High Spatial Resolution Observation of Single-Molecule Dynamics in Living Cell Membranes

Joshua B. Edel,* Min Wu,† Barbara Baird,‖ and Harold G. Craighead*
*School of Applied and Engineering Physics, and †Department of Chemistry and Chemical Biology, Cornell University, Ithaca, New York

ABSTRACT  Self-organized lipid bilayers together with proteins are the essential building blocks of biological membranes. Membranes are associated with all living systems as they make up cell boundaries and provide basic barriers to cellular organelles. It is of interest to study the dynamics of individual molecules in cell membranes as the mechanism of how biological membranes function at the single molecule remains to be elucidated. In this letter we describe a study in which we incubate rat basophilic leukemia cells with a fluorescently labeled cell membrane component on a surface containing zero-mode waveguides (ZMWs). We used the ZMW to confine fluorescent excitation to an ~100-nm region of the membrane to monitor lipid diffusion along the cellular membrane. We showed that confinement with a ZMW largely reduced fluorescent contributions from the cytosolic pool that is present when using a more standard technique such as laser-induced confocal microscopy. We show that optical confinement with ZMWs is a facile way to probe dynamic processes on the membrane surface.

Received for publication 24 February 2005 and in final form 4 April 2005.

Address reprint requests and inquiries to Harold G. Craighead, Tel.: 607-255-8707; E-mail: hgc1@cornell.edu.

Over the past few years a number of optical techniques have been developed with sufficient sensitivity to detect single molecules. For single-molecule detection in fluids, optical methods such as laser-induced fluorescence have proved successful. This approach affords detection probe volumes on the order of $10^{-12}$–$10^{-16}$ L useful for nanomolar or more dilute sample concentrations. Recently, Levene et al, circumvented the need for dilute solutions by utilizing ZMWs to perform single-molecule detection at micromolar concentrations with microsecond temporal resolution (1). The ZMW, as used in this work, incorporates 100-nm diameter holes in a 100-nm-thick aluminum film on a fused silica coverslip (fabrication procedure is described in Levene et al. (1)). Because the diameter of the hole is much smaller than the excitation wavelength, the hole acts as a ZMW, and the light generates an evanescent field with a decay length of <50 nm into the cavity, producing a zeptoliter-scale effective observation volume. By utilizing single-molecule fluorescence correlation spectroscopy (FCS), we show that ZMWs can be used to monitor diffusion of single lipids within a cell membrane with high spatial resolution in all three dimensions.

FCS analysis is an extremely sensitive method for analyzing fluorescence photon bursts in single-molecule experiments. This analysis method measures the relative fluctuation of a signal traveling across the detection probe volume (2). The concept relies on analysis of local concentration fluctuations in a small volume, and is a measure of the temporal behavior of a dilute system. Although FCS-based confocal spectroscopy is inherently sensitive, the minimum depth of field in a confocal setup is typically 500 nm. By utilizing ZMWs as shown in Fig. 1, the depth of field is reduced to 10–50 nm depending on the width of the hole. Scanning near-field optical microscopy can be used for obtaining subwavelength resolution (20–200 nm), but ZMWs are optically much simpler and adaptable to longer duration studies of living systems (3). Total internal reflection fluorescence microscopy has also recently been developed to study the behavior of individual protein molecules within living mammalian cell membranes (4). This method produced an evanescent depth of field comparable to that of ZMWs (~50 nm). However, the lateral excitation region can be on the order of microns, greatly increasing the detection probe volume compared to ZMWs.

In our experiments we formed an array of ZMWs on a fused silica coverslip on which we placed a 10-μL drop of solution containing suspended rat basophilic leukemia (RBL) cells that contained Dil-labeled C18 in their cell membranes (5). Laser-induced fluorescence confocal microscopy was used to monitor the photon statistics of the fluorescently labeled cells. Single fluorescently labeled molecules are detected as they diffuse or move through the probe volume. The diffusion time is directly related to the solvent viscosity and hydrodynamic radius, which is in turn determined by the shape and size of the molecule. It should be noted that RBL cells are of mucosal mast cell origin and can adhere to surfaces in the absence of any stimulation via cell surface integrins. In addition, the surface of unstimulated RBL cells includes large numbers of microvillus-like projections, ~80 nm in diameter (6). It is possible that these projections are making their way into the ZMW. After 30 min, the RBL cells not only adhered to the top metal cladding of the ZMW but also conformed to the shape of the hole. This was determined...
by monitoring the fluorescence intensity over an extended period of time within the ZMW. Directly after incubation of the cells ~2 photon bursts were recorded within a 20-s period. After 1.5 h, the rate of events was increased to 22 bursts over 20 s. This implies that not only does the cell over time conform to the shape of the ZMW hole but also the cell extends deep enough into the ZMW for excitation of the fluorophore in the membrane to be possible. A single-molecule photon-burst scan, recorded using a conventional 500-nm diffraction limited focused laser beam integrated with a ZMW, for the diffusion of DiI-C18 within the RBL cell membrane is shown in Fig. 2. Each photon burst is associated with single fluorescently labeled lipids diffusing across the detection probe volume within the ZMW cavity. The average single-molecule intensity within the ZMW was 614 photon counts at an incident power level of ~1 mW. All photon burst scans and FCS curves were obtained using power levels ranging from 1 mW to 100 μW.

Fig. 3a shows normalized fluorescence correlation curves for DiI diffusion in the RBL cellular membrane, using confocal fluorescence excitation FCS, without a ZMW. Two diffusive components were associated with the correlation curves. The quicker component had diffusion times on the order of 1–4 ms (free diffusion, possibly due to the formation of dye micelles) whereas the longer component had diffusion times between 300 and 500 ms (diffusion along the membrane). The diffusion coefficients (~1 × 10⁻⁶ cm²/s and ~5 × 10⁻⁹ cm²/s, respectively) are comparable to what has been reported in the literature for similar systems (7). Fig. 3b shows FCS curves using the ZMW for the same cell system. In this case there was only one diffusive component in the autocorrelation curve. A single component is a direct consequence of the evanescent decay of the excitation light within the ZMW as only the fluorophores within or close to the cellular membrane are probed. It should be noted that the transit time of the lipid through the probe volume was at least a factor of 10–100 times quicker giving diffusion coefficients ranging from 5 × 10⁻⁹ to 6 × 10⁻⁸ cm²/s. Transit times within the ZMW would be expected to be even quicker due to such small excitation volumes; however, it is possible that surface effects between the membrane and the walls of the ZMW are inducing a change in diffusive characteristics (this is currently being investigated and will form the basis of a future publication).
ZMWs appear to be a powerful tool for the monitoring of diffusion along living cellular membrane. For cells that conform to the shape of the waveguide, the limited depth of field associated with ZMWs insures that what is being detected is on the membrane. This opens the door to new experimental possibilities, such as studying dynamics of receptor ligand binding, binding of localized regions within a cell to a target within the ZMW, and studying dynamics of protein trafficking within the cell membrane. There has been a lack of suitable methodology to visualize nanometer-size organization in living cells. The use of the ZMW optical nanostructures creates new possibilities in performing such experiments on cell surfaces.

SUPPLEMENTARY MATERIAL

An online supplement to this Biophysical Letter can be found by visiting BJ Online at http://www.biophysj.org.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation through the Nanobiotechnology Center.

REFERENCES and FOOTNOTES


(5) Details of the experimental procedure can be found in the Supplementary Material.
