X-Cite®
Fluorescence Illumination • In Control

Sensitized emission FRET measurements using white-light TIRF microscopy

Challenge

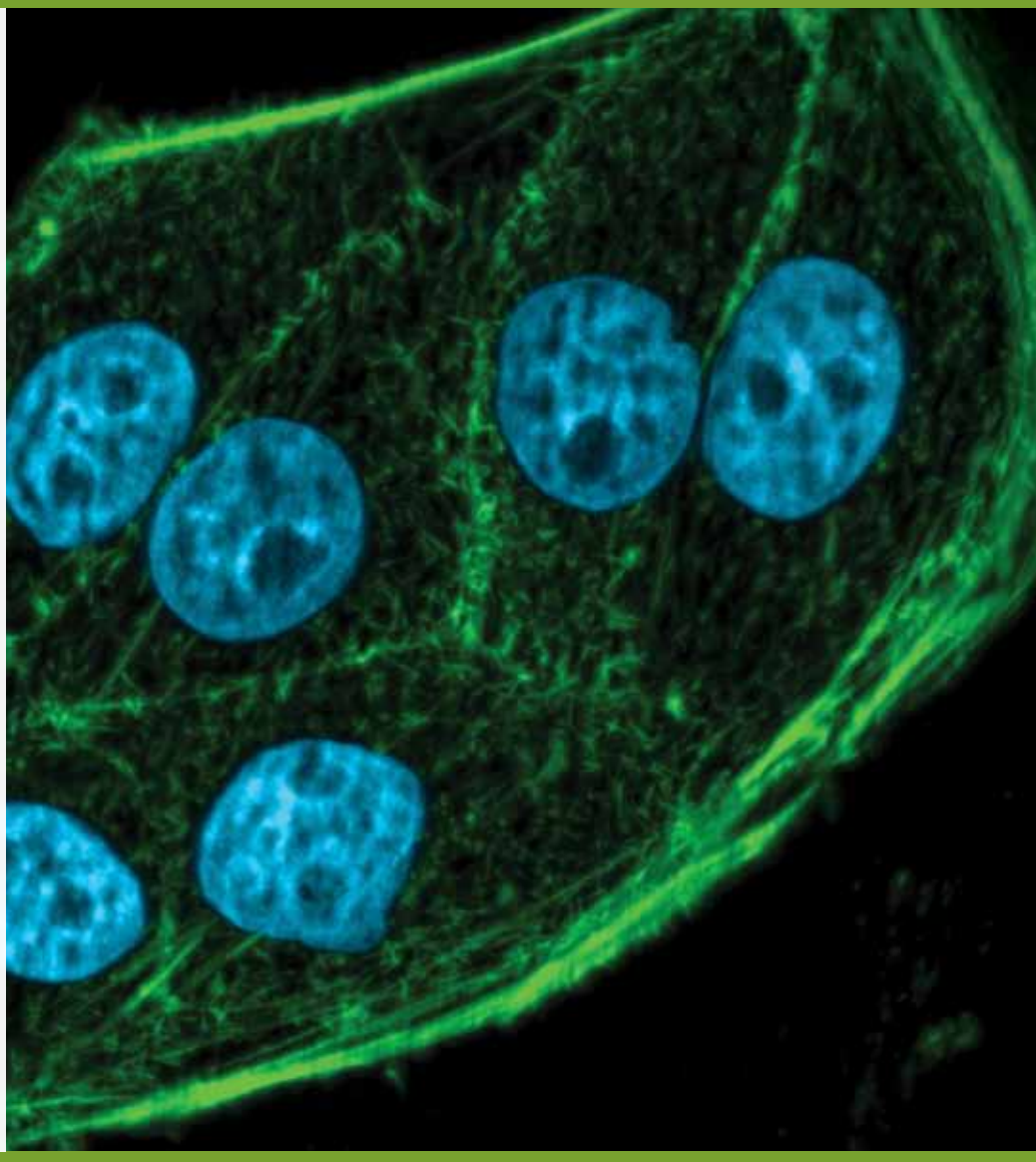
TIRF imaging with low photobleaching and without the cost of a laser system

Solution

X-Cite® 120 used in TIRF microscopy instead of a laser

Benefit

Affordable TIRF microscopy with minimal photobleaching



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Abstract

Fluorescence resonance energy transfer (FRET) has been a unique system in measuring the dynamics of protein structure in living cells, as it is the only biological assay to directly speculate conformational mobility. However, employing epi-fluorescence microscopy to detect fluorophores of donor and acceptor fluorescence-coupled proteins in biological phenomena at the cell surface has been challenging due to the inability to exclude fluorescence originating from intracellular regions. Therefore, total internal reflection fluorescence (TIRF) microscopy is advantageous to selectively monitor protein dynamics at the plasma membrane cell-subtract contact zone (1). By measuring fluorescence at the plasma membrane with a white-light TIRF microscope equipped with a mercury arc lamp of the X-Cite® 120 system, sensitized emission FRET analysis in live cells allows us to define protein dynamics contributing to cell motility.

BACKGROUND

Integrins are heterodimeric adhesion molecules expressed at the cell surface and involved in cell mobility through interactions with ligands. Activation occurs upon binding of the integrin to its respective ligand (outside-in signaling), inducing subsequent structural rearrangement of both the extracellular and cytoplasmic domains – a process during which the cytoplasmic tails of the α and β subunits become distantly located. Here, we describe a method for monitoring integrin conformational activity using a sensitized FRET method coupled with TIRF microscopy. We used this system to determine conformational status of integrin VLA-4 during cell mobility on a substrate for VLA-4, VCAM-1 (2).

TIRF microscopy is a valuable tool for studying the molecular mechanisms involved in cell migration because the technique allows visualization of optical events within 100 nm of the plasma membrane, where cell-substrate traction occurs. Since the thickness of plasma membrane in mammalian cells is 5 - 10 nm and the extended form of integrin extracellular domains at the cell surface is 15 – 20 nm, it is feasible to determine conformational change of VLA-4 proximal to the cell membrane during cell mobility through dynamic FRET on a TIRF microscope. We developed a VLA-4 FRET sensor, in which α_4 and β_1 subunits of VLA-4 were fused to monomeric CFP (mCFP) and mYFP, respectively. By transiently transfecting the VLA-4 FRET sensor on VLA-4 deficient GD25 cells, we observed active VLA-4 localizing at the lamellipodia during cell migration on VCAM-1. A caveat in dynamic FRET is how efficiently photobleaching is constrained during time-lapse image capture. Therefore, it is critical to minimize photobleaching of fluorophores which results from exposure to the light source used for fluorophore excitation. Using the X-Cite® 120 system, we could minimize photobleaching of the fluorophores (CFP and YFP) in the sensitized FRET method, and gain reliable FRET data during 30 min time-lapse cell migration.

MATERIALS AND METHODS

Delta T dishes (Fisher Scientific) were coated with 1 µg/ml CXCL12 and 100 µg/ml human VCAM-1-Ig fusion protein. Twenty-four hours after transient transfection of VLA-4 FRET sensor to GD25 cells, the transfected cells were resuspended in L15 medium with 2 mg/ml glucose and then added to the CXCL12/VCAM-1 coated delta T dish (2). Dynamic FRET imaging using a TIRF microscope was conducted with a dual-view image splitter (Photometrics) and CFP/YFP dual-band filter set (Chroma) by the sensitized emission method (3). Fluorescence images were acquired every 10 sec under a ×100 objective lens at 37°C during cell spreading or migration. TIRF imaging was performed with a white-light TIRF aperture diaphragm coupled to a Nikon Eclipse TE2000-E microscope and a ×100 TIRF 1.49 aperture oil immersion objective attached to a QuantEM charge-coupled device camera (Roper Scientific) using a 120-W mercury arc lamp of the X-Cite® 120 system (Lumen Dynamics) (2). Image processing for FRET analysis was performed with Nikon NIS Element software, Autodeblur (Autoquant Imaging), and MATLAB (MatWorks).

RESULTS

White-light TIRF microscopy for sensitized FRET method

TIRF microscopy exploits an optical phenomenon called "evanescent wave generation", which penetrates ~100 nm into the cell (Figure 1). To attain the superficial visualization limited to plasma membrane imaging, TIRF microscopy quantitates the fluorophores fused to proteins at the cell surface. Sensitized emission FRET between donor and acceptor fluorophores fused to target proteins is measured by the increase in sensitized YFP emission over the decreased intensity in CFP emission by energy transfer. By obtaining fluorescence intensities in a time-lapse manner, sensitized FRET exhibits dynamic conformational changes of target proteins during biological process. Therefore, we coupled the sensitized FRET method with TIRF microscopy to specifically analyze a cell surface protein, VLA-4 (α_4/β_1) integrin, in close contact with an adhesive substrate.

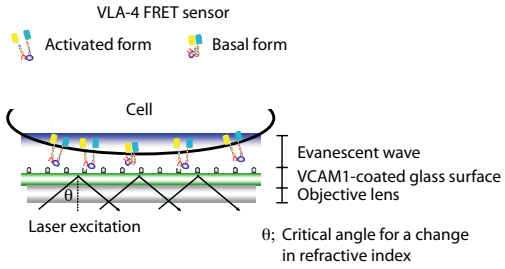

Figure 1: FRET on TIRF microscope

For typical TIRF microscopy, multiple lasers have been mainly used for excitation of fluorophores at 488, 532, 561 and 635 nm (4). Although it is possible to use multiple lasers for simultaneous acquisition of multiple fluorophores from the same cell, the cost of multiple lasers and related equipment for TIRF-FRET can run very high. As we show here, we are able to bypass costs associated with purchasing or renting laser equipment by performing white-light TIRF microscopy, in which mercury lamp (X-Cite® 120) is used effectively for TIRF illumination. The white-light TIRF microscopy enabled us to accomplish cost-effective sensitized FRET system for studying structural and functional study of VLA-4 integrin in contact with an adhesive substrate.

Minimal bleaching of fluorescence in time-lapse image captures

Repetitious exposure of fluorescent proteins expressed in host cells to fluorescence light tends to induce bleaching of fluorophores, which results in an artificial influence of FRET data interpretation. For reliable FRET data to interpret conformational status of target proteins, it is crucial to minimize bleaching of fluorescence during fluorescent imaging. Using white-light TIRF microscope equipped with a mercury arc lamp of the X-Cite® 120 system (as opposed to conventional LSM - TIRF), we could acquire time-lapse fluorescence images of VLA-4 FRET sensors expressed on the plasma membrane of GD25 cells that excluded intracellular fluorescence without bleed-through between CFP and YFP (Figure 2). Since β_1 subunit of integrins are paired with other indigenous a subunits of integrins, including α_1, α_3, α_5, and α_v, the fluorescence images of Figure 2 shows that β_1-mYFP was expressed more strongly than α4-mCFP at the cell surface. Time-lapse fluorescence image acquisition for FRET analysis at a frequency of each 10 sec for 30 min did not cause significant photobleaching and fluorescence intensities of CFP and YFP remain constant (2). Thus, illumination of fluorophores by the X-Cite® 120 system was enough to excite VLA-4 FRET sensor for FRET value acquisition. We were able to collect reliable fluorescence images for dynamic FRET data to reflect conformational changes of VLA-4 during cell mobility.

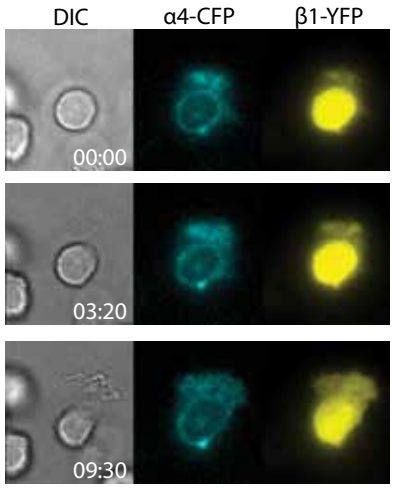

Figure 2: Stable fluorescent images from VLA-4 FRET sensor

To view the full time-lapse video, visit
www.ldgi-xcite.com/applications-movies.php

FRET analysis to determine activation status of VLA-4 during cell spreading

We performed sensitized emission FRET analysis with white-light TIRF microscopy to determine conformational changes of integrin VLA-4 on the plasma membrane during cell spreading on VCAM-1 for 20 min (Figure 3). In the cell spreading experiments, time-lapse analysis of FRET on TIRF microscope indicated that low levels of FRET occurred at the leading edge, which finely reflects that VLA-4 is structurally extended and its α and β subunits become distantly located. CFP and YFP images were acquired every 10 sec for 20 min from a cell expressing VLA-4 FRET sensors, and the data showed no obvious bleaching of fluorescent proteins. We conclude that it was efficacious to use the X-Cite® system for CFP and YFP excitation of VLA-4 FRET sensor.

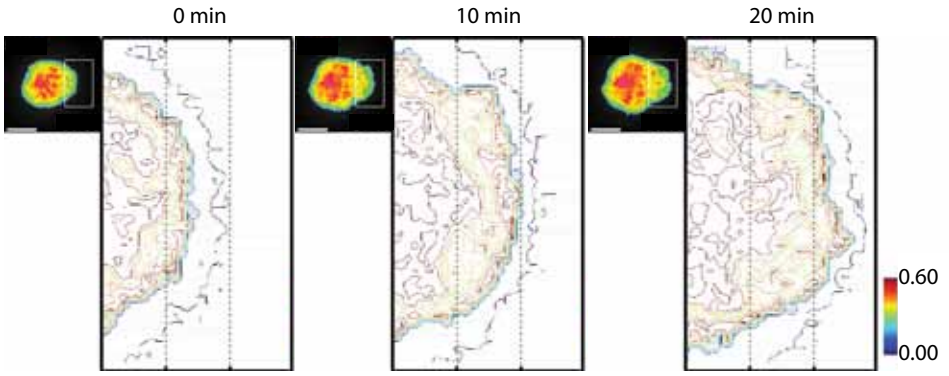

Figure 3: FRET during cell spreading on VCAM-1

SUMMARY

For accurate calculation of the sensitized dynamic FRET signal, it is critical to acquire fluorescence images from the target cells without photobleaching. Unless properly controlled, photobleaching results in inaccurate FRET data values and affects normal cell physiology. Therefore, it was of importance to our work to ensure limited photobleaching occurred during fluorescent excitation and data collection. In this note using white-light TIRF system-coupled FRET method, we established a method for acquiring time-lapse fluorescent images for dynamic FRET calculation without significant photobleaching for 20 min using VLA-4 FRET sensors. Using an X-Cite® 120 mercury lamp instead of a laser, we were able to minimize photo bleaching and acquire reliable FRET data. This experimental design proved to be a stable and accurate method for direct visualization and quantitation of protein dynamics.

REFERENCES

1. I. Riven, E. Kalmanzon, L. Segev, E. Reuveny, Conformational rearrangements associated with the gating of the G protein-coupled potassium channel revealed by FRET microscopy. Neuron 38, 225 (Apr 24, 2003).
2. Y. M. Hyun, H. L. Chung, J. L. McGrath, R. E. Waugh, M. Kim, Activated integrin VLA-4 localizes to the lamellipodia and mediates T cell migration on VCAM-1. J Immunol 183, 359 (Jul 1, 2009).
3. G. W. Gordon, G. Berry, X. H. Liang, B. Levine, B. Herman, Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. Biophys. J. 74, 2702 (1998).
4. N. Ruthardt, D. C. Lamb, C. Brauchle, Single-particle Tracking as a Quantitative Microscopy-based Approach to Unravel Cell Entry Mechanisms of Viruses and Pharmaceutical Nanoparticles. Mol Ther, (Jun 7, 2011).

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