Journal of Visualized Experiments

Analyzing the Interaction of Fluorescent-labeled Proteins with Artificial Phospholipid Microvesicles Using Quantitative Flow Cytometry

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63459R2
Full Title:	Analyzing the Interaction of Fluorescent-labeled Proteins with Artificial Phospholipid Microvesicles Using Quantitative Flow Cytometry
Corresponding Author:	Nadezhda Podoplelova, Ph.D. Center for Theoretical Problems of Physico-Chemical Pharmacology RAS: Centr teoreticeskih problem fiziko-himiceskoj farmakologii RAN Moscow, Moscow RUSSIAN FEDERATION
Corresponding Author's Institution:	Center for Theoretical Problems of Physico-Chemical Pharmacology RAS: Centr teoreticeskih problem fiziko-himiceskoj farmakologii RAN
Corresponding Author E-Mail:	podoplelovan@yandex.ru
Order of Authors:	Nadezhda Podoplelova, Ph.D.
	Polina Soloveva
	Andrei Garzon Dasgupta
	Aleksandra Filkova
	Mikhail Panteleev
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biochemistry
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Moscow, Russia
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release
Please provide any comments to the journal here.	

TITLE:

Analyzing the Interaction of Fluorescent-labeled Proteins with Artificial Phospholipid Microvesicles Using Quantitative Flow Cytometry

AUTHORS AND AFFILIATIONS:

Nadezhda Podoplelova[1,2], Polina Soloveva[1,4], Andrei Garzon Dasgupta[1,2,3], Aleksandra Filkova[1,2], Mikhail Panteleev[1,2,3,4]

[1]Center for Theoretical Problems of Physicochemical Pharmacology of the Russian Academy of Sciences, Moscow, Russia
[2]National Medical Research Center of Pediatric Hematology, Oncology and Immunology named after Dmitry Rogachev, Moscow, Russia
[3]Faculty of Physics, Lomonosov Moscow State University, Moscow, Russia
[4]Faculty of Biological and Medical Physics, Moscow Institute of Physics and Technology, Dolgoprudnyi, Russia

Email addresses of co-authors:

Polina Soloveva (polina.soloveva@phystech.edu)
Andrei Garzon Dasgupta (garzon.ak15@physics.msu.ru)
Aleksandra Filkova (filkova.alexandra@ctppcp.ru)
Mikhail Panteleev (mikhail.panteleev@fccho-moscow.ru)

Corresponding author:

Nadezhda Podoplelova (podoplelova.nadezhda@ctppcp.ru)

SUMMARY:

Here, we describe a set of methods for characterizing the interaction of proteins with membranes of cells or microvesicles.

ABSTRACT:

In the human body, most of the major physiologic reactions involved in the immune response and blood coagulation proceed on the membranes of cells. An important first step in any membrane-dependent reaction is binding of protein on the phospholipid membrane. An approach to studying protein interaction with lipid membranes has been developed using fluorescently labeled proteins and flow cytometry. This method allows the study of protein–membrane interactions using live cells and natural or artificial phospholipid vesicles. The advantage of this method is the simplicity and availability of reagents and equipment. In this method, proteins are labeled using fluorescent dyes. However, both self-made and commercially available, fluorescently labeled proteins can be used. After conjugation with a fluorescent dye, the proteins are incubated with a source of the phospholipid membrane (microvesicles or cells), and the samples are analyzed by flow cytometry. The obtained data can be used to calculate the kinetic constants and equilibrium K_d. In addition, it is possible to estimate the approximate number of protein binding sites on the phospholipid membrane using special calibration beads.

INTRODUCTION:

Biomembranes separate the inner contents of animal cells and extracellular space. Note that membranes also surround microvesicles formed during the cell's life cycle and organelles. The cell membrane is predominantly composed of lipids and proteins. Membrane proteins perform signaling, structural, transport, and adhesive functions. However, the lipid bilayer is also essential for the interrelation of the animal cell with the extracellular space. This paper proposes a method for studying the peripheral interaction of external proteins with the lipid membrane.

The most striking example of reactions occurring on the outer membrane layer of an animal cell is the blood coagulation reaction. An important feature of blood coagulation is that all the main reactions proceed on the phospholipid membranes of cells and microvesicles arising from these cells and not in the plasma[1–3]. Membrane-dependent reactions include the process of starting coagulation (on the cell membranes of the subendothelium, inflamed endothelium, or activated immune cells, with the participation of a tissue factor), all reactions of the main cascade—activation of factors IX, X, prothrombin; activation of factor XI by thrombin (on the membranes of activated platelets, erythrocytes, lipoproteins, and microvesicles); reactions of the protein C pathway; inactivation of coagulation enzymes (on the membranes of endothelial cells with the participation of thrombomodulin cofactors, endothelial protein C receptor, heparan sulfate); and contact pathway reactions (on membranes of platelets and some microvesicles with the participation of unknown cofactors). Thus, it is impossible to investigate blood coagulation without studying the interaction of various plasma proteins with the membrane of blood cells.

This paper describes a flow-cytometry-based method for characterizing the interaction of proteins with lipid membranes of cells or microvesicles. This approach was initially proposed to study the interaction of blood plasma with platelets and artificial phospholipid vesicles. Moreover, most of the studied proteins interact directly with negatively charged membrane phospholipids, particularly with phosphatidylserine[4, 5]. Additionally, there are proteins whose interaction with the membrane is mediated by special receptors[6].

An important ability of flow cytometry is discriminating between free and bound ligands without additional separation. This feature of cytometry allows the study of ligand equilibrium binding at the endpoint and helps perform continuous kinetic measurements. The technique is unsophisticated and does not require complex sample preparation. Flow cytometry is actively used to quantitatively study the dynamics of interaction between fluorescent peptides, receptors, and G-proteins in intact and detergent-permeable neutrophils[7]. This approach is also applicable for exploring protein–DNA interactions and the kinetics of endonuclease activity in real time[8]. Over time, this method was used to quantitatively study high-affinity protein–protein interactions with purified lipid vesicles[9], or, more generally, with membrane proteins expressed in a highly efficient Sf9 cell expression system[10]. Quantitative methods have also been described for characterizing protein–liposome interactions using flow cytometry for transmembrane proteins[11].

This technique uses self-made calibration beads to avoid using commercially available beads[7]. The calibration beads used previously[7] were intended to work with fluorescein, which

substantively restricted the assortment of accessible fluorescent ligands on the proteins. In addition, this paper offers a new way to acquire and analyze kinetic data for reasonable time resolution. Although this method is described for artificial phospholipid vesicles, there are no obvious limitations for its adaptability to cells, natural vesicles, or artificial phospholipid vesicles with a different lipid composition. The method described herein allows the estimation of the parameters of interaction (k_{on}, k_{off}) and equilibrium (K_d) and facilitates quantitative characterization of the number of protein binding sites on the membrane. Note that this technique provides an approximate estimate of the number of binding sites. The advantages of the method are its relative simplicity, accessibility, and adaptability to native cells and natural and artificial microvesicles.

PROTOCOL:

1. Fluorescent protein labeling

1.1. Material preparation

1.1.1. Prepare 1 M Sodium bicarbonate buffer, pH 9.0, store it at 4 °C, and use it within one week.

1.1.2. Prepare M hydroxylamine hydrochloride, pH 8.5, immediately before use.

1.1.3. Prepare a 10 mg/mL solution of fluorescent dye (see the **Table of Materials**) in dimethylsulfoxide.

NOTE: This solution can be stored for a month at -20 °C in the dark.

1.1.4. Prepare solutions of purified antibodies or other proteins at 1–10 mg/mL.

NOTE: Avoid buffers containing ammonium ions or primary amines. Replace the buffers containing Tris or glycerol with phosphate-buffered saline (PBS) by dialysis. Neither sodium azide (≤3 mM) nor thimerosal (≤1 mM) will significantly affect the conjugation reaction.

1.1.5. Incubate the gel filtration medium (see the **Table of Materials**) for protein purification in PBS overnight at room temperature or for 2 h at 60 °C. Apply the gel filtration medium onto spin columns with 0.2 µm membranes.

1.2. Calculate the amount of reactive dye to be used for each reaction according to the concentration of protein to be labeled by using Eq (**1**).

$$\mu L\ dye\ stock\ solution = \frac{Concentration\ protein\ mg\ /\ ml\ \times\ V\ protein\ ml}{MW\ protein\ g/mol} \times MW\ dye \frac{g}{mol} \times 100 \times MR \quad (\mathbf{1})$$

Where *100* is a unit conversion factor; *MR* is the molar ratio of dye to protein in the reaction mixture.

NOTE: The following MRs are recommended for IgG labeling reactions: MR = 40 if the antibody is at 1–3 mg/mL or MR = 30 if the antibody is at 4–10 mg/mL. For coagulation factors, MR = 5 is usually used.

1.3.Conjugation reaction

1.3.1. In a reaction tube, mix the protein solution with a 10x lower volume of 1 M bicarbonate solution.

1.3.2. Add the required amount of fluorescent dye (see step 1.2) with continuous stirring.

1.3.3. Incubate the reaction mixture at room temperature for approximately 1 h, protected from light and with continuous stirring.

1.3.4. For each 200 µL of protein solution, add 5 µL of 1.5 M hydroxylamine hydrochloride.

1.3.5. Incubate the reaction mixture at room temperature for approximately 30 min, protected from light and with continuous stirring.

1.4. Prepare the spin column.

1.4.1. Add 500 µL of gel filtration medium for protein purification to a column. Centrifuge the column for 3 min at 1,000 × *g*.

1.4.2. Discard the buffer from the collection tube. If the column is not full, add more gel filtration medium, and centrifuge the column for 3 min at 1,000 × *g*. Repeat this step until the column is full.

1.5. Purification

1.5.1. Centrifuge the reaction mixture (from step 1.3.5) for 5 min at 17,000 × *g* and remove the precipitate.

1.5.2 Transfer the supernatant to the spin column with gel filtration medium. Allow the solution to absorb into the gel bed.

1.5.3. Use an empty collection tube for the spin column and centrifuge it for 5 min at 1,000 × *g*. After centrifugation, collect the labeled protein from the collection tube.

1.6. Determination of the degree of labeling

1.6.1. Correct for the contribution of the dye to the absorbance at A_{280} by measuring the absorbance of free dye at 280 nm (A_{280}) and the λ_{max} for the dye (A_{max}) (see Eq (**2**)).

$$CF = \frac{A_{280}\ free\ dye}{A_{max}\ free\ dye} \quad (\mathbf{2})$$

1.6.2. Measure the absorbance of the protein–dye conjugate at 280 nm (A_{280}) and the λ_{max} for the dye (*Amax*) using Eq (**3**).

$$protein\ concentration\ (M) = \frac{A_{280} - A_{max} \times CF}{\varepsilon} \quad (\mathbf{3})$$

Where ε is the molar extinction coefficient of the protein at 280 nm.

1.6.3. Calculate the degree of labeling (DOL) using Eq (**4**).

$$DOL = \frac{A_{max} \times MW_{protein}}{protein\ concentration \times \varepsilon\ dye}, \quad (\mathbf{4})$$

2. Preparation of phospholipid vesicles

2.1. Preparation and storage of the lipid mixture

2.1.1. Combine the lipids in the appropriate ratio (phosphatidylserine:phosphatidylcholine at a ratio of 20 mol%:80 mol%).

2.1.2. Dry the lipid mixture after lyophilization or evaporation and store it under an inert atmosphere in glass ampules.

2.2. Lipid film production

2.2.1. Open the ampule, and resuspend the lipid mixture in a small amount (~100 µL) of chloroform.

NOTE: Do not use too much chloroform as it does not evaporate completely.

2.2.2. Add DiIC16(3) in ethanol at 0.2 mol%. Transfer the lipid mixture to a round bottom flask. Spread the mixture thinly over the sides of the flask by rotating it. Dry the lipid mix for 30 min under an argon stream.

2.3. Hydration of the lipid mixture

2.3.1. Add a suitably warm (~55 °C) aqueous buffer (HEPES 20 mM, NaCl 140 mM, pH 7.4) in a volume corresponding to the expected lipid concentration to the flask with the lipid film. Incubate the mixture with vortexing at 55 °C for 30 min for complete hydration.

2.3.2. Placing the sample tube in a freezer or warm thermostat to make the lipid suspension undergo 3–5 freeze-thaw cycles.

2.4. Formation of lipid vesicles by extrusion

2.4.1. Prepare the extruder according to the manufacturer's instructions. Warm up all the extruder components to the phase transition temperature of the lipid mixture.

2.4.2. Fill one of the extruder syringes with the hydrated lipid mixture. Wait for 5–10 min for the temperature of the lipid suspension to equilibrate with the temperature of the extruder.

2.4.3. Extrude the lipid mixture through the membrane at least 10 times. For the final extrusion, place the lipid suspension in the alternate syringe and look for a change in appearance from a slightly nebulous to a clear solution.

2.4.4. Store the resulting mixture of lipid vesicles at 4 °C, preferably in an inert atmosphere of argon or nitrogen, for 3–4 days. Do not freeze.

3. Isolation of platelets from whole blood

3.1. Collect whole blood from healthy donors in tubes containing 3.2% sodium citrate.

3.2. Add prostaglandin E1 (PGE1) (1 µM) and apyrase (0.1 U/mL) to the blood, followed by centrifugation at room temperature at 100 × *g* for 8 min.

3.3. After centrifugation, take the platelet-rich plasma and add sodium citrate solution (106 mM, pH 5.5) to a plasma: citrate ratio of 3:1. Centrifuge the plasma at room temperature at 400 × *g* for 5 min.

3.4. Remove the supernatant, and resuspend the pellet in 300 µL of Tyrode's buffer without BSA (20 mM HEPES, 150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 5 mM glucose, pH 7.4). Purify the platelets from plasma proteins by gel chromatography on the gel filtration medium for platelet purification (see the **Table of Materials**).

4. Detection of protein–lipid interaction by flow cytometry

4.1. Kinetic binding experiments

4.1.1. Dilute phospholipid vesicles (from step 2.4.4) in Tyrode's buffer (20 mM HEPES, 150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 5 mM glucose, 0.5% BSA, pH 7.4) to a concentration of 1 µM and total volume of 250 µL.

4.1.2. Mix fluorescent-labeled coagulation factor X (fX-fd) from step 1 at a concentration of 500 nM with the phospholipid vesicles from step 4.1.1 in a 1:1 ratio (final vesicle concentration 0.5 µM, fX-fd concentration is 250 nM of fX) to a total volume of 500 µL.

4.1.3. Immediately inject the 500 µL of the mixed suspension (~20 min for analysis with a low flowing rate) into the flow cytometer. Use a low flow rate and ensure that the threshold for channel FL2 is as follows: excitation 488 nm, emission filter 585/42 nm. Measure the mean fluorescence in channel FL4 (excitation 633 nm, emission filter 660/20) for the fluorescence dye from the **Table of Materials**.

NOTE: Choose a cytometer without an autosampler. This will speed up the process of injection of the sample into the measuring cell.

4.1.4. When saturation of binding is achieved (no significant increase in fluorescence within 5 min), rapidly dilute the sample 20-fold with Tyrode's buffer, and monitor the dissociation until baseline fluorescence is reached (complete dissociation) or until a plateau is reached (no significant decrease in fluorescence within 5 min).

NOTE: As a control, add 10 µM EDTA and monitor complete dissociation for 5 min.

4.2. Equilibrium binding experiments

NOTE: Use the kinetic curve of binding to determine the time to reach saturation; the time for saturation for fX-fd and artificial vesicles is 20 min.

4.3. Incubate artificial phospholipid vesicles (5 µM) for the binding assay with different concentrations of fX-fd (from 0 to 1,000 nM) in Tyrode's buffer for 20 min.

4.4. Dilute each sample from step 4.3 by 20x to a final volume of 200 µL with Tyrode's buffer. Immediately analyze the diluted sample by flow cytometry within 30 s. Use settings from step 4.1.3.

NOTE: As a control for nonspecific binding, use similar samples with EDTA (10 µM) and incubate them for 5 min.

5. Analysis of flow cytometry data

5.1. Export experiments in FSC format from cytometry data acquisition software to cytometer software for data analysis (see the **Table of Materials**). Choose **File | Export | FCS files**. Open FSC

files in cytometer software for data analysis by selecting the files on the computer and dragging them to the workspace of the program.

5.2. For gating of the microvesicles, identify the region of microvesicles by the fluorescence of the lipophilic dye $DiIC_{16}(3)$. Use menu commands or **plot** button in the worksheet to create dot plot SSC from FL2 (dye $DIiC_{16}(3)$) in log coordinates. Choose the **Rectangular Gate** button to draw a gating region so that events from a sample without vesicles are not included in this region (**Figure 1B,C).**

5.3. Analyze the kinetic experiments.

5.3.1. Create a dot-plot using the coordinates of fluorescence (FL4) over time for the region of the vesicles (double-click in the region of vesicles in step 5.2)

5.3.2. Export the data on the change in fluorescence over time in csv format. **Choose Sample | Right-click | Export | Choose FL4** and **Time in Parameters | Select directory for saving | Select CSV format | Export**.

5.3.3. Open the CSV file in any statistical software (see the **Table of Materials**). Calculate a simple moving average of fluorescence and time for every 1,000 events.

5.3.4. Approximate a graph of the dependence of the simple moving average fluorescence on time under the assumption of exponential dependence (Analysis > Fitting >Nonlinear Curve Fit) and use this to calculate the kinetic association constant using Eq (**5**).

$$\left[X^B\right] = [X]_{\max} \cdot (1 - e^{(-k[X]\cdot t)}) \qquad (\mathbf{5})$$

Where $[X^B]$ is the bound factor concentration at each moment of time (user-defined units) according to the simple moving average from step 5.3.3; $[X]$ is the added factor concentration; $[X]_{max}$ is the maximum bound factor concentration; k is the association constant; t is the time.

5.3.5. Repeat the same set of actions (5.3.1–5.3.4) to calculate the kinetic dissociation constant using Eq (**6**).

$$\left[X^B\right] = [X]_0 \cdot e^{(-k\cdot t)} \qquad (\mathbf{6})$$

Where $[X^B]$ is the bound factor concentration at each moment of time; $[X]_0$ is the bound factor concentration at the initial moment of time; k is the dissociation constant; t is the time.

5.4. Equilibrium binding assay

5.4.1. Determine the average fluorescence of fX-fd in the region of the vesicles for each selected concentration of fX-fd.

5.4.2. Approximate the dependence of the bound factor fluorescence from the concentration of the added factor in the assumption of simple single-site binding. Calculate the average binding parameters using Eq (**7**) from three independent repeats at a minimum.

$$\left[X^{B}\right]=\frac{n^{X}\cdot\left[X\right]}{K_{d}+\left[X\right]} \qquad (7)$$

Where $[X^B]$ is the bound factor concentration; $[X]$ is the added factor concentration; n^x is the apparent number of binding sites per vesicle; K_d is the apparent dissociation constant.

6. Converting fluorescence intensity to the mean number of binding sites

6.1. Prepare calibrated beads.

6.1.1. Incubate gel-filtered platelets (see step 3.3) with A23187 (10 µM) in the presence of $CaCl_2$ (2.5 mM) for 10 min at room temperature.

6.1.2. Add to the activated platelets the various concentrations of fX-fd (0 to 1,000 nM). Add 2% v/v formaldehyde and incubate for 1 h. Stop the reaction by incubating the platelets with 3 M glycine and 5% BSA for 30 min at room temperature.

6.1.3. Purify the mixture from the unreacted dye. Centrifuge the platelets for 5 min at 400 × *g*, remove the supernatant, and resuspend the pellet in Tyrode's buffer (containing 0.5% BSA).

NOTE: Repeat step 6.1.3 three times.

6.2. Measure the fluorescence level of the calibration beads in each sample first using a spectrofluorometer (for fluorescent dye from the **Table of Materials**, excitation 633 nm, emission 670 nm) and then using the flow cytometer (in channel FL4: excitation 633 nm, emission filter 660/20). Using a cell counter, determine the number of beads in each sample.

6.3. Convert the fluorescence intensity of each respective bead sample to the concentration of soluble fluorescent dye using a spectrofluorometer. Recalculate the fluorescent dye concentration for the number of fluorophore molecules using Eq (**8**).

$$N_x = C \times V \times N_A \qquad (8)$$

Where N_x is the number of fluorophore molecules; C is the fluorescent dye concentration; N_A is Avogadro constant; $N_A = 6.02214076\times10^{23}\ mol^{-1}$.

6.4. Create a dependence graph of the average fluorescence of the beads in a flow cytometer (step 6.2) on the number of fluorophore molecules (see step 6.3) for each sample using any

statistical software (see the **Table of Materials**). Approximate this dependence by line proportionality (**Analysis | Fitting | Fit linear**). From the approximation in Eq (**9**), calculate the conversion factor of the mean fluorescence to binding sites.

$$MF = CF \times N^x + b \qquad (9)$$

Where MF is the mean fluorescence of beads by flow cytometry; Nx is the number of fluorophore molecules per bead; CF represents the conversion factor of the mean fluorescence to binding sites. CF and b are obtained from the results of fitting the graph by linear proportionality.

6.5. Calculate the apparent number of binding sites per vesicle of interest using Eq (**10**).

$$n^x = \frac{MF-b}{CF} \qquad (10)$$

Where nx is the apparent number of binding sites per vesicle of interest; MF is the mean fluorescence of the vesicles of interest by flow cytometry; CF and b are conversion factors from the Eq (8).

REPRESENTATIVE RESULTS:

The flow cytometry method described herein is used to characterize the binding of plasma coagulation proteins to activated platelets. In addition, phospholipid vesicles PS:PC 20:80 were applied as a model system. This paper mainly focuses on artificial phospholipid vesicles as an example. The parameters of the cytometer, in particular, the photomultiplier tube (PMT) voltage and the compensation must be selected for each specific device, the object of study (cells, artificial or natural microvesicles), and the dyes used. **Figure 1B,C** show an example of gating artificial phospholipid vesicles that are ~1 µm in size with the incorporated lipophilic fluorescent dye DiIC16 (3). Large vesicle size and lipophilic fluorescent dye helped detect vesicles using a cytometer. The gate was set based on a sample containing the same-size artificial lipid vesicles but without the fluorescent dye (**Figure 1B**). Only events inside this gate were used in the analysis.

The kinetics of protein binding to vesicles was analyzed at the first stage. The sample for this was collected continuously as described in step 4.1. A typical dot-plot is shown in **Figure 1D–F**. The data obtained were analyzed using the flow cytometry software. The resulting curve is shown in **Figure 1G**. Solid lines show the curves of approximation, from which the kinetic constants of association (k_{on}) and dissociation (k_{off}) were obtained. As factor X binds to phospholipid vesicles reversibly and Ca^{2+}-dependently, samples with EDTA controlled the specificity and reversibility of this binding. The resulting constants are shown in **Table 1**.

Based on the kinetics of binding, a time of 20 min was chosen for further equilibrium experiments to describe saturation in binding accurately. The mean fluorescence intensity of the factor was subsequently determined in the region of the vesicles. Each sample was analyzed in the presence and absence of EDTA. The fluorescence intensity in the presence of EDTA was taken as

background and subtracted from the total signal as the binding of fX to the membrane in the absence of Ca^{2+} ions is considered nonspecific. The resulting fluorescence was converted to the number of binding sites per vesicle using the calibration beads.

FIGURE AND TABLE LEGENDS:

Figure 1: Specific binding of fX to artificial phospholipid vesicles. (**A**) Scheme of experiment. (**B, C**) Typical dot plots of phospholipid vesicles without (**B**) or with (**C**) lipophilic fluorescent dye DiIC16 (3). (**D–F**) Typical dot plots of factor X interaction with phospholipid vesicles before (**D**) or after (**E**) 20-fold dilution and in presence EDTA (**F**). (**G**) Kinetics of FX (250 nM) binding and dissociation to phospholipid vesicles. (**H**) Equilibrium interaction of factor X to phospholipid vesicles. Results are the means ±SD for n=3 different samples. Abbreviations: FX = Factor X; Ph vesicles = phospholipid vesicles; SSC = side scatter; a.u. = arbitrary unit).

Table 1: Parameters of fX interaction with artificial phospholipid vesicles. Parameters were determined from the curves (see **Figure 1F,G**). Results are the mean ± SEM for n = 3.

DISCUSSION:

The proposed method can be adapted for a rough characterization of the interaction of proteins with phospholipid membranes from various sources and compositions. The quantitative flow cytometry described here concedes to surface plasmon resonance (SPR) in several parameters. In particular, it has a lower sensitivity and time resolution and requires fluorescent labeling of proteins. Fluorescent labeling can lead to a change in conformation and loss of activity for many proteins and therefore requires careful control. However, this technique has significant advantages over others. This method provides an opportunity to explore the interaction of proteins with the native cell membrane, which is not readily implemented using SPR. Moreover, the approach allows the estimation of the number of protein binding sites on the membrane surface and can be efficient for some analysis tasks.

Commercially available beads are available for some fluorescent dyes to count binding sites. However, there are no such beads for many widely used dyes. Hence, self-made beads are the best way to resolve this. The same cells were used to prepare these beads as for all other experiments. However, as the beads require high centrifugation speeds during washing, phospholipid vesicles cannot be used. However, cells or vesicles can be replaced with beads with amino-reactive groups, which can be conjugated to the chosen dye. The sequence of actions will be similar to those described in step 6.1.

The limitations of this quantitative flow cytometry method are related to the technical capabilities of the used cytometer. Three different models of flow cytometers (with the variable lasers, detectors, pumps) were applied to reproduce this technique without any complication. However, the selection of a fluorescent label suitable for the cytometer used must be carefully considered because the set of lasers, detectors, and optical filters differ from device to device, even within the same model. It is necessary to focus on the ability of the cytometer to measure microparticles of a specific diameter; not all instruments are equally capable of detecting particles at resolutions below 200 nm (to determine this, use the fixed-size calibration beads

supplied by the instrument's manufacturer). Additionally, some flow cytometers, which are sampled using a syringe pump, cannot measure continuous binding kinetics in principle. In this case, the kinetics can be recorded only point by point, taking separate samples for measurement at certain points in time[4,6].

Flow cytometry is used to investigate the expression of antigens on various cells—the presence/absence of an antigen and the percentage of cell populations expressing and not expressing this antigen. The ability of flow cytometry to concurrently discriminate free and bound ligands without additional separation procedures also affords the opportunity for quantitative assessment of ligand-binding dynamics. The method proposed herein describes the preparation of self-made calibration beads to quantify the binding of a fluorescent ligand to artificial phospholipid vesicles. This approach does not limit the choice of commercially available fluorophores. Beyond that, the technique described here for the acquisition and analysis of kinetic data enables improved time resolution. Thus, the method described herein can be used alone as a first step in characterizing protein–membrane interactions or in combination with other methods (for example, SPR or microscopy) to improve measurement accuracy.

ACKNOWLEDGMENTS:

The authors were supported by a Russian Science Foundation grant 20-74-00133.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

REFERENCES:

1. Hoffman, M., Monroe, D. M. A cell-based model of hemostasis. *Thrombosis and haemostasis*. **85** (6), 958–965 (2001).
2. Roberts, H. R., Hoffman, M., Monroe, D. M. A cell-based model of thrombin generation. *Seminars in Thrombosis and Hemostasis*. **32** (Suppl 1), 32–38 (2006).
3. Panteleev, M. A., Dashkevich, N. M., Ataullakhanov, F. I. Hemostasis and thrombosis beyond biochemistry: roles of geometry, flow and diffusion. *Thrombosis Research*. **136** (4), 699–711 (2015).
4. Podoplelova N. A. et al. Hysteresis-like binding of coagulation factors X/Xa to procoagulant activated platelets and phospholipids results from multistep association and membrane-dependent multimerization. *Biochimica et Biophysica Acta.* **1858** (6), 1216–1227 (2016).
5. Panteleev, M. A., Ananyeva, N. M., Greco, N. J., Ataullakhanov, F. I., Saenko, E. L. Two subpopulations of thrombin-activated platelets differ in their binding of the components of the intrinsic factor X-activating complex. *Journal of Thrombosis and Haemostasis*. **3** (11), 2545–2553 (2005).
6. Kotova, Y. et al. Binding of coagulation factor XIII zymogen to activated platelet subpopulations: roles of integrin αIIbβ3 and fibrinogen. *Thrombosis and Haemostasis*. **119** (6), 906–915 (2019).
7. Fay, S. P., Posner, R. G., Swann, W. N., Sklar, L. A. Real-time analysis of the assembly of

ligand, receptor, and G protein by quantitative fluorescence flow cytometry. *Biochemistry*. **30** (20), 5066–5075 (2002).
8. Nolan, J. P., Shen, B., Park, M. S., Sklar, L. A. Kinetic analysis of human flap endonuclease-1 by flow cytometry. *Biochemistry*. **35** (36), 11668–11676 (1996).
9. Sarvazyan, N. A., Lim, W. K., Neubig, R. R. Fluorescence analysis of receptor−G protein interactions in cell membranes. *Biochemistry*. **41** (42), 12858–12867 (2002).
10. Sarvazyan, N. A., Neubig, R. R. Analysis of molecular assemblies by flow cytometry: determinants of Gi1 and by binding. *Advances in Optical Biophysics*. **3256**, 122–131 (1998).
11. De Franceschi, N. et al. ProLIF – Quantitative integrin protein–protein interactions and synergistic membrane effects on proteoliposomes. *Journal of Cell Science*. **132** (4), jcs214270 (2018).

Figure

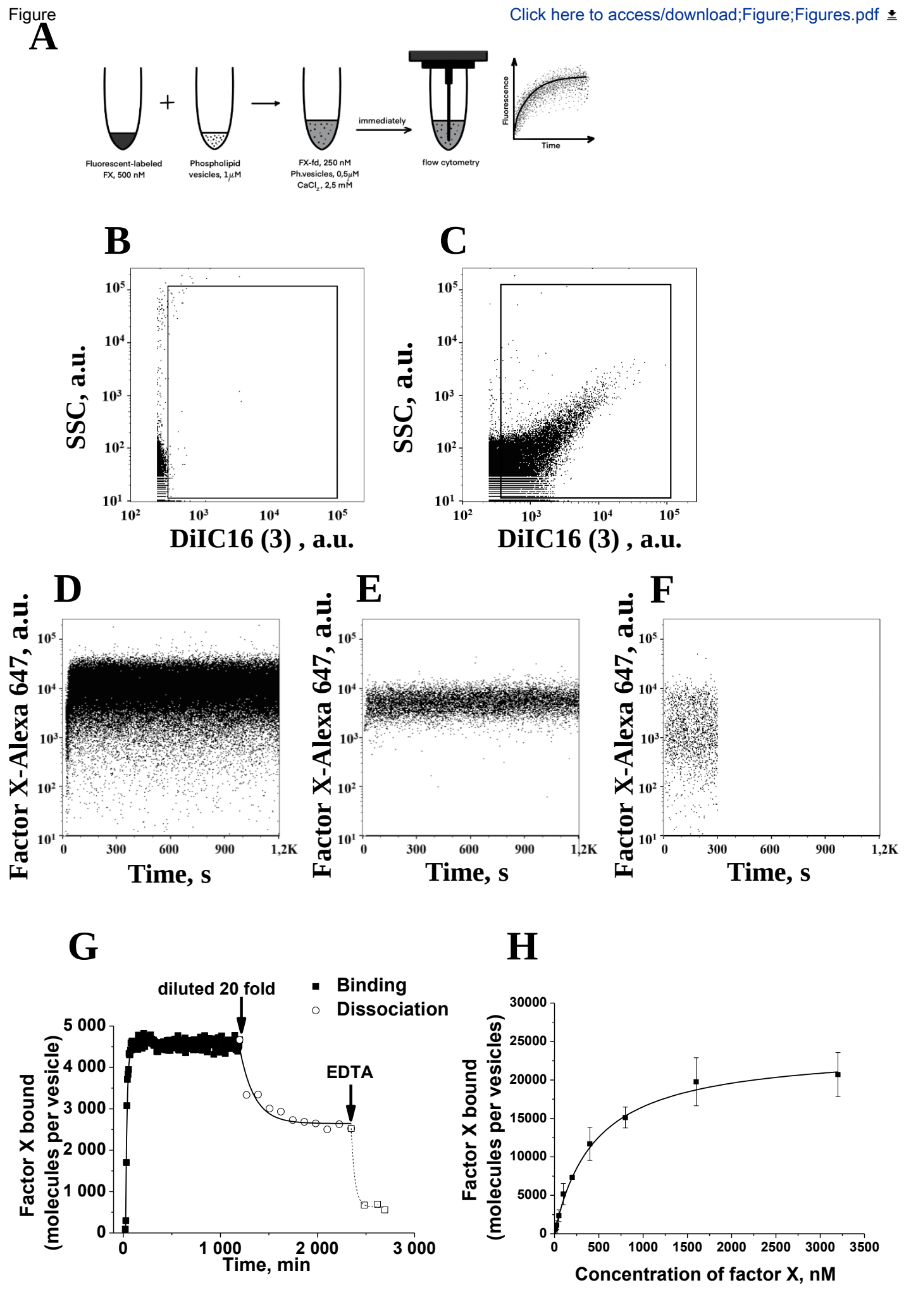

Table 1

Apparent K_d ± SEM (nM)	k_{on} ± SEM ($\mu M^{-1}s^{-1}$)	K_{off} ± SEM (s^{-1})	Apparent number of binding sites per vesicle ± SEM
400 ± 80	0.371 ± 0.012	0.019 ± 0.004	8,000 ± 800

Editor:

Thank you for your evaluation of the manuscript and your overall favourable opinion. Our responses to specific comments are provided below, preseded by your original comments in bold.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Thank you for your reading of the manuscript. We double-checked the manuscript and corrected the grammatical errors. All essential modifications are marked with green background in the revised manuscript.

2. Please use a professional copyediting service to improve your manuscript since several sentences have grammatical and typographical errors and the meaning conveyed is not clear to the reader.
Thank you. We carefully corrected the text and hope that the readability of the revised version is significantly improved.

3. Please revise your protocol to avoid overlap with published content. See the attached similarity report for your reference.
Thank you. This was corrected in the revision

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Thank you. This was fixed

5. Please remove citations within the Abstract.
Thank you. This was corrected in the revision

6. Please revise the Introduction to include all the following:
a) The advantages over alternative techniques with applicable references to previous studies
b) A description of the context of the technique in the wider body of literature
c) Information to help readers to determine whether the method is appropriate for their application

Thank you! Following your suggestion, we included such comments in the Introduction. The added text is marked with green background in the revised manuscript.

7. For in-text formatting, corresponding reference numbers should appear as numbered superscripts without brackets after the appropriate statement(s), but before the punctuation.
Thank you. This was fixed

8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: AlexaFluor488, AlexaFluor647, Sephadex G25, Avanti Polar Lipids, Vacuette tubes, BD FACSCantoII (BD Bioscience), FlowJo, FACSDiva (BD Bioscience), etc.)
Thank you. This was fixed

9. Please use SI units and abbreviate all units: L, mL, µL, cm, kg, etc. ml should be mL. Use h for hours, min for minutes and s for seconds. Please include a single space between the numeral and the unit, e.g., 24 h, 3 days, 20 mol% etc.
Thank you. This was corrected in the revision

10. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.
Thank you. This was fixed.

11. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.
Thank you. This was corrected in the revision

12. Please use a single line spacing between steps and sub steps of the protocol and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please do not highlight calculations, derivations or math equations. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
Thank you. This was fixed. The essential steps of the protocol for the video Was highlight up yellow

13. Figure 1C-E: please give units for the axes.
Thank you. This was corrected in the revision

14. Figure 1G: please define error bars in the legend
Thank you. This was corrected in the revision

15. Please submit the figure as a vector image file (.pdf, .psd, ai, .eps.).
Thank you! Figure will be submitted to .pdf

16. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Thank you for suggesting it. We included these details in Discussion. The added text was highlighted in green

17. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 5 authors, list only the first author followed by et al. Please do not abbreviate journal names.
Thank you. This was fixed

Reviewer #1:

Manuscript Summary:

In this protocol, the authors explained the procedure of fluorescently labeling proteins and applying flow cytometry to characterize their binding with lipid membranes. It can be potentially a useful virtual method for membrane studies. However, the language of the manuscript has to be further improved and there are also some overstatement need to be revised.

Thank you for your evaluation of the manuscript and your overall favourable opinion. Our responses to the specific comments are provided below, preseded by your original commends in bold.

Major Concerns:

1. The title is too broad which can not really be covered in this brief method paper. It can be just "a fluorescent-labeled protein-based flow cytometry method". Meanwhile, the method is not really studying "protein-lipid interactions", it is more like protein interactions (or actually attachment or binding) with lipid membranes. It is not studying a specific type of lipid, and also cell membrane composition contains way more than just lipids.

Thank you for your suggestion! We try change the title to be more specific.

Analysis of the interaction fluorescent-labeled proteins to artificial phospholipid microvesicles by Quantitative Flow Cytometry

2. I understand that the novelty of the method is not that important for JoVE, however, the authors should have much better citations in explaining many previous studies on using very similar techniques of fluorescently labeled proteins and flow cytometry for studying membrane binding. The current references are really limited only in the thrombin field which I think the authors may have some expertise on. However, since the title and scope of this method is beyond thrombin and blood cells, the current list of references should definitely be revised.

Thank you for your offer. We have added text to the Introduction and Discussion to highlight the situation beyond blood clotting. The list of references has also been expanded.

3. The language used in the manuscript should be very carefully revised.

Thank you. We carefully corrected the text and hope that the readability of the revised version is significantly improved.

Minor Concerns:

1. "All interaction of living cells with the environment happens through membranes" is an overstatement and not correct.
2. "pH = 8.5M", "4C" (rather than 4oC), "antybodies", "100 g" (rather than 100x g) places should use subscript: "A280", "Amax", equations 2,3,4, "MgCl2" "NaH2PO4", etc. "Annalise", "[XB]" vs. "[Xbeta]"
3. "In further only events that fall inside this gate were analyzed" "the conversion factor of the mean fluorescence."
4. the reference is missing for "artificial phospholipid vesicles were prepared with a protocol recommended"

Thank you. This was corrected in the revision

Reviewer #2:

Manuscript Summary:

The manuscript by Podoplelova et al. describe a method to determine the soluble protein-lipid membrane interaction. The technique makes use of a protein modified with fluorophore (could also be a genetic fusion) and flow cytometry to monitor protein-lipid membrane interactions. The method is interesting and will be of interested to those studying peripheral membrane proteins and their interactions with membranes.

Thank you for your in-depth analysis of our submission and valuable comments. To address them, we revised the text of the manuscript. All essential modifications are marked with green background in the revised manuscript and are discussed in detail below. For convenience, we present your original comments in bold followed by our responses point-by-point.

Major Concerns:

The discussion of how kinetics of protein binding is a too vague. A bit more detail on the actual process would be useful to the general reader. For example, the protocol section could be written to include steps with more detailed information. A schematic highlighting each step that shows the workflow would help readers understand this method.

Thank you for suggesting to include the scheme. Following your suggestion, we include an additional panel to figure 1A

The preparation of protein samples would benefit from the inclusion of more details.

Thank you! We have significantly redacted the Protocol section to improve readability.

Provide more details on how beads can be used to count binding sites on protein. Add a section that explain the methodology in here or in the introduction.

Thank you. Following your suggestion, we include additional information in section 6.4 of Protocol.