.

Image analysis and NBI calculation ● TIMING 5 h for each array of 120 spots
70| Browse the Atto 647 images and remove all low-quality images (out of focus, no liposomes on the image and so on). Use this filtered data set for further analysis.
▲ **CRITICAL STEP** If an Atto 647 image is removed, the corresponding GFP image(s) must also be removed. If you suspect that protein precipitation or cross-contamination of lipids during spotting occurred, the GFP images also need to be checked so that images with precipitates or cross-contamination can be removed.

Alternatively, you can skip Step 70, directly proceed with Step 71 and prepare a .csv file containing a list of all the low-quality images (defined by a number assigned to each image by CellProfiler in Step 71; see below, and also see **Supplementary Data 3** for a template) to be removed from the analysis. This file is then used by the rLiMA script (**Supplementary Data 3**), which automatically removes the data from the low-quality images and directly provides a filtered final output file (see Step 73).

To simplify systematic image browsing and annotation, a web interface that automatically creates the .csv file with a list of low-quality images can be built. A snapshot of the web interface is provided (**Fig. 7b**). Please contact the authors for more information. Alternatively, images can be inspected using CellProfiler Analyst.

71| Start the CellProfiler software, adjust the processing pipeline according to the acquired images (i.e., exposure time(s)) and specify the output directory in which the processed images and output .csv file will be saved.
▲ **CRITICAL STEP** Use our example CellProfiler pipeline provided in **Supplementary Data 3,** and adjust it to your data (i.e., image names, exposure time(s), format of saved processed images).
▲ **CRITICAL STEP** For advice on running the CellProfiler analysis in a Linux cluster environment to increase analysis speed, please contact the authors.

72| If only one GFP exposure time was used, the NBI value of each spot is given directly in the output .csv file in a column called Math_Ratio. In the case of multiple exposure times in the GFP channel, continue with Step 73 to obtain final NBI values.
▲ **CRITICAL STEP** Each experiment will generate one .csv file.

73| If multiple GFP exposure times were used, process the output .csv file with the R package called rLiMA (**Supplementary Data 3**) to calculate the final mean NBI. In addition to the TAB file summarizing the final NBIs, rLiMA also compares the NBIs of all protein–lipid combinations tested in the experiment.
▲ **CRITICAL STEP** All the failed experiments must be removed from the downstream data analysis process.

Data quality control ● TIMING 2 d
▲ **CRITICAL** Scripts used in the following steps are available on the tutorial webpage http://www.bork.embl.de/Docu/LiMA_protocol/.
74| Concatenate all .csv files generated from Step 72 or 73 in one master data set using the script 'LiMA-dataset-generation.r'.

75| Add the manual annotation information (Step 73) using the script 'LiMA-dataset-clean.r'.

76| Add the information concerning the protein concentration using the script 'LiMA-add-protein-concentration-to-dataset.r'.

77| Import the data into the R workspace. Open RStudio and choose 'R script' by clicking on 'File' and then on 'New File' at the toolbar. Import the data set into a data.frame using the following line code: *data = read.table ('dataset.tsv',header=T,sep='\t')*.

78| Test for reproducibility using the script 'LiMA-reproducibility.r'.

79| Check for spatial effects using the script 'LiMA-spatial-effects.r'.

80| Check for further potential bias. The script 'LiMA-protein-concentration-effects.r' will test for potential concentration effects. The script 'LiMA-liposome-number.r' will measure the influence of liposome numbers on the NBI (the second part of the script makes use of a multiple Wilcoxon test and needs to be completed by a false discovery rate correction test using the R function 'p.adjust()' from the standard package 'stats').

81| Generate ROC curves as described in the script 'LiMA-roc-analysis.r' and determine the NBI threshold that gives optimal sensitivity and sensibility. The script first removes the data point annotated as dubious and performs the predictions on the data points annotated as binding and nonbinding events, and the performance is then computed on the basis of those predictions.

? TROUBLESHOOTING

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

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
7	Agarose layer is not homogeneous	Glass slides are not hydrophilic enough	Increase the washing time in Hellma solution
10	Lipids form a turbid solution	Lipids are not soluble	Heat for a few minutes at 60 °C and/or add acid dropwise to the solution
28	NOA81 injection leads to bubbles	Bad connection between the syringe and the tube	Reconnect the syringe and the tube
29	Protective stickers are not sticky	UV illumination time is too high	Decrease the UV illumination time
	Protective stickers are too sticky	Underillumination	Increase the UV illumination time
34	Protective stickers not sticking to the TAL	Protective stickers are too old	Prepare new protective stickers
49	Hardened adhesive	Heating time too long, temperature too high	Decrease the heating time or check the heating temperature
59	Bubbles present in the channels	Bad connection between the syringe and the tube	Make sure that the connection between the syringe and the tube is tight
	Dust in the channel	Buffer not filtered	Prepare the buffer again and filter using Millex GV filter of size 0.22 µm
	Leakage	Time for adhesion is too short	Allow at least an overnight period between binding and the experiment
	None or few liposomes	Degraded lipids	Use freshly made lipids
		Devices are too old	Device should not be stored more than 2 months
		Lipid incompatible with the liposome formation method	Try to add PE-PEG2000 up to 5% to force the formation of liposomes
	Detachment of liposomes	Microfluidic channel is not tall enough	Height of the microfluidic channel should be >600 µm
		Too strong of an injection flow rate	Reduce the injection flow rate
	Cross-contamination between spots	Misalignment of the spotting platform	Check that the spotting coordinates are correct

● TIMING

Steps 1–9, preparation of the TALs: overnight
Steps 10–14, lipid mixture preparation: 6 h
Steps 15–20, preparation of microfluidic channels: 1 d
Steps 21–30, protective sticker preparation: 2 d
Steps 31–36, spotting platform preparation: 2 h hands-on time + overnight adhesion
Steps 37–43, spotting: 10 h
Steps 44–52, device assembly and storage: 3 h
Steps 53–56, low-volume cell extract preparation: 1 h (excluding time needed for protein expression)
Steps 57–64, protein–lipid interaction assay: 1 h
Steps 65–69, image acquisition: 50 min
Steps 70–73, image analysis and NBI calculation: 5 h
Steps 74–81, data quality control: 2 d

ANTICIPATED RESULTS

This protocol enables researchers to measure the recruitment of proteins to membranes in a high-throughput manner. In our laboratory, LiMA has been used to study the recruitment of Pleckstrin homology domains to more than 120 different types of liposomes[20] comprising 26 different signaling lipids and representing more than 32,000 experiments overall. The amount of data generated by LiMA has required the implementation of a web interface to easily annotate the data (**Fig. 7b**); all together, <30% of images had to be removed from the analysis (because of protein precipitation, failure to produce liposomes, or failure to acquire focused image), which means that the technical success of the experiments was >70%, showing the robustness of the assay and demonstrating the feasibility of lipidome and proteome screening using LiMA. The image annotation has been used to determine the best conditions in terms of sensitivity and specificity using ROC and to determine an NBI threshold discriminating between interaction and noninteraction (see tutorial at http://www.bork.embl.de/Docu/LiMA_protocol/). The measurement of the NBI allows the relative affinity of interacting pairs to be quantified[20,23], and the use of dose-response curves enables LiMA to estimate affinities[20]. LiMA has also been used to probe how mutations in lipid-binding domain residues can change lipid-binding specificities[20,23]. However, the values obtained with LiMA need to be complemented with orthogonal *in vivo* experiments in order for the real pathological potential of a mutation to be determined. Overall, because the complexity of cellular membranes can be incorporated into the assay, LiMA sets a new paradigm for studying the many and varied interactions between proteins and lipids.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS We are grateful to the EMBL Advanced Light Microscopy Facility (ALMF), C. Gehin and C. Merten for expert help. We also thank other members of P.B.'s, J.E.'s and A.-C.G.'s groups for continuous discussions and support. This work is partially funded by the DFG in the framework of the Cluster of Excellence, CellNetworks Initiative of the University of Heidelberg (ExIni, EcTop). A.-E.S. is supported by the European Molecular Biology Laboratory and the EU Marie Curie Actions Interdisciplinary Postdoctoral Cofunded Programme.

AUTHOR CONTRIBUTIONS A.-E.S., A.-C.G. and J.E. designed the research. A.-C.G. directed the research. A.-E.S. developed the experimental protocol with the help of I.V. and S.C. A.-E.S., I.V., K.G.K. and C.T. developed the image analysis pipeline. S.D. and P.B. developed the bioinformatics tools.

COMPETING FINANCIAL INTERESTS The authors declare competing financial interests: details are available in the online version of the paper.

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