Vertical Sectioning of Molecular Assemblies at Air/Water Interface Using Laser Scanning Confocal Fluorescence Microscopy

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Laser scanning confocal microscopy was applied to collect vertical fluorescence intensity profiles of fluorescent supramolecular assemblies at the air/water interface. For monolayers, fluorescence was collected in the range of a couple of tens of micrometers according to the theoretical thickness of optical section. This technique provides an *in situ* possibility to monitor supramolecular assemblies and assembling at the air/water interface even without resolving in molecular dimensions.

Fluorescence microscopy has become more versatile with laser scanning confocal microscopy (LSCM). Confocal imaging (1) reduces interference from out-of-focus structures, (2) is capable of electronic contrast enhancement, and furthermore (3) offers the possibility of surface profiling.[1] In this work we introduce this methodology to investigate vertical distribution of fluorescent probes at the air/water interface and outline its validity by an example of well investigated interactions between biotin or its derivatives and streptavidin.[2] Furthermore we demonstrate also adsorption behavior of cholesterol-bearing pullulan (CHP), a hydrophobized polysaccharide as developed by ourselves,[3] to lipid monolayers.

The LSCM used in this study was a BioRad MRC-600 in combination with a Nikon Diaphot inverted microscope equipped with a long distance objective (Nikon CF Plan Achromat 10X, NA 0.3). A five-phase stepper motor drive controlled by the MRC-600 software was connected to the fine focus control of the microscope. An argon ion laser operating at 488 nm and a krypton/argon mixed gas laser (both lasers were from Ion Laser Technology Inc. Salt Lake City, UT) operating at 488 and 568 nm were used as excitation sources. Automatic gain and black level settings of the MRC 600 control unit were switched off. For one vertical scan, a stack of two dimensional (2-D) images in x-y planes was recorded with increasing z-value (Figure 1). The stack of intensity integrals of the 2-D images yields in a vertical (z-direction) fluorescence intensity profile of the scanned volume element. With the current set up the x-y plane can be as small as a single line scan (1416 μm) or up to 1416 x 944 μm^2. Therefore, the recording of a stack of 100 2-D images consumes from several minutes down to less than a second for a single line scan. For scanning the air/water interface, a miniaturized Langmuir trough with a quartz bottom (USI-System, Fukuoka, Japan) was mounted on the microscopic stage.

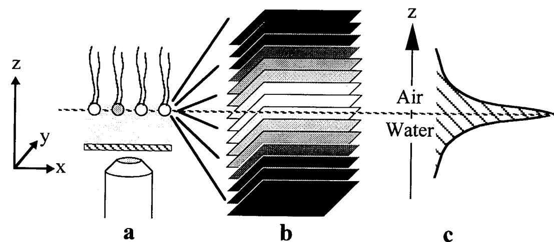

Figure 1. General procedure for obtaining a vertical fluorescence intensity profile. a) Scanning with LSCM; b) stack of 2-D images; c) processing of stack of 2-D images into a fluorescence intensity profile $I(z)$.

As shown before,[4] streptavidin can bind to biotin-conjugated lipids and spontaneously self-organizes to give a thin two-dimensional crystalline layer of the protein at the lipid-water interface. Specific binding of streptavidin to the biotin-lipid can be achieved by injecting the protein underneath the biotin-lipid monolayer. In a desthiobiotin-lipid system, the protein can be replaced by addition of free biotin into the subphase, since the affinity of streptavidin to desthiobiotin is lower than that to biotin.[5] We followed this process by mixing a monolayer of DMPE-desthiobiotin-lipid with a fluorescent lipid N-(Lissamine™ rhodamine B sulfonyl)-1,2-dihexadecanol-sn-glycero-3-phosphoethanolamine (B-DHPE). FITC-streptavidin was injected into the

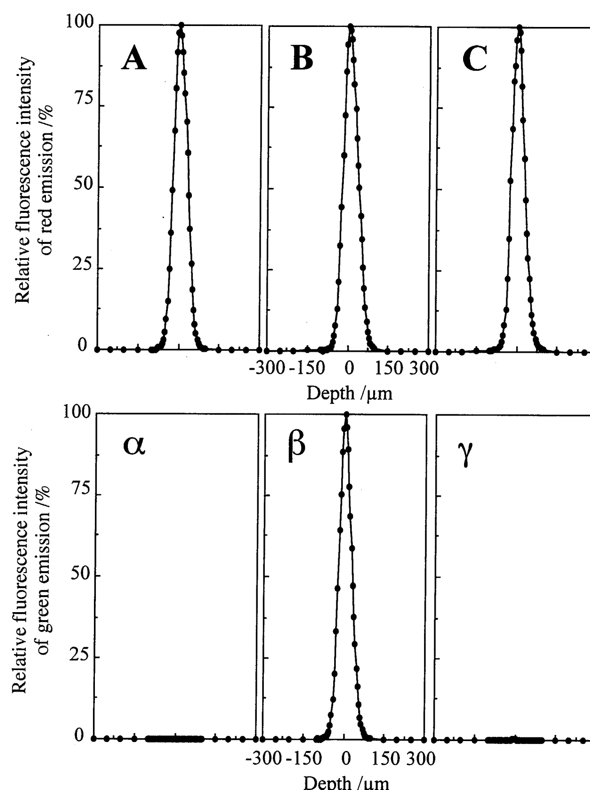

Figure 2. Fluorescence intensity profiles of DMPE-desthiobiotin-lipid monolayer containing 1% (by wt.) of Rhodamine B-DHPE (A, B, C) and FITC-streptavidin (α, β, γ). Profiles A and α, before the FITC-streptavidin injection; profiles B and β, after the injection; and profiles C and γ, after the addition of free biotin (scanned area, 1416 x 944 μm^2).

subphase. Finally, the streptavidin bound to the lipid layer was replaced by the addition of free biotin. The resulting fluorescence intensity profiles of Rhodamine B-DHPE and FITC-streptavidin could be independently detected by two photomultipliers with one vertical scan using the 488 and 568 nm lines of the krypton/argon mixed gas laser. The ternary interaction of the desthiobiotin-lipid/streptavidin/biotin system is carefully documented by the LSCM vertical scans as seen in Figure 2.

Figure 2 represents an example of intensity profiles those we obtained in any scan of fluorescent probe-containing monolayers. Those manolayers can be formed by basically any kind of preparation technique. Fluorescence is detected in the range of a couple of tens of micrometers along the monolayer's vertical section. The point with the highest fluorescence intensity was almost coincident with the air/water interface as it could be seen by focusing onto the water surface. Therefore, the peak position was assigned as zero-point of the vertical scan-depths. The optical section thickness (defined as the distance $Z_{1/2}$ between the focus positions at which the collected intensity is 50% of the peak value) was found to be between 20-25 μm under the present system. This section thickness is well above the confocal effect that can be theoretically expected[6] to be approximately 5 μm with our experimental set-up. This discrepancy is not surprising since the theory assumes a perfectly transparent sample, an ideal lens with perfectly flat field, and no spherical or chromatic aberration. To increase the signal intensity, the confocal aperture in our system was usually set rather large, reducing further the confocal effect. This was, however, unavoidable since caution had to be taken about photo bleaching of the fluorescent probe by the scanning laser beam. Therefore, neutral density filters were used for cutting the laser light up to only 1% transmission leading consequently to weaker emission signals. Photobleaching can additionally be reduced by limiting both the number of single scans per entire vertical scan and the scanned area. In principle, more fluorescent dye in the monolayer leads to higher emission signals as long as any fluorescence quenching does not occur. Therefore, it can be nicely seen that a decrease in the surface area of a given monolayer leads to consecutively stronger emission signals with the vertical scan of the LSCM. In this sense, our new methodology directly applies to what is found by any conventional epifluorescence microscopy.

To demonstrate the versatility of our new methodology, another experiment, the initial interaction between CHP self-aggregate and lipid monolayer, is shown. CHP-50-1.6 (DS, 1.6 per 100 glucose units and Mw 50000 g/mol) was further labeled with fluorescein (DS 0.49 per 100 glucose units, FITC-CHP-50-1.6-0.49) and injected into the subphase of a mixed D_{14}DPC/DMPC lipid monolayer.[7] An increase in the fluorescence intensity at the air/water interface certainly indicates the interaction between the CHP aggregates and the monolayer. In the case that the corresponding parent polysaccharide before hydrophobization (FITC-Pullulan) was used, no increase in the fluorescence intensity was observed at the air/water interface. Clearly from Figure 3 for FITC-CHP, there is an increase in the fluorescence intensity at the air/water interface with time. This reflects a capability of our methodology to monitor the time resolved adsorption of the fluorescent substrate to the air/water interface.

With another investigation of the same system using surface pressure and surface potential measurements,[8] we observed a slow penetration of CHP to the DMPC lipid monolayer. However, our present new methodology could reveal a rather rapid preadsorption of the FITC-CHP-50-1.6-0.49 to the lipid

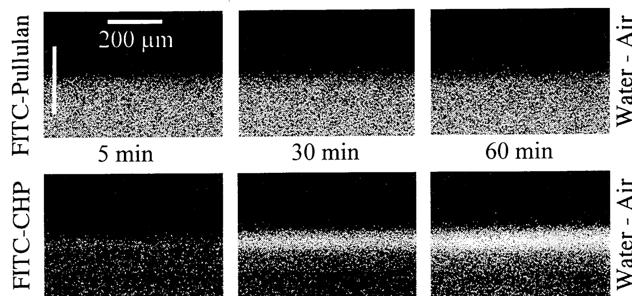

Figure 3. Interaction of FITC-Pullulan (top) and FITC-CHP-50-1.6-0.49 (bottom), (subphase concentration, 5 x 10^{-8} M) with D_{14}DPC/DMPC (60/40 mol%) monolayer (surface pressure, 30 mN/m). Each picture is a reconstruction of a stack of single line scans with a *z*-step wide of 2.5 μm.

monolayer interface. We are currently evaluating this interesting phenomenon and further experimental evidence will be published.

We could reveal that fluorescence from lipid monolayer can be vertically sectioned by scanning laser confocal microscopy over a range of several tens of micrometers. Of course, this method can not directly compare with other analytical tools that can resolve in the molecular range. However, this method offers a striking simple possibility to gain information about the vertical distribution of fluorescent molecules at the air/water interface. The existence of a vertical concentration gradient of fluorescent molecules can be monitored, and quantitative comparisons of different interfacial situations can be also directly studied by means of fluorescence intensity at the air/water interface. Furthermore this method can be easily combined with conventional epifluorescence studies, and any fluorescent molecules are applicable. To our knowledge, such an application of confocal laser microscopy to study monolayer at the air/water interface should be the first in this field.

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References and Notes

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