Chapter 16

Single Particle Tracking Assay to Study Coronavirus Membrane Fusion

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Abstract

Single particle tracking (SPT) of individual virion fusion with host cell membranes using total internal reflection microscopy (TIRFM) is a powerful technique for quantitatively characterizing virus–host interactions. One significant limitation of this assay to its wider use across many types of enveloped viruses, such as coronavirus, has been incorporating non-lipid receptors (proteins) into the supported lipid bilayers (SLBs) used to monitor membrane fusion. Here, we describe a method for incorporating a proteinaceous viral receptor, feline aminopeptidase N (fAPN), into SLBs using cell blebbing of mammalian cells expressing fAPN in the plasma membrane. This receptor binds feline coronavirus (FECV 1683). We describe how to carry out single particle tracking of FECV fusion in this SLB platform to obtain fusion kinetics.

Key words Cell bleb, Single particle virus fusion, Single particle tracking, Supported lipid bilayers, Microfluidics, Fusion kinetics

1 Introduction

A versatile approach for quantitatively studying virus–host cell interactions and viral entry kinetics is single particle imaging using total internal reflection fluorescence microscopy (TIRFM) [1–3] combined with microfluidic handling and fluid supported lipid bilayers (SLBs). There are a number of advantages of using this platform. First, the TIRFM imaging approach allows one to obtain unique single particle data that can be used to discriminate individuals within populations and to identify intermediate mechanistic steps of the entry process that are often masked by ensemble approaches. Second, microfluidic handling enables unique control over the temporal sequence of fusion triggers such as viral binding, exposure to proteases, and pH drop. Third, the supported lipid bilayer that coats the walls of microfluidic channels and acts as a host membrane mimic [4, 5] preserves lipid mobility in the bilayer while its planar geometry removes many experimental complications imposed by live cells. These features facilitate the study of

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virus–cell interactions and the membrane fusion processes required for viral infection. This convenient platform enables quantitative data collection used for statistical analysis of stochastic virus fusion events and the measurement of membrane fusion kinetics.

One of the biggest drawbacks of the SLB platform for single virion studies of virus entry is the limited range of viruses that are compatible with it. This limitation is imposed by the complexity of the receptor that can be incorporated into the SLB. As such, these platforms have been limited to the study of a few viruses, such as influenza virus [2, 3, 6], Sindbis virus [3], and vesicular stomatitis virus (VSV) [7], that are all known to interact with specific lipids that are easily incorporated into SLBs. However, the receptors for many enveloped viruses, including those in the *Coronaviridae*, are membrane proteins. In this chapter, we describe a method to incorporate membrane proteins into supported planar bilayers for the study of coronavirus fusion using single particle tracking by TIRFM. Here, we focus on one of the best-characterized receptors used by many coronaviruses in the alphacoronavirus genus [8–10], aminopeptidase N (APN).

To provide some perspective, we summarize standard procedures for creating proteoliposomes that can be used to form supported bilayers. Proteins are typically incorporated into vesicles using detergent to solubilize the membrane protein of interest, which is then reconstituted into a vesicle called a proteoliposome [11]. When membrane proteins are solubilized, they are extracted from their native lipid environment, which can expose the hydrophobic transmembrane domains to an aqueous environment. To minimize these energetically unfavorable interactions, the proteins may refold and lead to the incorporation of misfolded proteins into proteoliposomes. The reconstitution process can also lead to randomly oriented proteins in the bilayers. These non-native changes have major implications for pathogenesis: protein conformation in the membrane and its glycosylation are critical to controlling the host–pathogen interaction. To overcome these limitations, we have developed a method of embedding functional, enzymatically active membrane proteins in supported bilayers by using chemically induced cell blebbing [12–15] to create proteoliposomes composed of plasma membrane constituents [16, 17].

Chemical induction of cell blebs results in the productions of proteoliposomes that have never been subjected to detergent solubilization and are ideal for use in biomimetic systems to study virus–host interactions. Cells are first transfected with receptor proteins specific for coronaviruses (or any desired protein), grown to confluency, and then chemically induced to form blebs. To form the planar bilayers, the cell blebs are first adsorbed to a glass surface, and then incubated with liposomes devoid of proteins, but closely matching the lipid composition of the host cell. The rupturing of the liposomes in spaces in between adsorbed blebs induces the

rupture of the cell blebs on the surface to form a single planar bilayer [16]. We showed previously [17] that APN in the SLB made from blebs is enzymatically active, oriented properly, and competent to bind CoV prior to membrane fusion. Note that the blebbing procedure applies to many cell types and we have successfully expressed other proteins and incorporated them into SLBs using this approach, including DPP4, the receptor for MERS-CoV.

2 Materials

2.1 Cell Culture, Cell Blebbing, and Proteoliposome Preparation

1. Giant plasma membrane vesicle (GPMV) buffer: 150 mM NaCl, 10 mM HEPE, 2 mM calcium chloride. Adjust pH to 7.4 with hydrochloric acid (HCl).
2. Blebbing buffer: GPMV buffer, 2 mM dithiothreitol (DTT), 25 mM formaldehyde.
3. Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4{\cdot}2H_2O$, 1.8 mM KH_2PO_4. Adjust to desired pH with HCl.
4. Biotechnology grade chloroform and methanol.
5. 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (POPC).
6. 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphoethanolamine (POPE).
7. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC).
8. Cholesterol
9. Sphingomyelin.
10. Oregon Green DHPE.
11. Baby hamster kidney (BHK-21) cells.
12. pCAGGS-fAPN (Feline aminopeptidase N plasmid, generous donation from Prof. Kathryn Holmes of University of Colorado).
13. Sonicating water bath.
14. Zetasizer NanoZ (Malvern).

2.2 Microfluidic Setup, Virus Labeling, and SPT Assay

1. Glass coverslips (25 mm × 25 mm; No. 1.5).
2. Hydrogen peroxide (50 % solution).
3. Sulfuric acid.
4. Polydimethylsiloxane (PDMS).
5. Scotch tape.
6. Tygon Microbore tubing (outer diameter: 0.06″, inner diameter: 0.02″).
7. Tube connector (outer diameter: 0.025″, inner diameter: 0.013″, 0.300″ long).

8. 1 ml hypodermic syringes with flat ends.
9. 23 Gauge luer stubs.
10. Syringe pump.
11. Feline Enteric Corona Virus (FECV) 1683.
12. L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) Trypsin.
13. Lipophilic fluorophores such as octadecyl rhodamine B chloride (R18), 1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), or octadecyl-rhodamine (Rh110C18) (*see* **Note 1**).
14. Sulforhodamine B (SRB) powder.
15. Lipex extruder, 10 ml (Northern Lipids Inc).
16. 50 and 100 nm polycarbonate filters.
17. Ceramic boat.
18. Plasma cleaner.
19. Inverted Zeiss Axio Observer.Z1 with an α Plan-Apochromat 100× oil objective with a numerical aperture (NA) of 1.46. This microscope is equipped with a Laser TIRF 3 slider (Carl Zeiss, Inc.) and two-channel dual-view imaging system (DV2, Photometrics).
20. Semrock 74 HE GFP/mRFP filter cube.
21. Electron multiplying CCD camera (Hamamatsu ImagEM C9100-13, Bridgewater, NJ).
22. Index-matching liquid (Carl Zeiss, Inc.).
23. Image processing software such as Axiovision and Image J.
24. Data analysis software such as Matlab.

3 Methods

3.1 Cell Culture and Cell Blebbing

1. Cell seeding: Thaw BHK-21 mammalian cells and grow to confluency. Passage and seed cells at a density of 1.5 cells/ml in a 10 cm dish for 24 h at 37 °C and 5 % CO_2 (*see* **Note 2**).
2. Transfection: Following standard transfection protocols, transfect cells with 6 μg of PCAGGS-fAPN and incubate for at least 18 h at 37 °C and 5 % CO_2.
3. Blebbing: Wash cells with 6 ml of GPMV buffer (per 10 cm dish). Add 4 ml of blebbing buffer to each dish. Place dishes rocking gently at 37 °C for 1 h or at room temperature for 2 h (*see* **Note 3**).
4. Place dishes on an inverted microscope and using bright field, look for blebs floating in solution at 100× magnification. Blebs appear as small floating dark spheres.

5. To ensure maximum bleb yield, tap the sides of the dishes gently to help any undetached blebs release into the supernatant. Gently collect the supernatant in a test tube. Place test tube on ice for 15 min to allow any detached cells to the settle to the bottom. Remove all but last 500 μl from the tube and add to a new tube.
6. Dialyze the blebs against two 1-l volumes of GPMV buffer for 24 h to remove DTT and HCHO.

3.2 BHK Fluid (BHKF) Liposome Preparation

1. To form liposomes, mix the appropriate amounts of each POPC–POPE–sphingomyelin–cholesterol–Oregon Green DHPE in the ratio 37.3:34.2:5.7:22.8:0.1. This ratio is chosen to match as close as possible to the lipid content of the BHK cell. Dissolve all components except sphingomyelin in biotechnology grade chloroform before mixing in a scintillation vial. Dissolve sphingomyelin in a 4:1 mixture of chloroform–methanol.
2. Remove the bulk solvent from the vial under a stream of high purity nitrogen gas and then place in a desiccator under vacuum overnight to ensure complete evaporation of all solvent. Protect the lipids and fluorophores from the degradation by room lighting, by wrapping the dessicator in aluminum foil or a dark cloth.
3. Add phosphate-buffered saline at pH 7.4 to the dried lipid film and gently resuspend in a sonicating bath for twenty minutes on the lowest setting. The final lipid concentration should be approximately 2 mg/ml.
4. Extrude the liposomes five times through a 100 nm polycarbonate filter and then twice through a 50 nm polycarbonate filter. The average liposome diameter is typically 100 nm as determined by dynamic light scattering (Zetasizer Nano Z, Malvern).

3.3 Virus Labeling

1. Internal virus labeling: Working in a biosafety hood thaw a vial of coronavirus on ice. Add 10 μl of 20 mM SRB dye to 20 μl of coronavirus. Allow the virus/SRB mixture to incubate for 16–20 h (*see* **Note 4**).
2. Dilute the SRB-labeled coronavirus with 250 μl of GPMV buffer in a microcentrifuge tube. Vortex gently for 30 s to mix. Add 2 μl of TPCK-trypsin (2.5 μg/ml) to the virus/buffer mixture and vortex again for 30 s. Place the tube in a water bath or heat block at 37 °C for 15 min.
3. Viral membrane labeling: Remove trypsin treated virus from the water bath. Add 3 μl of lipophilic fluorophore, e.g., R110C18, to virus–buffer mix and place in a sonicating bath for 1.5 h. Filter out unincorporated dye by centrifuging using a G-25 spin column for 2 min at a speed of $3 \times 1{,}000 \times g$ (*see* **Note 5**).

3.4 Microfluidic Device Setup

1. Place glass coverslips in slots in a ceramic boat and then put the boat into a glass beaker. Working in a chemical safety hood and wearing appropriate personal protection equipment, measure 43 ml of hydrogen peroxide (50 wt%) in a 1 l-graduated cylinder and pour into the beaker, completely covering the slides. In the same graduated cylinder measure 100 ml of sulfuric acid and then add to the beaker. This mixture is typically called "piranha solution," and the reaction proceeds vigorously for the first few minutes.
2. Allow the reaction to proceed for 10 min. Then very carefully add about 50 ml of deionized water. There will be some bubbling from the beaker as the reaction is quenched.
3. Once the vigorous bubbling has ceased, using a Teflon mitt, pick up the beaker and very carefully decant some of the liquid from the beaker into a chemical waste bottle specifically for piranha waste. Only decant enough liquid so that the glass slides are always covered in liquid.
4. Repeat this rinsing step until approximately 500 ml of deionized water has been used and then transfer the beaker to a sink and rinse with a constant stream of deionized water for ~15 min.
5. Microfluidic devices are formed using polydimethysiloxane (PDMS) in a molding process. In a clean plastic container weigh out the elastomer and cross-linker in a 10:1 ratio (i.e., to make 22 g of PDMS use 20 g of elastomer and 2 g of cross-linker). Using a spatula, mix thoroughly, but be careful not to scratch any plastic from the container into the mixture.
6. After thorough mixing, the sample will be very aerated and the bubbles must be removed by degassing prior to complete cross-linking. Place the plastic container in a clean dessicator and place under vacuum. The PDMS mixture will rapidly climb the sidewalls of the container as the gas escapes. Periodically shutting the dessicator off from the vacuum and allowing slow pressure equalization will prevent the mixture from rising above the container sides. When all the larger gas bubbles are removed, leave the container under vacuum until the mixture is completely degassed.
7. Pour the PDMS mixture gently over the silicon wafer containing the microfluidic channel patterns (*see* **Note 6**). The layer of PDMS poured over the silicon wafer should be no more than 0.5 in. thick.
8. Bake for 3 h at 80 °C.
9. After sufficient cooling has taken place, cut the devices out of the mold and punch holes in the inlet and outlet of the channels using a 23 gauge luer stub.
10. Wash the device with water and then ethanol, especially the inlet and outlet ports to remove any obstructions created during the hole-punching process.

11. Dry the device completely and clean with scotch tape to remove any dust/particulates.
12. Place a dry piranha cleaned slide and PDMS microfluidic device into the chamber of the plasma cleaner. Use oxygen plasma on the highest setting at 600 μm pressure for ~25 s.
13. Following treatment, equalize the pressure in the chamber, then once opened very quickly place the device, channel side down, on top of the glass slide, as close to the center of the glass slide as possible (i.e., far away from the glass edges). Using a pair of tweezers, gently push down on the device to ensure complete contact with the glass.
14. Bake the device at 80 °C for 10 min. This process should result in the glass slide being irreversibly bound to the PDMS mold. If it does not, the device was not clean enough and the above steps should be repeated.
15. Allow the device to cool for 5 min after baking.
16. Cut 2 lengths of Tygon tubing for each channel in the device: 1×23 cm (inlet) and 1×30 cm (outlet).
17. Using a pair of pliers or tweezers insert metal tube connectors into one end of the inlet and outlet tube. Insert the metal end of each tube into the inlet and outlet ports in the assembled microfluidic device. Take care not to apply too much force, which may crack the glass coverslip. Do not insert the tube so that it is touching the bottom of the channel so that the liquid may flow freely into the channels.
18. Place the other end of the inlet tube in a vial containing filtered GPMV buffer and attach a syringe/luer stub to the end of the outlet tube.

3.5 Microscopy and SPT Assay

1. Membrane fusion assays are conducted with total internal reflection fluorescence (TIRF) microscopy using an inverted Zeiss Axio Observer.Z1 with an α Plan-Apochromat 100× oil objective with a numerical aperture (NA) of 1.46. This microscope is equipped with a Laser TIRF 3 slider and two-channel dual-view imaging system to split the image for simultaneous imaging of two emission signals on one CCD chip. In this setup, two lasers are used simultaneously to excite different color fluorophores. Here 561 and 488 nm excitation wavelengths from solid-state lasers are used excite red and green fluorophores, respectively. Excitation laser light is band-pass filtered through a Semrock 74 HE GFP/mRFP filter cube, and then combined with a dichroic mirror before being focused on the outer edge of the back aperture of the objective. The fluorescence emission signal is filtered through a 525/31 and 616/57 nm dual band-pass emission filter and then sent to an electron multiplying CCD camera.

2. Couple the glass coverslip of the microfluidic device to the 100×-objective for TIRF imaging using index-matching liquid.
3. Place device on 10× objective of microscope (or another low magnification).
4. Attach syringes to a syringe pump. Flow buffer through the channels at a flow rate of 100 µl/min for 2 min to set the walls of the device and clear any debris. Wait for 5 min, then transfer the inlet tubes into a vial containing BHK21-fAPN blebs.
5. Visually inspect the tubing inlet to ensure no bubbles or plugs of air have formed. If bubbles do form, run the syringe pump in reverse at 50 µl/min until the air has been pushed back into the vial of buffer and then transfer tubing to the vial containing the cell blebs.
6. Flow blebs at dilution of 1:4 (blebs–GPMV) buffer (*see* **Note 7**) into the channel at a flow rate of 30 µl/min for 5 min.
7. Allow the blebs to incubate on the glass for at least 30 min.
8. Rinse the microchannel with GPMV buffer for 2 min at 100 µl/min.
9. Flow a 0.5 mg/ml solution of BHKF liposomes into the channel for 2 min at 100 µl/min.
10. Incubate the liposomes in the channel with the cell blebs for at least 1 h. Repeat the aforementioned rinsing step with GPMV buffer.
11. Inspect the bilayer under 100× magnification. If the bilayer appears patchy, further incubation with BHKF liposomes may be required (*see* **Note 8**).
12. Dilute 250 µl of fluorescently labeled virus in 800 µl of PBS buffer at pH 7.4.
13. Flow the diluted virus solution into the microfluidic channel at 30 µl/min for 5 min. Allow the virus to bind to the membrane for at least 20 min.
14. Rinse excess virus from the channel with buffer at a flow rate of 100 µl/min for 2 min.
15. Locate a region in the channel that has a uniform bilayer and a high density of bound virions in the green channel (*see* **Note 9**). Switch the camera view to dual-view mode and turn on the red laser. In this setting, virions that have taken up both SRB and R110C18 should be visible in the green and red channels. Ensure that the focus is maintained in both channels.
16. Switch off the red and green lasers once an appropriate location has been found to avoid unnecessary photobleaching. Carefully switch the inlet tubing for the channel from neutral PBS buffer into acidic PBS buffer (<pH 5.5).

17. Flow acidic buffer into the channel at 100 μl/min for 2 min and set up recording software to run for 4–5 min. Set the camera exposure to a maximum of 100 ms. A drop in background intensity in the green channel indicates that the acidic buffer has reached the channel. Hemifusion follows and is marked by a sharp increase in fluorescence of the punctate fluorescent virions and then diffusion of the green fluorophores into the supported bilayer, radiating away from the punctate dot. A sharp drop in intensity in the red channel marks pore formation in the same particle.

3.6 Imaging Processing and Data Analysis

1. Import the image sequence into an image processing software such as Image J.
2. Determine the approximate time at which acidification occurs by tracking Oregon Green intensity.
3. Create a substack video from the original file. The time at which the Oregon Green intensity drops becomes the first frame of the video substack (time zero). For experiments where only hemifusion is being monitored, track the intensity of each particle that fused over the course of the entire video. For experiments monitoring both hemifusion and pore formation identify and track the intensity only of virions that undergo both processes.
4. To analyze data and obtain fusion kinetics, import this data into data analysis software such as Matlab to determine the time at which the maximum intensity (hemifusion) or intensity drop (pore formation) occurs for each fusing particle. These data can then be analyzed in various ways to determine kinetics parameters associated with the fusion process. One commonly used method plots the data as cumulative distributions and fits with a gamma distribution [6]. In this approach, the number of steps in the kinetic process can be resolved and the rate constant for the limiting step. For pH sensitive fusion, these values can vary with the pH of buffer used to initiate fusion.

4 Notes

1. Hemifusion experiments can be carried out using commercially available lipophilic fluorophores such as octadecyl Rhodamine B chloride (R18) or 1′-Dioctadecyl-3,3,3′, 3′-Tetramethylindocarbocyanine Perchlorate (DiI) from Invitrogen. Synthesis of R110C18 is described in Floyd et al. [2] and Costello et al. [6].
2. Cell Culture. Cell blebbing is not restricted to BHK-21 cells; a variety of cell lines can be used.

3. Cell blebbing. There are a number of ways in which the cells can be incubated during blebbing. Agitation is not strictly required and blebs can be produced at 37 °C if the incubation time is increased to 2 h. Another very effective method is to place the plates in a thin layer of water shaker/heating bath at 37 °C and incubate for 1 h.
4. Timing of experiment. Microfluidic devices and BHKF liposomes may be prepared days or weeks in advance of the experiment so long as they are stored appropriately. Piranha cleaned glass coverslips should not be used more than 24 h after cleaning. For dual labeling experiments with the coronavirus should be incubated with SRB at least 16 h before co labeling with a lipophilic fluorophore such as R110C18. Once the virus has been labeled, the fluorescent probes will only stay stably associated with the virus for several hours.
5. Optimization of virus labeling. When a new batch of dye is synthesized or new bath of virus is obtained it can be necessary to re optimize the amount of R110C18 required to achieve sufficient dequenching at a single particle level. Typically to optimize the dye concentration, bulk dequenching events should be carried out in a fluorimeter. Incubate the virus with varying amounts of dye and filter. Add the labeled virus to a cuvette and take a baseline reading at the appropriate wavelength for the dye being used. To check if the virus is quenched, add 100 μl of a 10 % solution of Triton-X detergent. A significant and rapid increase in fluorescence indicates that the virus was quenched before solubilization of the membrane by the detergent. Once the bulk quenching concentration has been obtained the optimal quenching conditions in the microfluidic should be close to that obtained in the fluorimeter. Further optimization may be required as a result of photobleaching.
6. Microfluidic master. The microfluidic master silicon wafer used for these experiments was designed in the Cornell Nanoscale Facility (CNF). Each channel was designed to be 135 μm wide and 70 μm deep.
7. Bleb concentration. The ratio of blebs to GMPV buffer recommended in this chapter can be subject to change. Depending on the yield of blebs this ratio may need to be changed in order to optimize adsorption in the microfluidic device. It should be noted that completely saturating the channel with blebs will result in an immobile bilayer upon incubation with BHKF liposomes, presumably because there is no space left for the liposomes to fuse between adsorbed blebs.
8. BHKF incubation. The concentration of BHKF liposomes for bleb incubation is recommended to be 0.5 mg/ml; however, increasing the concentration may help the bilayer form faster.

Depending on the concentration of blebs adsorbed it may also be necessary to increase the BHKF incubation time from 1 h to 2 or 3 h.

9. Laser power. Photobleaching can greatly impair visualization of bound dual-label virions. The lowest laser power possible should be determined, typically by trial and error, so that photobleaching can be avoided but the intensity of the bound virions is above background noise in both red and green channel. The Zeiss microscope used in our experiments, the laser power is modulated to 20 % of total intensity available from the lasers. Within this modulation, the laser power was typically further reduced to between 5 and 10 % in both the red and green channels. These values may vary depending on the microscope set up and software used.

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