Video Article

Molecular Diffusion in Plasma Membranes of Primary Lymphocytes Measured by Fluorescence Correlation Spectroscopy

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Abstract

Fluorescence correlation spectroscopy (FCS) is a powerful technique for studying the diffusion of molecules within biological membranes with high spatial and temporal resolution. FCS can quantify the molecular concentration and diffusion coefficient of fluorescently labeled molecules in the cell membrane. This technique has the ability to explore the molecular diffusion characteristics of molecules in the plasma membrane of immune cells in steady state (*i.e.*, without processes affecting the result during the actual measurement time). FCS is suitable for studying the diffusion of proteins that are expressed at levels typical for most endogenous proteins. Here, a straightforward and robust method to determine the diffusion rate of cell membrane proteins on primary lymphocytes is demonstrated. An effective way to perform measurements on antibody-stained live cells and commonly occurring observations after acquisition are described. The recent advancements in the development of photo-stable fluorescent dyes can be utilized by conjugating the antibodies of interest to appropriate dyes that do not bleach extensively during the measurements. Additionally, this allows for the detection of slowly diffusing entities, which is a common feature of proteins expressed in cell membranes. The analysis procedure to extract molecular concentration and diffusion parameters from the generated autocorrelation curves is highlighted. In summary, a basic protocol for FCS measurements is provided; it can be followed by immunologists with an understanding of confocal microscopy but with no other previous experience of techniques for measuring dynamic parameters, such as molecular diffusion rates.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54756/>

Introduction

Many immune cells functions rely on molecular diffusion and interactions within membranes. Biological membranes are complex, and many factors that may be important for the function of immune cells can influence the speed of translational diffusion of proteins within cellular membranes¹. We recently showed that natural killer (NK) cells, lymphocytes belonging to the innate immune system, exhibit differential diffusion of two studied proteins at the cell membrane depending on the state of NK cell activation².

Fluorescence correlation spectroscopy (FCS) is a technique that is capable of quantifying molecular diffusion rates within biological membranes. It reports the average diffusion rate of fluorescently labeled molecules in a fixed volume, typically the focus of a confocal microscope. It is based on the measurement of the fluctuations in fluorescence that occur upon molecular movement in a system in steady state. FCS has been widely used for studying the diffusion of fluorescent dyes and proteins, both in solution and within lipid membranes. Other output parameters affecting the diffusion rate can also be indirectly studied in this fashion (*e.g.*, conformational changes of proteins or interactions of molecules on cell membranes)^{3,4}. FCS stands out compared to other techniques due to its high sensitivity, allowing the possibility for single-molecule detection. It works well for molecular concentrations in the nanomolar to millimolar range, which is typical for endogenous expression levels of most proteins⁵. Furthermore, FCS can give an approximation of the absolute number of proteins within the studied volume, while most other techniques only give relative information about protein expression levels. Other methods to measure molecular diffusion rates within membranes include fluorescence recovery after photobleaching (FRAP), single particle tracking (SPT), multiple pinhole FCS, and image correlation methods. FRAP and image correlation methods are ensemble techniques, which generally do not give information about the absolute number of molecules¹⁰. Compared to SPT, the throughput of FCS is higher in regard to characterizing the population average. The analysis is also less demanding since the average diffusion rate of the molecules present within the laser focus is measured, rather than the rate of single molecules. Also, unless specialized microscopes are available¹¹, SPT cannot give any information about concentrations, since standard SPT labeling must be very low to allow for the identification of single molecules. On the other hand, FCS requires the molecules under study to be mobile. It will simply not detect any putative immobile fractions or molecules moving very slowly. The diffusion rate of molecules that reside within the focus longer than approximately one tenth of the acquisition time will not be correctly represented in FCS measurements^{3,12}. Therefore, diffusion coefficients recorded by FCS tend to be faster than diffusion rates reported from techniques like FRAP and SPT, where the close-to-immobile and very slow fractions are taken into account as well. SPT will also give a more detailed description of the variability of diffusion rates within the molecular population than FCS will.

FCS quantifies the fluctuation of fluorescence intensity over time within the excited volume. In the case of membrane measurements, this translates to the illuminated area of the membrane. In this paper, we utilize the fact that such fluctuations are induced by molecules exhibiting Brownian diffusion and are thus moving in and out of the excitation volume. There are also several other possible sources for the fluctuations in the fluorescence signal, such as blinking or the presence of a triplet state in the fluorophores, environmental effects, binding-unbinding of the ligand, or movement of the entire cell membrane. These putative error sources need to be taken into consideration when designing an FCS experiment in order to accurately interpret the results^{12,13}. Typically, lateral diffusion rates in biological membranes are low due to crowding and interactions, both between membrane proteins and between proteins and the cytoskeleton. Historically, the use of FCS in membranes has thus been hampered by the lack of photo-stable fluorophores, which are required to avoid bleaching during the extended transit times through the excitation focus¹⁴. However, today, there are plenty of options for suitable photo-stable dyes. Significant improvements in detectors and other hardware also allow the detection of fluorescent proteins and dyes of lower brightness. Here, a basic protocol for the application of FCS using murine primary lymphocytes, where the protein of interest is labeled with a fluorescently tagged antibody, is described. An approach to fit the autocorrelation curves in order to extract the diffusion coefficient and the molecular density is also shown. The protocol aims at being easily followed by immunologists with no previous experience of techniques to study the diffusion of molecules. However, a basic understanding of confocal microscopy is expected (to gain this basic understanding, see reference¹⁵). This protocol can relatively easily be adapted to other suspension cells, both cell lines and primary cells. For more experienced FCS users, more refined analysis methods exist, some of which are described in the discussion.

Protocol

1. Staining for FCS

1. Isolate murine NK cells from spleen lymphocytes using magnetic bead labelling, as per the manufacturer's protocol¹⁶. Use $2-3 \times 10^5$ cells per sample for the following steps.
NOTE: Use negative selection to enrich the target cell population, leaving it untouched, so that the cells can be labelled using primary antibodies alone. See reference² for more detailed information on cell isolation from mice².
2. For murine cells expressing Fc receptors, block the Fc receptors with the antibody clone 2.4G2 at $5 \mu\text{g}/\mu\text{l}$ in $25 \mu\text{l}$ of phosphate-buffered saline (PBS) and 1% fetal bovine serum (FBS) per sample. Incubate for 15 min at room temperature.
3. **Preparation of antibody mixture.**
 1. Use antibodies directly conjugated to a photo-stable fluorophore. If two antibodies against two different proteins are used, use fluorophores that have well-separated emission spectra to minimize the cross-talk (e.g., excited by the 488 and 633 lasers², later in the protocol referred to as the "green" and "red" lasers and fluorophores, respectively).
NOTE: If commercial antibodies labeled with photo-stable fluorophores are not available for the selected biomarkers, conjugate unlabeled antibodies to the fluorophores according to the manufacturer's instructions. See **Materials Table** for the antibodies used in this study.
 2. Prepare an antibody mixture by diluting fluorescently labeled antibodies against the protein or proteins of interest in PBS + 1% FBS. Add the antibody mix to each sample to a final volume of $50 \mu\text{l}$ per sample. Incubate on ice for 45 min in the dark.
NOTE: Optimize the concentration of the antibodies in advance to ensure that the antibody is used at a saturating concentration, typically $1-10 \mu\text{g}/\text{ml}$.
4. After incubation, wash the cells by adding $250 \mu\text{l}$ of PBS and centrifuge them at $150 \times g$ for 3 min. Discard the supernatant.
5. Repeat step 1.4.
6. Resuspend the cells in $200 \mu\text{l}$ of PBS + 1% FBS. Keep on ice and in the dark until acquisition.
NOTE: Murine NK cells can be kept on ice for up to 10 hr.

2. Preparation of Microscope Chambered Coverglass Slides

NOTE: Use chambered coverglass slides or dishes with the cover glass thickness that the microscope has been optimized for, either #1 or #1.5. If the microscope is not aligned for a certain thickness, or if it is unknown, the microscope collar ring needs to be optimized for the current coverglass thickness¹⁷.

1. Dilute poly-L-lysine with distilled water in a 1:10 ratio. Add $200 \mu\text{l}$ of diluted poly-L-Lysine to the chambers and incubate for 20 min.
2. Remove the poly-L-lysine by aspiration and let the chamber air dry by leaving it without a lid at room temperature for at least 20-30 min.

3. Starting the FCS System

NOTE: This protocol refers to a specific microscope system and software (see **Materials/Reagents Table**), although other microscopy setups and software packages can also be used.

1. Turn on the confocal laser scanning microscope with the FCS correlator. Open the software in "Expert mode". Select the 40X water immersion lens.
2. Under the "Acquire" tab, press the "Config" and "Scan" buttons to open the "Configuration Control" and the "Scan Control" windows (windows A and B in **Figure 1**, respectively). Under the "Confocal" tab, press "Measure" to open the "Measurement" window (window C in **Figure 1**).
3. Create a folder to save the FCS measurements. Start a new image database by clicking "New" under the "File" tab.
4. Using the "Laser" button, turn on the halogen lamp and the relevant lasers for exciting the fluorophores (for instance, 488 nm for the "green" excitation laser and 633 nm excitation for the "red" excitation laser).
5. **Select suitable excitation and emission filters and activate the corresponding detectors.**

1. Specify settings for image capture, including the excitation laser, excitation and emission filters, light path, and detectors to be used, in the "Configuration Control" window.
2. Similarly, specify the corresponding settings for the FCS measurements in the "Measurement" window, under the "System Configuration" tab. Use as similar settings as possible for imaging and FCS.
3. Activate the detection channels for FCS recordings by checking the "Ch1" box for the green channel and "Ch2" for the red channel in the "Measurement" window.
NOTE: The settings should be optimized for the fluorophore(s) used, as in a standard confocal imaging experiment. In subsequent experiments, the same FCS settings can be reused by opening an old FCS file, using the "Confocor" pull-down menu at the top of the user interface, and choosing "Open file." Press "Reuse" in the emerging acquisition window. Similarly, image acquisition settings can be loaded by opening a saved image and pressing "Reuse."
6. Wait until the laser is stable before performing FCS measurements. This can take up to 30 min for gas lasers, while diode lasers stabilize faster. Adjust the pinhole while waiting.

4. Pinhole Adjustment

1. Prepare 0.2-0.5 μM solutions of fluorophores or fluorescent dyes corresponding to the excitation and emission spectra of the labels on the antibodies. The fluorophores must have known diffusion coefficients^{18,19}.
2. Put a 50 μl drop of solution with the fluorophore excited by the green laser in the center of a well in a chambered coverglass slide. Put a drop of distilled water on the 40X water objective and position the slide. Press the "Vis" button to start visible light and use the ocular to find and focus on the fluorophore drop.
NOTE: Use the same slide as for later cell measurements, since putative slight variations in glass thickness between slides can affect the microscope alignment.
3. Place the focus well inside the liquid drop (10-100 μm from the bottom).
4. In the "Measurement" window, select the "Acquisition" tab. Press "Current position" under the "Positions" (window C in **Figure 1**).
5. Return to the "System Configuration" tab in the same window. Set a high power for the green laser.
NOTE: A high laser power refers to the saturating power (*i.e.*, the fluorescent signal does not increase linearly if the laser power is increased further). Around 1 mW of system settings power, or 5-10% of the max laser power, is typically enough.
6. Test the stability of the signal by pressing "Start." This will open a separate window displaying the time trace and autocorrelation curve for the current measurement. Check that the fluorescent signal is high, stable over time, and showing fluctuations in the kHz rather than the Hz range.
7. Select the "O Adjust" button in the "Measurement" window. Check if the right detection channel (Ch1 or Ch2) is selected. Press "AutoAdjust X." If the resulting curve does not show a clear maximum, or the maximum is at the edge, mark the "coarse" option and press "AutoAdjust X" again. When the resulting screen in the x-direction is finished, do the same for Y by pressing "AutoAdjust Y."
8. De-select "coarse" and alternate between adjusting X and Y by pressing "AutoAdjust" until both have clear maxima and the values are not changing between measurements.
9. If cells are labeled with two different fluorophores, move to a chamber where red fluorophore solution has been added. Select a high laser power for the red laser and repeat steps 4.6-4.8 for the red excitation and detection channel.
NOTE: If X and Y values deviate between the channels in systems where only one pinhole is used for both detection channels, select intermediate values and press OK to accept the changes. Here, for the 488 nm laser with maxima at X = 79 and Y = 189 and the 633-nm laser with maxima at X = 80 and Y = 188, values X = 80 and Y = 189 were selected. The combination of the 488 nm and 633 nm lasers for excitation of the two antibodies labeled with fluorophores excited by 488 nm or 633 nm is a good choice, since the risk for cross-talk is minimized if the emission spectra do not overlap².

5. Measure the Transit Time of the Free Fluorophore

NOTE: By determining the transit time through the focus (TauD) of a fluorophore with a known diffusion coefficient, the size of the detection volume, and therefore the area of the cell membrane that is within the focus, can be calculated. The calculation of TauD is described in step 7.2.

1. Using the same solutions used for the pinhole adjustment, set the acquisition time to 30 sec x 2 repeats under the "Acquisition" tab in the "Measurement" window (**Figure 1**, window C).
2. **Start a measurement by clicking on "Start" in the "Measurement" window (Figure 1, window B).**
 1. Perform a laser power series for each excitation laser and the corresponding fluorophore in solution, starting at or near saturating power and decreasing by half for each measurement. Change the laser power in the "System Configuration" tab of the "Measurement" window (**Figure 1**, window C). Press the "Start" button to start a measurement.
NOTE: Include the laser power for the upcoming cell measurements in the lower range (*e.g.*, measure at 16, 8, 4, and 2 μW power, before the objective)²⁰. System laser settings are in general higher than the output excitation and can vary largely depending on the microscope and the quality of alignment. In this system, the above power range corresponds to approximately 60-500 μW of system settings power. It is advised to use a power meter to measure the power output before the objective the first time a new microscope is used. The current maximum power of the laser is found under the "Info" button of the "Measurement" window.
 2. Write down the counts per molecule (CPM, unit: kHz) from each laser power measurement.
 3. If two detection channels are used, check the window displaying the autocorrelation curve for cross-talk. Use a solution containing only green fluorophores to measure detected FCS autocorrelation in the red detection channel and a solution with only red fluorophores to assess detection in the green detection channel. As a rule of thumb, keep the CPM detected from the "wrong" fluorophore below 5% of the specific signal from the proper fluorophore.
 4. Check that the values scale linearly with the laser power used. Saturation of CPM at the highest laser power (*i.e.*, slightly lower values than expected from a linear relationship) is acceptable.
NOTE: CPM should be well above 10 for the highest laser power for almost all microscope systems and common fluorophores and can be significantly higher for sensitive systems. The first time a newly conjugated fluorescent antibody is used, perform an FCS

measurement of the antibody freely diffusing in solution to quantify the expected CPM. Prior to measurement, coat the measurement chamber with 200 μ l of poly-L-lysine grafted with polyethylene glycol (diluted to 0.5 mg/ml in PBS) for 1 hr at room temperature. Remove the liquid with a pipet and allow the chamber to air dry. Save the stock solution in the fridge (up to 2 weeks) and the powder stock in the freezer. At the microscope, dilute the antibody to 1–10 μ g/ml in PBS. Follow steps 4.2–4.4 and 6.7 in this protocol. If the surface is not coated with a non-stick solution, antibodies will interact with the glass surface and be rapidly depleted from the solution.

6. Cell Measurements

1. Add 125 μ l of cells in PBS + 1% FCS from the antibody-labeled cell suspension (step 1.5) and 150 μ l of transparent Roswell Park Memorial Institute medium 1640 (RPMI) into one well of an 8-well chambered coverglass slide. Leave the cells in the dark at measurement temperature for at least 20 min before starting FCS measurements.
NOTE: This is to allow the cells to settle and attach to the bottom of the wells and to equilibrate the cells and solution to the measurement temperature.
2. In the "Scan Control" window (**Figure 1**, window B), set zoom 1 and start a fast X-Y scan by pressing the "Fast XY" button. Modify the laser power so that it is as low as possible while the cells are still clearly visible. Center the image on a round cell of average brightness, where the membrane is clearly defined and there is no fluorescent signal in the cytoplasm. Zoom in until the cell covers most of the image.
3. Select a new cell if the current cell is moving or displays tendrils, extrusions, or extremely bright spots. Keep the same zoom and laser power for all cells captured during the same experiment.
NOTE: A ruffled membrane can also be a sign of upcoming apoptosis.
4. Focus on the top of the cell membrane. In the "Acquisition" tab of the "Measurement" window (**Figure 1**, window C), select a position for FCS measurement by activating "Crosshair." Alternatively, select the "LSM Image" tab, mark the wanted measurement spot in the lsm image, and press "Add position."
NOTE: The top of the cell membrane will not be influenced by fluorescent antibodies or fluorophores unspecifically bound to the poly-L-lysine. However, it is important to ascertain that the cell do not move during the measurement (see the results).
5. In the "Acquisition" tab of the "Measurement" window (**Figure 1**, window C), set the number of repeats to 6–10, with the "Measure Time" set at 10 sec per repeat.
6. Return to the "System Configuration" tab of the "Measurement" window. Adjust the laser power for the FCS measurement under the "Laser" button. Before the objective, use low power for cell measurements, in the range between 1 and 10 μ W²⁰.
NOTE: The recommended values are a trade-off between signal detection and photodamage to cells. See step 5.2.1 Note the percentage of laser power these values correspond to.
7. Start an FCS measurement by pressing "Start" in the "Measurement" window (window C). If the cell bleaches significantly in the beginning of the measurement, as detected by a drop in the intensity trace by more than 30% during the first 10 sec (an example is shown in **Figure 3A**, lower panel), move to another cell and lower the laser power for future measurements.
 1. If the signal is lost, adjust the focus in the Z-direction. After the measurement is complete, use the "Scan Control" window (**Figure 1**, window B) to check that the cell is still centered and that the cell membrane was measured. If the cell has moved, discard the measurement.
 2. In the auto-correlation window where the measurement is displayed, manually delete individual repeats that contain zooming maneuvers, bleaching, large clusters, or other artifacts (see examples in **Figure 3**). Do this by marking them, right-clicking, and choosing "Delete." Save the final file in .fcs format. Save at least four repeats per cell.
8. Optionally, acquire an image of the cell at the middle section (with the entire circumference of the membrane visible) using the "Scan Control" window. Capture the image after the FCS measurement to avoid bleaching. Press the "Single" button to start the image capture.
9. If using "Add position" for the FCS focus positioning, click "Remove position" before moving on to the next cell.
10. Change to a new aliquot of cell suspension after a maximum of 2 hr of measurement to keep the cells viable throughout the experiment.

7. FCS Analysis

1. Open the .fcs files in a software with curve fitting capability (see **Materials/Reagents Table** for the software used in this protocol).
NOTE: The FCS software that comes with the microscope is a good place to become familiar with basic fitting, but additional software is necessary to fit two-dimensional membrane diffusion.
2. First, determine the size of the excitation volume by analyzing the free fluorophore measurements. Fit a 3-dimensional free diffusion curve to the autocorrelation curves²¹.

$$G_{3D}(\tau) = \frac{1}{N} * \left(1 + \frac{\tau}{\tau_D}\right)^{-1} * \sqrt{\left(1 + S^{-2} * \frac{\tau}{\tau_D}\right)} * \left(1 + T1 * (1 - T1)^{-1} * \exp\left(\frac{-\tau}{\tau_T}\right)\right) + 1 \text{ (Eq. 1)}$$

NOTE: Definition of parameters: N: mean number of fluorescent entities in the focal volume, τ (Tau): correlation time, τ_D (TauD): average residence time in the focal volume, T1: probability for the fluorophores to be in the triplet state, τ_T : relaxation time for the singlet-triplet state transitions, S: ratio of height to width diameter for the focal volume.

1. Extract TauD, the transit time for molecules to transfer the focus.
2. Use the observed TauD and published diffusion coefficients (D) of fluorophores used for calibration^{18,19} to calculate the pinhole radius (ω).

$$\omega = \sqrt{\frac{\text{observed } \tau_D}{4 * D}} \text{ (Eq. 2)}$$

3. **Analysis of the autocorrelation curves on cell membranes.**
 1. To visualize the part of the curve representing molecular diffusion, select the time frame starting before the steepest slope of the autocorrelation curve and ending after the curve convergences at 1 (e.g., 0.001 to 5 sec for murine surface receptors; see **Figure 4**).
 2. Generate an average autocorrelation curve of the individual repeats saved in step 6.7.2.

3. Fit a 2-dimensional membrane diffusion curve to each averaged autocorrelation curve using Equation 3³.

$$G2D(t) = \frac{1}{N} * \left(1 + \frac{t}{\tau_D}\right)^{-1} * \left(1 + T1 * (1 - T1)^{-1} * \exp\left(\frac{-t}{\tau_T}\right)\right) + 1 \quad (\text{Eq. 3})$$

NOTE: Important output parameters are as follows: N is the average number of molecules residing within the excited membrane area. Recalculate to density ($N/\mu\text{m}^2$). τ_D (TauD): average residence time in the focal area, restricted by the two-dimensionality of the membrane (s). Recalculate the average residence time to a diffusion coefficient ($\mu\text{m}^2/\text{sec}$) via the determined pinhole radius (ω) and Equation 2. T1 is the probability for fluorophores to be in the triplet state. The brightness (CPM) is calculated by dividing the average overall intensity of the measurement by N.

4. If a good fit is not achieved at the first try, change the starting values and/or the upper and lower limits for the variables until this is achieved.

NOTE: Typical upper-lower limits for protein diffusion rates in primary cell membranes are 10-500 msec for TauD, 0-100% for T1, and 0.1-5 msec for TauT1. Select starting values for the fitting in the middle of these intervals. The part of the curve representing fluorescence fluctuations derived from diffusion is typically located between 1 msec and 1 sec (see **Figure 4**). Depending on the fluorophore, there can sometimes be a second process present, giving rise to autocorrelation at shorter time scales than the diffusion parameter. This is caused by a transient dark state (triplet) or blinking (as often occur for fluorescent proteins). A triplet state is accounted for in Equation 3, and the presented model should thus also provide a good fit in these situations.

Representative Results

A typical result will generate an autocorrelation curve with a transit time in the range of 10 msec to 400 msec for membrane proteins. The number of molecules can vary between 0.5 to around 200 per μm^2 for endogenously expressed proteins. Check carefully that the CPM is not lower than expected. This may mean that there is an influence of the background signal. As a rule of thumb, the CPM signal on cells that is accepted for analysis should not be lower than 33% of the CPM for freely diffusing antibodies at the same laser power. The CPM should scale linearly with the laser power. The autocorrelation curve for primary immune cell surface receptors is expected to be smooth, with the steepest part between 0.001 and 1 sec. See **Figure 2** for a representative autocorrelation curve and its time trace.

As mentioned briefly in the introduction, there are several cases where FCS is not suitable for measuring molecular diffusion due to the influence of other sources of fluorescence fluctuations, which contribute to confounding the signal. **Figure 3** shows examples of cases in which a particular cell or measurement repeat should be discarded. It has already been mentioned that an extremely low signal (very low CPM) is cause for discarding the cell. Another reason for discarding the individual cell is that the cell is moving (**Figure 3A**). In the case of bleaching (**Figure 3B**) or if large clusters are present (**Figure 3C**), the measurements should be discarded if the effect is considerable or present throughout the whole measurement. If this feature is only present in one or two repeats, these individual repeats may be discarded, but the remaining repeats may still be used.

It is important to both use the correct model to fit the data and also to check carefully that the fit closely overlaps with the autocorrelation curve. In **Figure 4**, examples of good and bad curve fits are shown. Pay close attention to the part of the curve representing diffusion (the most steeply sloping part). It is also important to ascertain that both the start and the end of the sloping part of the curve are well-fitted by the model. If the fit is bad, without apparent problems such as moving, bleaching, or clusters, modify the starting values and/or the upper and lower limits (within a reasonable range) before making a final decision to discard the cell.

Figure 1: FCS software interface. Shown here are the main windows necessary to operate the software tool for FCS acquisition and imaging, as described in the protocol. Window A, the "Configuration Control" window is the control window for the beam path, laser channels, and filters for imaging. Window B is the "Scan Control" window for imaging and shows the scanning settings. Window C is the "Measurement" window, the window for the setup of the FCS measurement settings. [Please click here to view a larger version of this figure.](#)

Figure 2: Representative trace and autocorrelation curves of diffusing H-2D^d major histocompatibility class I surface molecules in freshly isolated murine NK cells. The upper panel in the figure shows the fluorescence fluctuation as a function of time throughout the whole time trace of 7x 10 sec measurements. The lower panel represents the averaged autocorrelation curve from these seven repeats. The height of the autocorrelation curve is inversely proportional to the concentration of mobile fluorescently labeled H-2D^d entities within the focal volume. The x-axis value at the steepest part of the slope of the curve, half of the amplitude, represents the average residence time of fluorescently labeled H-2D^d molecules within the focal volume. The measurement presented is a part of the data set published in reference². [Please click here to view a larger version of this figure.](#)

Figure 3: Examples of problematic cell traces. (A) A moving cell, as exemplified by the trace not having a stable basal level. The top panel shows an example where the whole cell has moved out of focus after approximately 35 sec, as represented by the very low signal after this time point. In the lower panel, the effect is more subtle, with undulations apparent at an interval of several seconds. (B) Cell displaying bleaching, the height of the trace decreasing with time. Compare the height of the trace at the start of measurement to that at the end of the measurement. (C) The presence of large clusters, represented by spikes in the trace. In the upper panel, one large cluster at 20-25 sec is present. In the lower panel, several clusters are present throughout the measurement. The measurements presented are a part of the data set published in reference². [Please click here to view a larger version of this figure.](#)

Figure 4: Representative autocorrelation curves with curve-fitting and residuals. The red and blue vertical lines represent the limits of the time window used for fitting. **(A)** Example of a representative fit of a 2-dimensional diffusion model to a sample autocorrelation curve. In the upper panel, the green curve (the model) overlays the blue curve (the acquired autocorrelation), indicating a good fit. The lower panel shows the residual from the curve fitting. The fluctuations around 1 sec, which are not fitted well, are typical for cell measurements and are tolerable. **(B)** Example of a bad fit of 2-dimensional diffusion to the same autocorrelation curve. The fitted model does not overlay the autocorrelation curve, and it does not converge at 1. The lower panels in (A and B) show the residual from the curve fitting. The residuals are significantly larger for the bad fit, especially for the diffusion part of the curve. The measurement presented is a part of the data set published in reference². [Please click here to view a larger version of this figure.](#)

Discussion

This protocol for FCS can be used for the assessment of the molecular dynamics of surface molecules on all types of immune cells (murine, human, or other species). FCS measures spatio-temporal molecular dynamics down to single-molecule resolution in live cells. The molecular density, as well as the diffusion rate and clustering dynamics of the proteins of interest, can be extracted from the autocorrelation curves.

The fluorescent labeling is of pivotal importance for successful FCS experiments. Proteins of interest on the cell membrane have traditionally been labeled using antibodies, and antibodies are often most convenient for use on surface proteins in naïve immune cells. Unlabeled antibodies must be directly conjugated to photo-stable fluorescent dyes with a high molecular brightness. The selection of the fluorophore for antibody conjugation is important for the quality of the measurements. Standard labels for flow cytometry, such as fluorescein and phycoerythrin-based dyes, are not recommended due to rapid bleaching. Photo-stable dyes for direct conjugation to antibodies are currently provided by several companies. The manufacturers usually provide satisfactory protocols for conjugation and purification of the resulting conjugated antibody, and this can be done in a few hours. It is also possible to perform FCS using fluorescent proteins, but special care should be taken to minimize the laser power, since fluorescent proteins are in general more prone to bleaching than the most photo-stable chemical fluorophores. According to previous experience, over-expression of proteins often leads to a considerable decrease in the diffusion rate due to crowding, which makes the use of fluorescent proteins unsuitable.

Pinhole calibration prior to the measurements is also a critical step. The fluorophores used for determining the size of the focal volume must have known diffusion coefficients (see Equation 2). Use free fluorophores corresponding closely to the emission spectra of the antibody labels to determine the size of the focal volume. This must be done to assure the optimal signal efficiency and proper detection of potential cross-correlations between color channels. Perform an FCS laser power series of the freely diffusing fluorophores to ensure that the system gives the expected output signal over a range of powers. Compare the CPM at the same laser power between experiments to ensure consistency between experiments.

In the analysis step, it is essential to put reasonable ranges and starting values for the variables when fitting the models. Use previously published knowledge from similar cell systems to find good starting points. Theoretically, FCS is a quantitative technique, but since optimal conditions seldom occur, a certain degree of caution has to be taken when interpreting the results. All types of fluctuations will be recorded, regardless of the origin of such fluctuations. Therefore, it is useful to have as much information as possible about the experimental system in order to exclude possible error sources. For instance, movement of the whole cell membrane will give rise to fluctuations with longer TauD (Figure 3A), whereas putative contaminants of the free fluorophores in the solution will diffuse with at least ten times shorter TauD .

This basic protocol can be modified in several ways. If two proteins are labeled with different fluorophores, co-diffusion (an indirect measure of interaction) can be assessed by expanding the methodology to detect the cross-correlation. The extent to which these proteins bind to each other in the cell membrane is represented by the height of the cross-correlation curve relative to the height of the autocorrelation curves of the individual color channels. Cross-correlation is denoted as Ch1-Ch2 in the measurement software. The precise analysis of cross-correlation requires controls for cross-talk and is thus somewhat more complicated than the protocol presented here. A detailed description of how to proceed, as an extension to this basic protocol, can be found in Strömquist *et al.*¹³. To optimize the positioning in the z-direction, z-scanning FCS can be used²². According to previous experience, it has been satisfactory to do this manually. It is also possible to fit multiple diffusion coefficients to an FCS autocorrelation curve²³. This requires previous knowledge of the number of subpopulations with different diffusion rates and of the approximate diffusion rates of at least one of the subpopulations. Otherwise, there will be too many free variables, which will render the fitting unreliable. A typical example would be a surface protein whose diffusion rate is considerably slowed down by the binding of a ligand. Finally, cleaning the trace of outliers without entirely removing repeats is possible²⁴.

The lack of a fluorescent signal is a common problem to troubleshoot. For freely diffusing fluorophores, use the halogen lamp to check if the drop is fluorescent. If the drop is not fluorescent, mix a new batch of fluorophores with a slightly increased concentration (within the nM range) and try again. Check that the focus is within the fluorescent drop, the lasers are turned on in the software, the laser power is sufficient, the correct emission filters and detectors are chosen, and the right channels in the "FCS Measurement" window are activated (see step 3.6). Start the laser and look from the side to visually confirm that the laser light reaches the sample. Avoid looking straight into the laser source. If the selected emission filter encompasses the laser wavelength, a shutter will automatically block the light path to avoid damage to the detector. If all the settings are fine but no light arrives, re-start the lasers, the FCS system, and the computer. Ask an expert if the lack of a fluorescent signal persists. A simplified procedure applies if the labeled cells do not display fluorescence. Confirm the presence of fluorescence with the halogen lamp; turn on the lasers, select the laser channels, and adjust the laser power. Select a position on the cell membrane, either using the "Crosshair" or "Add position" option (see step 6.4). Switch to the next sample if the signal is still too low.

One important aspect of the fluctuation basis of the technique is that molecules have to be reasonably mobile to be detected. Very slowly moving fractions of molecules or molecules trapped within areas smaller than the focus during the whole measurement time can therefore not be measured. This leads to a possible underestimation of surface densities and an overestimation of the diffusion rate. Bleaching can also contribute to a putative underestimation of both the density and TauD , since molecules will have a higher probability of being bleached the longer time they spend in the laser-illuminated focal volume. Influence from the background (fluorescence from outside of the focal plane) would, on the other hand, artificially increase the number of detected molecules and decrease the measured CPM. Previously, the total maximal error in the absolute density determination was estimated to be around 40%²⁰. However, it is usually possible to compare different biological samples to each other, since the error sources are, in most cases, equal throughout the experiments. Another potential error source is that antibodies are bivalent, meaning that each antibody can potentially bind to two target molecules. The authors did not observe this phenomenon, but it cannot be guaranteed that this is a global feature for other antibodies. The potential influence of bivalence must therefore be tested individually for each antibody. The combination of specific primary and labeled secondary antibodies must never be used due to the high risk of inducing artificial clusters from two successive bivalent labels. If cells have instead been transfected with fluorescent protein-labeled versions of the protein of interest, every protein will have only one label. However, this requires transfection, which is not always desirable and may not even be possible (e.g., if using immune cells directly isolated from human blood). Finally, single-point FCS does not record images. If images are required for

publication or other purposes, these must be captured separately using the imaging part of the software. Always capture images after FCS measurements to avoid unnecessary bleaching of the cell surface.

Unlike FCS, traditional confocal-based microscopy, such as FRAP, and image correlation methodologies cannot quantify fluorescence fluctuations at the single-molecule level⁶. Image correlation methodologies measure the number of molecules indirectly and with lower resolution, but they can assess the variability of molecular diffusion and clustering over the entire imaged area²⁵. Standard SPT measured by confocal microscopy has an ideal level of labeling, orders of magnitude lower than FCS⁷. Therefore, the density of labeled proteins cannot be measured by standard SPT. The combination of SPT with other microscopy techniques (e.g., stochastic optical reconstruction microscopy or photoactivation localization microscopy) allows the density and movement to be assessed, but it requires very specialized dyes or proteins¹¹. Super-resolution techniques, such as structured illumination microscopy and stimulated emission depleted microscopy, often require fixed samples and a combination of primary and secondary antibodies for labeling²⁶. Localization is therefore very precise, while the dynamics of the system cannot often be assessed. In comparison to SPT and super-resolution techniques, FCS also requires significantly less computer power and time for the analysis and extraction of results. Flow cytometry is the workhorse of immunology and can be favorably combined with FCS. Cell sorting can, for example, be followed by subsequent measurements on selected cell populations if photo-stable dyes had been used prior to the sorting. FCS is thus a methodology that can be used alone or in combination with other established methods.

This protocol can be used to identify currently unknown features of immune cell dynamics and interactions within the cell membrane at the single-molecule level. Furthermore, features of whole populations versus selected subsets can be compared. It also has a potential role as a selection step for immune cell therapy. Since the measurements do not consume the cell, unlike most other methods for investigating the functionality of immune cells, individual cells can potentially be recovered and cultured. Thus, after the analysis of the FCS curves, promising cells can be extracted and expanded for additional applications, including putative clinical applications.

Disclosures

The authors have nothing to disclose.

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