CHEMISTRY LETTERS, pp. 1079-1082, 1987.

Dynamic Fluorescence Microprobe Method Utilizing Total Internal Reflection Phenomenon

Akira ITAYA, Akihiko KURAHASHI, Hiroshi MASUHARA,*
Naoto TAMAI,† and Iwao YAMAZAKI†

Department of Polymer Science and Engineering,
Kyoto Institute of Technology, Matsugasaki, Kyoto 606
†Institute for Molecular Science, Okazaki 444

An optical set-up for time-resolved total internal reflection fluorescence spectroscopy under a microscope was developed and its performance was examined by using specially prepared polymer films. This microspectroscopic method provides ps dyamic information for a microvolume of 0.1 μm (depth) × 5 μm (diameter).

Microprobe methods characterizing small sections are widely used as indispensable tool in the fields of material science, electronic device technology, and biological science. UV absorption, fluorescence, IR absorption as well as reflection, Raman scattering, and ESCA measurements are possible with commercial instruments where probing electron (or optical beam) is focussed or a microscope is incorporated for choosing a target position and limiting its size.1) Among these, fluorescence microprobe has the highest sensitivity, so that its limit of detection size is the smallest and the quantity the lowest. Depth-profile was also available by adjusting the focal point which gave $\sim$ 3 μm resolution for a time-correlated single photon counting technique.2) In the present work we propose a new fluorescence microprobe method which improves this depth-resolution by one-order of magnitude.

Recently, we reported a time-resolved total internal reflection (TIR) fluorescence spectroscopy for surface photophysics studies.3,4) The organic materials were contacted firmly to a sapphire plate whose refractive index is larger than that of the formers. A laser beam was led to the sapphire under TIR condition and its evanescent wave penetrating into the material was used as an excitation light pulse. The effective thickness of the interface layer excited is in the order of the excitation wavelength and even an information on the thickness of 100 Å was available under a specially selected condition. Monitoring fluorescence of the present surface areas, we could elucidate their photophysical processes and molecular motions in the ns - ps time domains. As an extention of this methodology, we perform this TIR fluorescence measurement under a fluorescence microscope, which enables us to obtain time- and space-resolved data on fluorescence characteristics.

A block diagram of the developed system is schematically shown in Fig. 1, where the TIR excitation condition is also illustrated. Fluorescence microscope Nikon XF-EFD equipped with an adaptor PFX or Olympus BHS-RFK-A was chosen, while their internal optics for excitation beam was not used, since it absorbs a UV beam to some extent. We selected a long-distance-working objective lens Nikon ELWD M Plan 40 or Olympus ULWDCDPL40X which gives a more space between the lens and the sample compared to the conventional lens system, and makes it possible to set the coated sapphire system. Incident angle θ was adjusted by inclining the laser mirrors and by sliding the rotating stage on which the mirror is mounted. The beam diameter was reduced to 1 mmϕ with an aperture. On the top of the microscope an X-Y stage with a 1 mmϕ diaphragm was set in order to choose the microsection whose fluoresence dynamics we probe. A HTV R2809U-01 microchannel plate photomultiplier was used as a fast-response detector. All these optical instruments were designed and constructed in the present work. When the two-dimensional resolution was examined, the film sample on the quartz plate was set instead of the sapphire sample and it was illuminated from the upper-right side. Excitation laser, detection system, preparation of polymer films, and other conditions were the same as used previously.[4)]

First we describe the depth information which was confirmed by examining the bilayer model systems. A thin surface (S) and a thick bulk (B) films had the thickness of 0.4 and a few μm, and were doped with N-ethylcarbazole and 9-ethylphenanthrene, respectively. As the refractiove indices of the sapphire and the polystyrene film are 1.81 and 1.68 at 313 nm, respectively, the critical angle (θ_C) of TIR phenomenon was calculated to be 68.15.

The fluorescence decay curves of the model film were measured by using a UV-33 filter whose results are shown in Fig. 2. The fluorescence lifetime of N-ethylcarbazole and 9-ethylphenanthrene in polystyrene matrix was obtained to be 12 and 40 ns, respectively. A deviation from a linear relation was ascribed to impurities contained in polymer film. Just above θ_C, the short decay component of the S film was slightly detected, while it was greatly enhanced by a little increase of θ. These decay curves of the model film were reproduced by a two-exponential function; $F(t)=F_S\cdot\exp(-t/\tau_S)+F_B\cdot\exp(-t/\tau_B)$. Here, τ_S and τ_B are the time-constants of S and B films and were confirmed to be identical to the lifetime of fluorophores doped. F_S and F_B represent the fluorescence contribution in the monitoring wavelength region and vary depending upon absorbance at the excitation wavelength and fluorescence yield. In order to obtain a more quantitative data, the values of F_B/F_S were plotted against θ in Fig. 3. A large decrease of F_B/F_S was observed in the angle region from $<\theta_C$ to θ_C +1° and an almost constant value was attained above θ_C+2°. Since this relation between F_B/F_S vs. θ is the same as obtained under the TIR condition without a microscope,[4)] We have come to the conclusion that the present optical system works well and the time-resolved TIR measurement combined with the fluorescence microscope is practically possible.

The two-dimensional resolution of the present optical system was examined by using a polymer film microfabricated with the 308 nm excimer laser. A cast

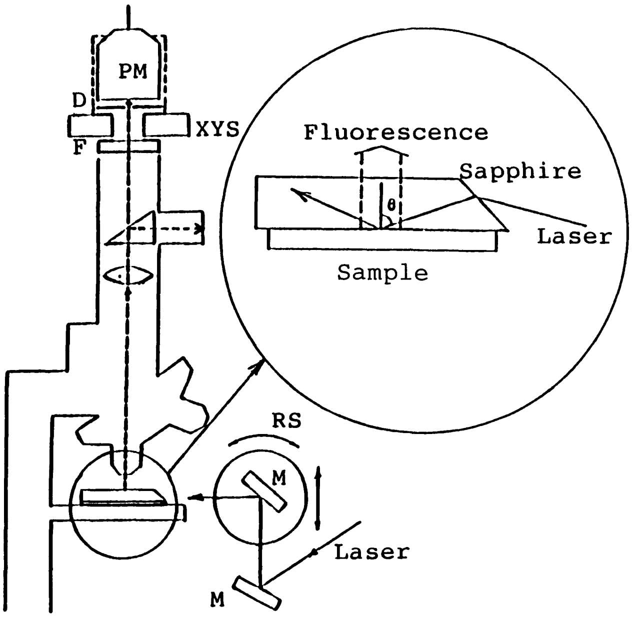

Fig.1. Schematic diagram of ps fluorescence microprobe apparatus.
PM : Photomultiplier,
D : Diaphragm, XYS : X-Y stage,
F : Filter, M : Laser mirror,
RS : Rotating stage

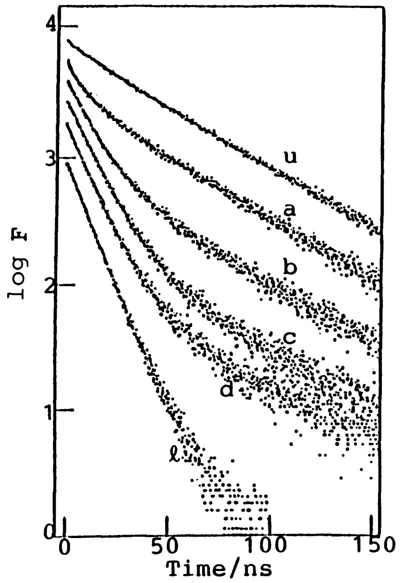

Fig.2. Fluorescence decay curves of the bilayer system of 0.4 μm S film doped with 4.8 $\times 10^{-3}$ mol N-ethylcarbazole per mol phenyl group and the thick B film doped with 5.0 $\times 10^{-3}$ mol 9-ethylphenanthrene per mol phenyl group. Incident angles θ are (a) θ_c-0.16°, (b) θ_c+1.09°, (c) θ_c+1.49°, and (d) θ_c+2.28°. The upper (u) and lower (ℓ) curves were due to B and S films, respectively.

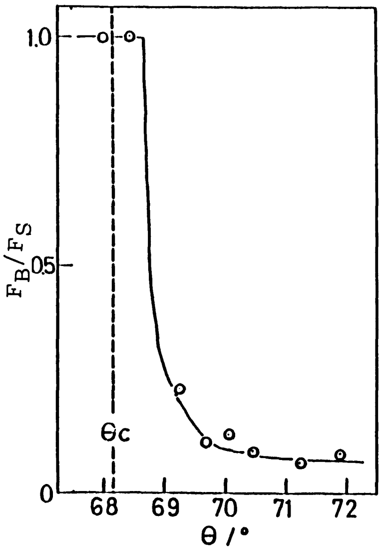

Fig.3. Relation between θ and the ratio of fluorescence intensity of the B film over that of the S film. The system is the same as that in Fig. 2.

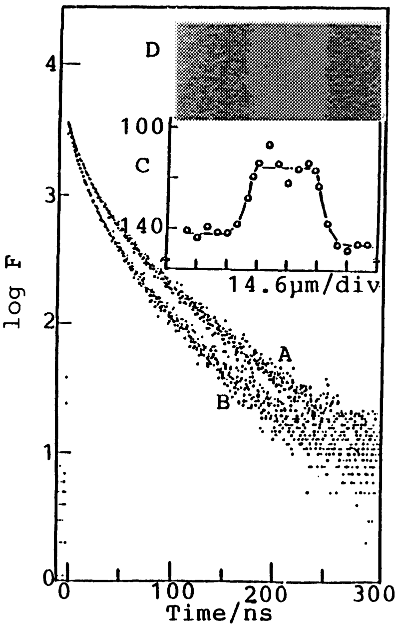

Fig.4. Fluorescence decay curves of ablated (A) and nonablated (B) areas of PMMA film doped with 7.75×10^{-2} mol pyrene per mol MMA unit. Cross section of ablated PMMA film as revealed by analyzing fluorescence data (C) and its optical micrograph (D).

poly(methyl methacrylate) film containing 1-ethylpyrene was ablated with a photo-lithographically prepared mesh mask in contact mode.[5)] In the ablated area, concentration and distribution of the dopant and its microenvironment were affected very much, which can be monitored by fluorescence measurement.[6)] Here the fluorescence decay curves were used for reproducing an etched pattern, although the molecular mechanism how laser ablation modify fluorescence dynamics of the film are being studied.

In Fig. 4 typical decay curves of 1-ethylpyrene aggregates in the film are given, where fluorescence was measured with Toshiba UV 33 and Hoya U 330 filters. It is clear that a difference was induced by laser ablation. By shifting the diaphragm of the microscope, fluorescence decay curves were measured as a function of the horizonal position. We plotted the time when the intensity became one-twentieth of the initial one against the position in Fig. 4 (C). The values below 120 ns and around 140 ns correspond to the masked and the ablated areas, respectively. Therefore, it is concluded that the present result represents the cross section of the masked area. The width of nonablated area was obtained to be $\sim$ 37 μm, which is in agreement with the mask dimension.

For the first time, we have demonstrated a time-resolved TIR fluorescence measurement under a microscope. The practical performance of the present method is summarized as follows; (1) depth-resolution, 0.1 μm, (2) two-dimensional resolution, 5 μm, (3) time-resolution, 10 ps, and (4) wavelength resolution, 10 nm by using an interference filter. Each resolution is easily improved if a measuring time longer than a few tens of minutes is permitted. We believe that the present new methodology will be fruitful for dynamic studies on microfabricated materials, biological tissues, cells, fabrics, and so on.

The present work was partly suppported by the Grant-in-Aid for Developmental Scientific Research from the Japanese Ministry of Education, Science, and Culture to HM and IY (59850146).

References

1) T. Hirshfeld, "Microbeam Analysis 1982," ed by K. F. J. Heinrich, San Francisco Press, San Francisco (1982).

2) T. Minami, M. Kawahigashi, Y. Sakai, K. Shimamoto, and S. Hirayama, J. Luminescence, 35, 247 (1986).

3) H. Masuhara, N. Mataga, S. Tazuke, T. Murao, and I. Yamazaki, Chem. Phys. Lett., 100, 415 (1983).

4) H. Masuhara, S. Tazuke, N. Tamai, and I. Yamazaki, J. Phys. Chem., 90, 5830 (1986).

5) H. Masuhara, H. Hiraoka, and K. Domen, Macromolecules, 20, 450 (1987).

6) K. Kurahashi, Mc Thesis, Kyoto Institute of Technology (1987).

(Received February 28, 1987)