Direct Observation of Single-Molecule Generation at a Solid–Liquid Interface

Peng Zhang and Weihong Tan*[a]

Abstract: Direct observation of single-molecule generation from a chemical reaction was achieved at a solid–liquid interface. The reaction between fluorescamine and immobilized *N*′-(3-trimethoxysilylpropyl)diethylenetriamine (DETA) was studied at the single-molecule level. Time-lapse fluorescence images of single-molecule products, excited by the evanescent field generated at a quartz–liquid interface, were recorded to follow the chemical reaction to its completion. The reactions were restricted to the approximately 1 nm thick layer nearest to the interface. Analysis of the photoelectron intensity of the fluorescent product of the reaction and its distribution shows that the reaction kinetics goes through a transition from zeroth-order to first-order as the reaction proceeds. This approach offered a novel means to study single-molecule reactions at the solid–liquid interface. It also enabled the investigation of reaction kinetics and chemical mapping of surface heterogeneity at the single-molecule level.

Keywords: DETA · evanescent wave · fluorescence · fluorescamine · single molecule

Introduction

Single-molecule studies have drawn considerable interest recently.[1, 2] A variety of single molecule detection (SMD) techniques have been developed by several groups.[1–5] The stochastic nature of molecular behavior and reactions in solution has been revealed by a number of studies using these techniques. Single enzyme molecule activities and their heterogeneity have also been observed.[6] Although single-molecule manipulation has been attempted,[7] chemical reactions at a single-molecule level have yet to be observed directly. The challenge for SMD in solution is to extract signal from high background due to light scattering and fluorescent impurities.[1] There are a number of ways to increase the signal-to-noise ratio. One approach is to improve the detection capability of SMD systems by using avalanche photodiode (APD),[1–3] intensified charge coupled device (ICCD) or a refinement of the arrangement of the optics.[5] Another approach is to work with a very small probe volume (less than 10^{-12} L) to reduce background signal. The observation or excitation volume can be confined optically by the use of a confocal microscope[4] or evanescent wave excitation.[5–7]

Evanescent wave field is usually generated by total internal reflection at an interface. Its intensity decays exponentially from the interface, resulting in a penetration depth of less than half of the wavelength of the incident beam.[5] The scheme of exciting single fluorophores in the evanescent field has been employed by a few research groups.[5] Funatsu et al. used a refined total internal reflection fluorescence microscopy to visualize single fluorophores in solution and observed ATP turnover reaction. Dickson et al. reported evanescent wave excitation at the boundary of a cover slip and a polyacrylamide gel for the detection of fluorophores diffusing in and out of the gel. With an ICCD, Xu and Yeung measured the diffusion and photodecomposition of single molecules in solution, and studied the electrostatic trapping of protein molecules at a solid–liquid interface. In all the methods mentioned above, an optical prism based system has been used effectively as the waveguide for the generation of evanescent field.

Since it was first introduced in 1972,[8] fluorescamine has been used extensively as an effective reagent for the fluorometric quantitation of primary amines. This non-fluorescent compound reacts with primary amines to form pyrrolinones, which upon excitation at 355 nm emit strong fluorescence from 475 to 490 nm. The reaction with fluorescamine proceeds efficiently in aqueous solutions and allows the assay of submicromolar concentrations of amines, notably those of biological importance. Since this reaction has two non-fluorescent reactants and a fluorescent product, it becomes an excellent tool for optically monitoring reaction at a single-molecular level.

Similar to the single-molecule detection, an extremely small reaction vessel will have to be developed, in which the

[a] Prof. W. Tan, P. Zhang
Department of Chemistry and UF Brain Institute
University of Florida
Gainesville, FL 32601 (USA)
Fax: (+1) 352-392-4651
E-mail: tan@chem.ufl.edu

  0947-6539/00/0606-1087 $ 17.50+.50/0

chemical reaction proceeds, in order to observe single-molecule reactions between two reactants efficiently. By immobilizing the amine molecules, *N*′-(3-trimethoxysilylpropyl)diethylenetriamine (DETA), onto a silica surface, which restricts the reaction volume for the reaction between DETA and fluorescamine to be sub-attoliter ($<10^{-18}$ L), one can greatly increase the probability of observing interactions between these two different molecules at a single-molecule level. We then monitor the progress of the reaction by detecting the fluorescence of the product at the interface. This was achieved by the well developed evanescent wave configuration.

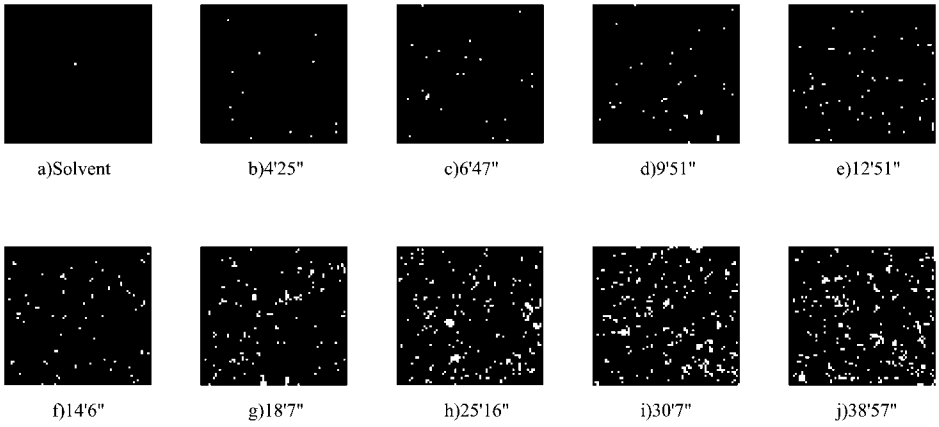

Figure 1. Subframe images (50×50 pixels) of fluorescence from the a) solvent and b)–j) reaction product. Concentration of fluorescamine solution was 1×10^{-5} M. Images were acquired with a 30 ms exposure time. Time zero corresponds to the moment the DETA-coated cover slip touched the fluorescamine solution. See text about the cutoff threshold selection.

Using the reaction between fluorescamine and DETA, direct observation of single-molecule generation from a chemical reaction was achieved at a solid–liquid interface. The reactions were restricted to the approximately 1 nm thick layer next to the interface. The fluorescent signals generated by the newly formed complex fluorophores are detected by an ICCD based microscope system. This approach offers a novel means to study single-molecule reactions at the solid–liquid interface and also enables the investigation of reaction kinetics at the single-molecule level.

Results and Discussion

Single-molecule generation at a liquid–solid interface by a chemical reaction: Figure 1 shows a series of fluorescence images taken (30 ms integration time each) at different times after the reaction of the 1×10^{-5} M fluorescamine solution and DETA-immobilized cover slip started. Figure 1a was taken under the same conditions with only the solvent (DMF). Figures 1b–1j were acquired at different times of the reaction. A photoelectron count distribution can be obtained from Figure 1a. A cutoff threshold (in this case 21 photoelectron counts) was chosen at a value of three times the standard deviation (3σ) above the mean value of photoelectron counts for this image of blank solvent.[9] This cutoff threshold was then applied to other images. Bright spots in the images indicate the photoelectron counts in those spots are higher than the cutoff threshold, and correspond to signal generated by single fluorescent product molecules by the reaction.

As shown in Figure 1, there were few bright spots in Figure 1a, which is expected. Assuming the background noise has a normal distribution, the threshold count (mean value plus 3σ photoelectron) should cut off 99.7% of the noise.[10] There are a few bright spots in Figure 1b, each corresponding to the fluorescent signal generated by one reaction product molecule. The number of bright spots increased with time and approached a constant value as the reaction proceeded. Fluorescence images in Figures 1b–1j represent the progress of the reaction from single molecular level towards completion.

Single-molecule criterion: There are a few reasons to support the conclusion that single fluorophore molecules were indeed imaged at the beginning of the reaction. The detection volume and the concentration only enabled at most one molecule at each pixel, with the vast majority of the pixels without any molecules. Each pixel in the ICCD images represents a square with 0.4 μm edges, as calibrated by a standard sample through the same microscope objective. The distance between the primary amine group of the immobilized DETA molecule and the silica surface is approximately 1 nm, which means that only fluorescamine molecules that come within this distance would be able to react with the immobilized DETA molecules. The reaction volume for each pixel can then be estimated to be 0.16 aL (0.16×10^{-18} L). With a fluorescamine concentration of 1×10^{-5} M, an expected number of fluorescamine molecules per pixel is 0.96. Thus, at a fluorescamine concentration of 1×10^{-5} M or lower, the molecules will be isolated from each other within the images taken when the reaction started. One important point worth noticing is that single-molecule detection for a chemical reaction is very different from single-molecule detection of a fluorophore. The existence of one reactant molecule (fluorescamine in this case) in the reaction volume does not guarantee one fluorescent product molecule. There is equilibrium between the reactant molecules and the product molecules,[11] that is, not every fluorescamine molecule would react to produce one fluorescent product molecule. Thus, statistically, even with one fluorescamine molecule per pixel, the fluorophore generated by the reaction will be much less than one per pixel. In addition, due to the low optical detection efficiency of about 5%,[9] the number of fluorophore molecules per pixel that can be detected is much smaller than the total number of generated fluorophores, that is, the probability of detecting one fluorophore in one pixel is less than 5%. Usually, the small detection volume and the fluorophore concentration are

0947-6539/00/0606-1088 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 6

used to claim single-molecule detection in solution,[3, 5, 6] which is mainly a statistical argument with a high confidence of the conclusion.

There is other evidence that single molecules are indeed generated and imaged in monitoring the progress of the chemical reaction. Similar experiments with a 1×10^{-6} M fluorescamine solution were also performed in the study. Notably, even though much fewer bright spots were observed at the same time intervals with the 1×10^{-6} M solution than that for the 1×10^{-5} M fluorescamine solution, the intensity of the bright spots are in the same photoelectric signal level. Figure 2, left, is taken at 4′55″ after the immobilized DETA

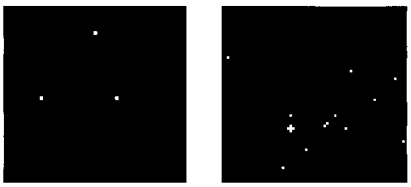

Figure 2. Subframe images (50×50 pixels) of fluorescence from the reaction product. Left: fluorescamine concentration of 1×10^{-6} M at 4′55″, and right: fluorescamine concentration of 1×10^{-5} M at 4′30″. Other experimental conditions and cutoff threshold selections remain the same as in Figure 1.

touched the 1×10^{-6} M fluorescamine solution, while Figure 2, right, is taken at 4′30″ after the immobilized DETA touched the 1×10^{-5} M fluorescamine solution, both with 30 ms exposure time. We choose to show the results obtained with 1×10^{-5} M fluorescamine solution because there were too few bright spots to perform reliable reaction kinetics analysis for the reaction with the 1×10^{-6} M fluorescamine solution.

A controlled experiment was done to confirm the above estimation. In the solution phase, different concentrations (1×10^{-5} M and 1×10^{-7} M) of fluorescamine solution and DETA solution were first mixed and placed on a clean cover slip sitting on the microscope stage. The DETA-coated cover slip in the previous experimental setup was then replaced by a clean cover slip in the same position, while all other experimental conditions remained the same as those for the solid-phase experiments. The results are shown in Figure 3. The threshold was determined the same way as previously described. Figure 3, left, showed that a solution with a fluorescamine concentration of 1×10^{-5} M led to many bright spots, in great contrast to those shown in Figure 1. Only when

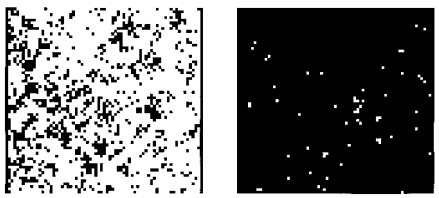

Figure 3. Subframe images (50×50 pixels) of fluorescence from the mixtures of fluorescamine and DETA by using a clean silica glass in place of the DETA-coated silica glass. Left: fluorescamine concentration of 1×10^{-5} M, and right: fluorescamine concentration of 1×10^{-7} M. Other experimental conditions and cutoff threshold selections remain the same as in Figure 1.

the fluorescamine concentration was decreased to about 1×10^{-7} M, were the bright spots corresponding to single fluorophore molecules in the image able to be resolved (Figure 3, right).

In this controlled experiment, the reaction was already completed before the fluorophore molecules were being observed. It is in essence a single-molecule detection experiment. The detection zone in this case is defined by the total internal reflection of the laser beam, which excites fluorescent product molecules within the depth of evanescent field generated at the liquid–solid interface. The estimated 150 nm depth (calculated based on the incident angle of the laser and the reflection indexes of the glass and the solution) of the evanescent field,[5] thus defines a detection zone of 25 aL (25×10^{-18} L) per pixel[5] (compared with the reaction volume of 0.16 aL per pixel in the immobilized DETA experiment). Following the similar estimation as before, the number of fluorophore molecules per pixel for both 1×10^{-5} M and 1×10^{-7} M fluorescamine solution will be 150 and 1.5, respectively. In this case, the bright spots in Figure 3, left, should not be taken as signals from single fluorophore molecules even after taking into account the 5% detection efficiency, while those in Figure 3, right, are likely from single fluorophores.

Note that the intensity of photoelectron counts for the bright spots in Figures 1 and 2 is approximately the same. This further supports the conclusion that these signals from the bright spots in the images were due to single fluorophore molecules. Since all these signals are relatively weak, much lower than the saturation level for the ICCD detector, it is impossible that the above observation is an artifact caused by the threshold settings. The intensity level of these signals is comparable to those SMD studies adopting a similar configuration, taking into consideration that the integration time for the ICCD detector was only 30 ms in our case versus 100–200 ms as reported in those studies.[5] The shorter integration time was chosen for this experiment in order to capture the dynamics of the reaction and to reduce photobleaching of the product molecules.

Single molecule reaction kinetics: Using the single-molecule images obtained in this experiment, we are able to study the reaction kinetics at a single-molecule level. Fluorescence images in Figures 1b–1j represent the progress of the reaction from single molecular events towards completion. This can be further elaborated by the histograms for these images, as shown in Figure 4. The distribution of the photoelectron counts of the images shift overall towards higher intensity as the reaction proceeds, which is as expected. However, the shape of the distribution also changes as the reaction proceeds. There is a peak at the value of 23 photoelectron counts being formed starting at the histogram corresponding to Figure 1e. The peak formation indicated that the histogram was likely the sum of two photoelectron distributions, one for single molecules and the other for two molecules.[9] Only when there are a significant number of two molecule events would there be a peak for two molecules appearing in the histogram.

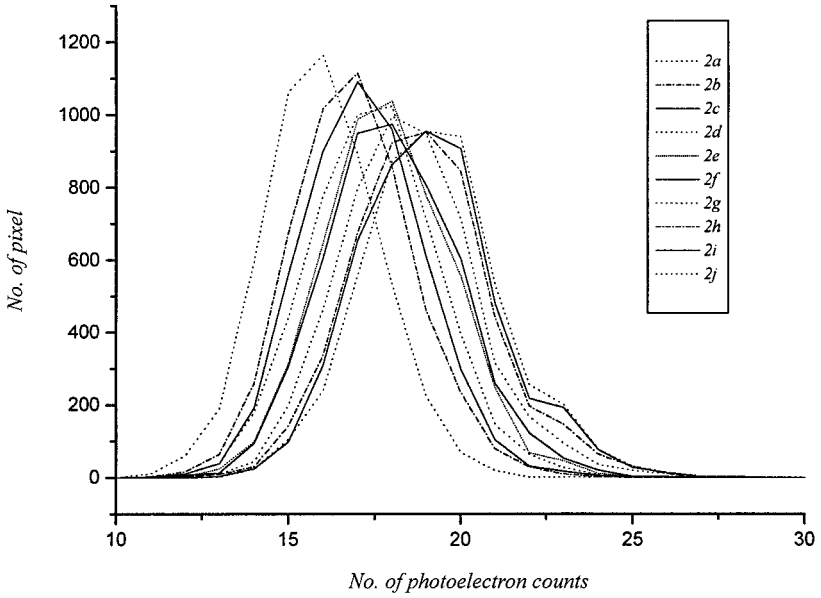

Figure 4. Histogram of photoelectron counts for images shown in Figure 1. Note that a peak started to form at 23 for the photoelectron counts after image e).

The reaction between fluorescamine and amines is believed to be rapid and reversible, proceeding through an intermediate that subsequently rearranges relatively slowly in a multistep sequence to the final fluorophore form.[8] In the solution reaction with fluorescamine in excess, the reaction kinetics was thought to be first-order with respect to amine.[8] This would lead to a linear relationship between $\ln(f_{max} - f_t)$ and time t, where f_{max} is the maximal fluorescence and f_t is the fluorescence at any given time t.

In our experiments, the situation could be somewhat different since the reaction takes place at the solid–liquid interface. Consider the reaction: F+A $\rightarrow$P, where F and A stand for fluorescamine and DETA, P for product. The total fluorescamine molecules in the solution, trapped between the two silica cover slip, are in excess relative to the DETA molecules immobilized in the area that has contact with the solution. Thus, we can assume the concentration of fluorescamine, [F], remains constant throughout the reaction. The integrated rate equation describing the amount of product P formed by time t after the start of the reaction is: $[P] = [A](1 - e^{-kt})$, where [A] is the surface density of immobilized DETA and k is the rate constant. This indicates a linear relationship between $\ln([A] - [P])$ and t.

If a photoelectron count cutoff value of 21 was chosen for the histograms in Figure 4, which was 3σ above the mean value of the photoelectron counts for the background image (Figure 1a) as described earlier, the total number of bright spots, N, with intensity above the cutoff can be obtained for each image. We take the maximal number of bright spots when the reaction completes as M in place of [A], N in place of [P], and plot $\ln(M - N)$ versus t, as shown in Figure 5, bottom. For comparison, N versus t is plotted in Figure 5, top. The correlation coefficients for the linear fits in Figure 6a and Figure 6b are 0.995 and 0.996, respectively.

It appears that data points in Figure 5, top, can be separated into two categories. The first four points exhibit a linear relationship versus reaction time, suggesting a zeroth-order reaction. Similarly, in Figure 5, bottom, the last five points have a good linear fit, suggesting a first-order reaction with regard to amines. This indicates that the reaction may progress through two types of reaction kinetic behavior. We suggest that the reaction is diffusion-limited at the early stage, and thus zeroth-order. As more and more fluorescamine molecules effectively diffuse to the interface, which results in pre-concentration of fluorescamine on the surface, the reaction conforms to first-order as suggested in ref. [8].

The existence of the two-molecule events and the transition from zeroth- to first-order reaction may not be coincidental. When the reaction is diffusion-limited, the density of the fluorophores at the interface is so low that the probability of having two molecules in the same area of one pixel is extremely small. Because of the stochastic nature of single-molecule behavior, reaction kinetics from the single-molecule domain reveals interesting information of the transition of the chemical reaction.

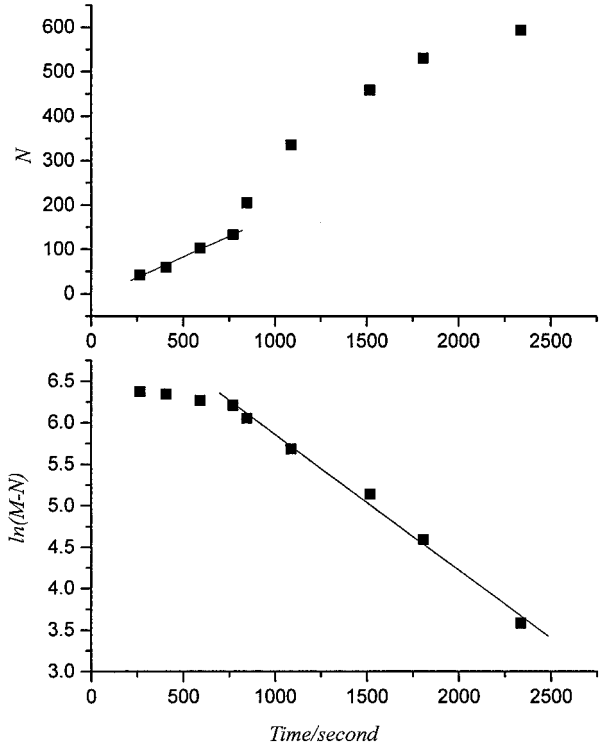

Figure 5. Plots of N vs. t (top) and $\ln(M - N)$ vs. t (bottom). Data were obtained from the histograms, Figure 4, with a photoelectron count cutoff of 21.

0947-6539/00/0606-1090 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 6

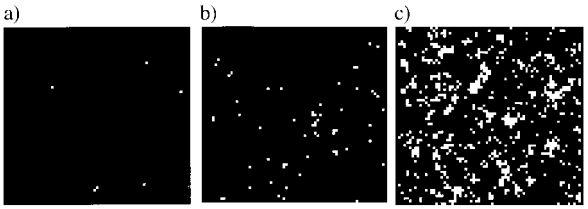

Figure 6. Subframe images (50×50 pixels) of fluorescence from the mixtures of fluorescamine and DETA. The integration times for the ICCD are a) 15 ms, b) 30 ms and c) 100 ms, respectively. Other experimental conditions were the same as in Figure 3.

Similar results were obtained with acetonitrile and DMSO as solvents for fluorescamine reaction. Overall, the reaction we observed at the interface was much slower (roughly 1000 times slower) than that in a solution.[8] This is probably due to a) different physical environments for the reaction, b) different reactivity since amine is covalently bound to a silica surface, and c) the solvents. Different solvents have great influence on the reaction rate of this reaction. The reaction rate was reduced several orders of magnitude when fluorescamine was dissolved in methanol.[8] Only organic solvents were used in our experiments, as compared with the aqueous/organic solvents mixtures described in the literature.[8] We found that the reaction proceeded much slower in DMSO than in DMF and acetonitrile. This is consistent with our observation of diffusion-limited reaction kinetics since fluorescamine diffusion in DMSO is slower than that in DMF.

Similar to the single-molecule detection of dye molecules,[12] the widening of pixel size due to the diffusion of fluorophores can be observed in some images, especially for those with longer exposure time. Such effect can be seen clearly from Figure 6, where three images were taken from the same solution with exposure times of 15 ms, 30 ms and 100 ms. Other experimental conditions were the same as those in Figure 3, right. While adjacent bright spots are barely seen in Figure 6a, bright spots occupying more than one pixel are common in Figure 6c. For the first few images in Figure 1, there was little widening of the bright spot since the product fluorophores were supposed to be fixed on the surface. The number of molecules in all images in Figure 6 increased with integration times. This is due to the fact that longer integration time results in lower detection limit. Therefore, more molecules were detected when a 100 ms integration time was used than that for the 15 ms.

Attempts have been made to follow the trajectory of the single fluorophores. We observed that some bright spots stayed in two consecutive frames. However, the majority of the bright spots disappeared from one image to another shown in Figure 1. Note that the images in Figure 1 are well separated in time sequence. There are two possible reasons for the above observation. First, it is likely that the fluorophore being in equilibrium with other forms of the reaction product. It was reported that only one form of the product is fluorescent.[8] This adds complexity to the continuous monitoring of the product fluorophores. Second, there was photobleaching of the product molecules. Even with 30 ms exposure time for imaging, a portion of the surface-bound fluorophores were probably photobleached or photodecomposed before the next frame was taken. Bulk solution experiments have shown photobleaching does occur under our experimental conditions.

The imaging of single-molecule reactions at the solid–liquid interface opens the possibility of studying surface heterogeneity in chemical reactivity and activity. As shown in Figure 1, there were certain areas of the surface, such as the one on the top right corner of the image, where no fluorescent product was observed. This indicates that there were no amine functional groups in these areas. On a microscopic level, we also observed certain areas where there have many amine groups, resulting in highly fluorescent product molecules formed on the surface. Together with kinetics studies, it would be feasible to map the reactivities of a variety of surface-immobilized molecules on a single molecular basis.

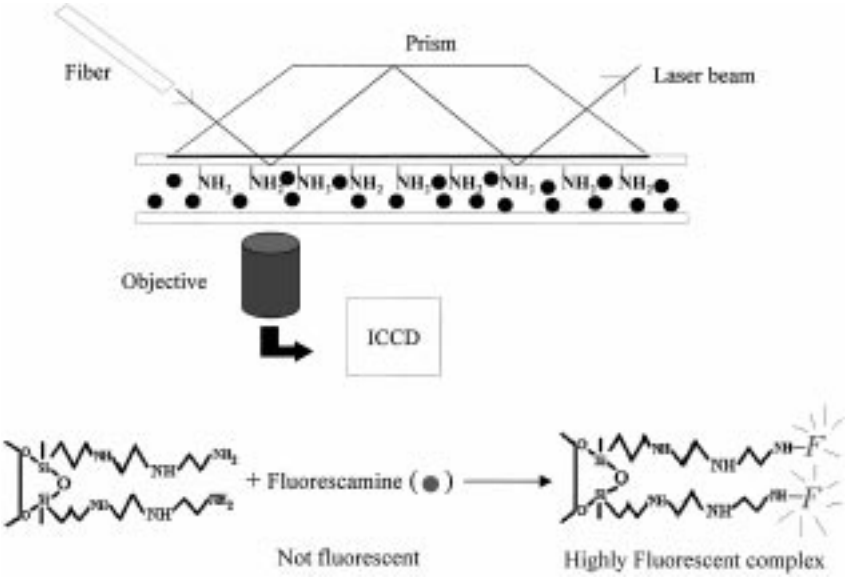

Figure 7. Schematic diagram of the instrumental setup.

Conclusion

In conclusion, we have developed a novel method for the study of chemical reaction kinetics at the single-molecule level. For the first time, we have directly observed single-molecule generation from a reaction between fluorescamine and immobilized DETA at a solid–liquid interface. The results suggest that there might be some unique properties that can only be studied by single molecule reaction kinetics. Our method could be extended to study biochemical reactions of interesting biological processes on a microscopic scale, and to map surface activities and interac-

tions on a single-molecule basis. Furthermore, if reactant(s) and product are both fluorescent, albeit at different emission wavelengths, trajectories of both species could be monitored, which should yield important insights into the stochastic nature of biochemical interactions and reactions at a single molecular level.

Experimental Section

The primary amine group (DETA) was immobilized onto a clean silica glass cover slip, following procedures developed in this lab.[13] In brief, the cover slip was first activated with concentrated sulfuric acid. A layer of DETA (United Chemical Technologies Inc., PA) was immobilized onto the silica surface by immersing the pre-activated glass in DETA solution overnight. The silica substrate was then dried and fixed at 100 °C in an oven for 5 min before being used. Fluorescamine (Acros, NJ) was used as purchased to prepare solutions with different concentrations. Because of the hydrolysis of fluorescamine in water,[8] non-aqueous solvents were used for this study. The results shown here were obtained with anhydrous DMF used as solvent.

Figure 7 shows the scheme of the experimental setup. The amine-immobilized silica glass cover slip was attached to a trapezoid-shape quartz prism (Harrick Scientific, NY) through an index matching oil. A homemade module that allows adjusting the position of the optical fiber and the prism with the amine-coated cover slip simultaneously was placed onto the microscope stage. An inverted microscope (Olympus, IX 70) was used for the optical measurements. The UV laser beam ($\lambda = 340-370$ nm) from an Innova 307 Ar^+ laser (Coherent Laser, Santa Clara, CA) was coupled to an optical fiber through an optical fiber coupler (Newport Corp, Irvine, CA), and entered from one side of the prism at an angle that allows total internal reflection (TIR) to take place in the bottom surface of the prism, which was used as the illumination source for fluorescent imaging. The laser power was 30–50 mW. The first spot of TIR with a diameter of approximately 400 μm was imaged by a microscope through a 60× objective (NA = 0.7). Fluorescence was detected by a cooled, 512 × 512 pixel ICCD (Princeton Instruments, NJ). A combination of filters (long-pass 400 nm and band-pass 455 nm, Oriel Corp.) was used to collect the desired signal. All measurements were carried out in dark room.

The reaction between DETA and fluorescamine started when the module was lowered such that the amine-coated cover slip was in touch with a drop of fluorescamine solution, held by a clear cover slip sitting on the microscope stage. Fluorescent images were collected at different times in respect to the moment when two reactants first got into contact. The laser beam was blocked when not collecting images in order to minimize the photobleaching of the fluorophore molecules (product). This setup has been successfully applied to achieve single-molecule imaging and detection, where single dye molecules (rhodamine 6G and fluorescein) and dye labeled biomolecules were imaged and investigated.[12]

Acknowledgement

We thank Drs. Xiaohong Fang and Dan Purich for helpful discussions. W.T. thanks the Beckman Foundation for a Beckman Young Investigator Award and Cortrell Scholar Award. This work is supported by NSF Faculty Career Award CHE-9733650 and by Office of Naval Research Young Investigator Award N00014-98-1-0621.

[1] T. Hirschfeld, *Appl. Opt*. **1976**, *15*, 2965; N. J. Dovichi, J. C. Martin, J. H. Jett, R. A. Keller, *Science* **1983**, *219*, 845; W. B. Whitten, J. M. Ramsey, *Anal. Chem*. **1991**, *63*, 1027; R. A. Keller, W. P. Ambrose, P. M. Goodwin, J. H. Jett, J. C. Martin, M. Wu, *Appl. Spectrosc.* **1996**, *50*, A12–A32; S. Nie, R. Zare, *Annu. Rev. Biophys. Biomol. Struct*. **1997**, *26*, 567; W. E. Moerner, P. F. Barbara, *Acc. Chem. Res.* **1996**, *29*, 561; C. Eggeling, J. Widengren, R. Rigler, C. A. M. Seidel, *Anal. Chem*. **1998**, *70*, 2651.

[2] E. Betzig, R. J. Chichester, *Science* **1993**, *262*, 1422; X. S. Xie, R. C. Dunn, *Science* **1994**, *265*, 361.

[3] B. Rotman, *Biochemistry* **1961**, *47*, 1981; A. Castro, F. R. Fairfield, E. B. Shera, *Anal. Chem*. **1993**, *65*, 849; Y. H. Lee, R. G. Maus, B. W. Smith, J. D. Winefordner, *Anal. Chem*. **1994**, *66*, 4142; U. Mets, R. Rigler, *J. Fluoresc*. **1994**, *4*, 259; S. Nie, D. T. Chiu, R. N. Zare, *Science* **1994**, *266*, 1018.

[4] J. J. Macklin, J. K. Trautman, T. D. Harris, L. E. Brus, *Science* **1996**, *272*, 255; S. B. Smith, P. K. Aldridge, J. B. Callis, *Science* **1989**, *243*, 203; F. R. Fan, A. J. Bard, *Science* **1995**, *267*, 871; M. M. Collinson, R. M. Wightman, *Science* **1995**, *268*, 1883.

[5] T. Funatsu, Y. Harada, M. Tokunaga, K. Saito, T. Yanagida, *Nature* **1995**, *374*, 555; R. M. Dickson, D. J. Norris, Y. Tzeng, W. E. Moerner, *Science* **1996**, *274*, 966; X. Xu, E. Yeung, *Science* **1997**, *275*, 1106; X. Xu, E. Yeung, *Science* **1998**, *281*, 1650.

[6] Q. F. Xue, E. Yeung, *Nature* **1996**, *373*, 681; D. B. Craig, E. A. Arriaga, J. C. Y. Wong, H. Liu, N. J. Dovichi, *J. Am. Chem. Soc*. **1996**, *118*, 5245; W. Tan, E. Yeung, *Anal. Chem*. **1997**, *69*, 4242.

[7] T. T. Perkins, D. E. Smith, S. W. Chu, *Science* **1994**, *64*, 819; P. Avouris, *Acc. Chem. Res.* **1995**, *28*, 95; W. Tan, X. Wang, *Thin Solid Film* **1998**, *331*, 189.

[8] S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber, M. Weigele, *Science* **1972**, *178*, 871; S. Stein, P. Bohlen, J. Stone, W. Dairman, S. Udenfriend, *Arch. Biochem. Biophys.* **1973**, *155*, 352; S. Stein, P. Bohlen, S. Udenfriend, *Arch. Biochem. Biophys.* **1974**, *163*, 400.

[9] M. D. Barnes, W. B. Whitten, J. M. Ramsey, *Anal. Chem*. **1995**, *67*, 418A.

[10] D. Skoog, F. J. Holler, T. A. Nieman, *Principle of Instrumental Analysis*, 5th ed., Saunders Brace, **1998**, pp. A9.

[11] S. Bernardo, M. Weigele, V. Toome, K. Manhart, W. Leimgruber, P. Bohlen, S. Stein, S. Udenfriend, *Arch. Biochem. Biophys.* **1974**, *163*, 390.

[12] X. Fang, W. Tan, *Anal. Chem*. **1999**, *71*, 3101.

[13] J. Cordek, X. Wang, W. Tan, *Anal. Chem*. **1999**, *71*, 1529.

Received: June 29, 1999 [F1880]

  0947-6539/00/0606-1092 $ 17.50+.50/0