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Characterization of a Novel Light Source for Simultaneous Optical and Scanning Ion Conductance Microscopy

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We have developed a novel light source suitable for imaging of biological samples. The method is based on the use of a micropipet filled with fluo-3. A fluorogenic complex is formed when fluo-3 meets calcium in the bath solution. The complex is excited by focusing a laser beam at the pipet tip to produce a submicrometer light source. This source is continually renewed at the tip, eliminating problems with photobleaching, and can be controlled by varying the applied potential. We first characterized the light source using fluorescence correlation measurements in order to optimize its properties. We then recorded an image of a model sample under buffer with submicrometer resolution using ion conductance distance control to demonstrate the feasibility of this approach.

Scanning probe microscopy has been widely applied to chemistry and material samples; however, its application to biological specimens is not straightforward because of the difficulties in controlling the probe distance over the soft cell membrane. One method to overcome these problems is scanning ion conductance microscopy (SICM),[1] in which an electrolyte-filled glass pipet is scanned over the surface.[2,3] The ion current provides the feedback mechanism to maintain a constant tip−sample distance, and this has been demonstrated to be a robust method to image living cells or follow cell contraction.[4] Scanning near-field optical microscopy (SNOM)[5,6] is a form of scanning probe microscopy that can be used for high-resolution optical imaging. To perform SNOM, it is first necessary to generate a sufficiently intense and small light source. This light source is then scanned over the sample, maintaining a constant subwavelength distance from the sample in order to obtain the near-field image. We have previously combined SICM and SNOM to enable the simultaneous topographic and optical imaging of living cells; the subwavelength-sized aperture was achieved by coupling light via an optical fiber into the metal-coated pipet.[7] Distance modulation has been introduced to further improve the reliability and robustness of the distance control for SNOM[8] and live cell imaging.[4] While the use of coated micropipets has extended SNOM to live cell imaging, this type of light source still has several problems. The long taper of the micropipet means that the intensity of light emerging at the tip was low, the coating thickness decreased the topographic resolution, and the amount of light emerging from the fiber optic was not reproducible between experiments. Thus, although SICM seems an excellent means to control the near-field probe, it is necessary to explore alternative ways to generate an intense near-field light source.

One approach for a near-field light source is to use a nanoscopic medium that emits light. The first work in this area used excitons in an anthracene crystal deposited in the tip of a micropipet in a plastic matrix;[9,10] however, the decrease in intensity caused by photobleaching limits the measurement time. Single molecules have been used as a near-field light source by use of a *p*-terphenyl crystal doped with terrylene molecules and deposited in a micropipet tip.[11] In this case, photobleaching was eliminated by operating at $T = 1.4$ K, but this is not compatible with biological imaging. At room temperature, diamond color centers have been used as a nanoscopic light source without photobleaching problems.[12] For imaging in solution, electrogenerated chemiluminescent light sources are also being developed.[13,14] An electrochemical reaction at an electrode surface produces continuous light[14] with shear force feedback used to control the probe−sample distance.[13]

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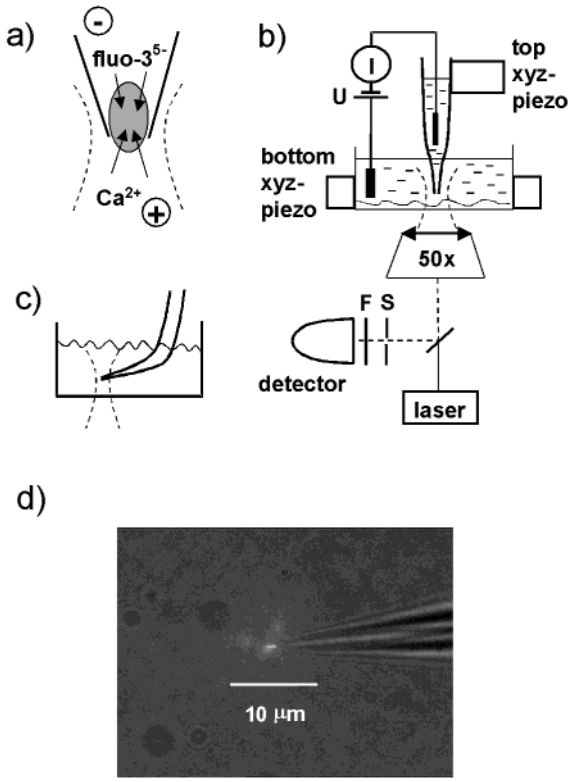

Figure 1. (a) Schematic representation of the light source. (b) Experimental setup for simultaneous optical and SICM experiments; F, filter; S, slits. (c) Detail of the experimental setup with the bent pipet, (d) microscope image of the light source in the bent pipet with 160 μW laser illumination.

Here we report a novel light source especially designed for use with the SICM distance control. The light source is generated by using the fluorescence produced under laser illumination when the calcium indicator fluo-3, confined within the pipet, binds with calcium in the scanning solution (see Figure 1a).

$$\text{fluo3}^{5-} + 2\ \text{Ca}^{2+} \rightarrow \text{fluo3(Ca)}_2^{-}$$

This results in a localized light source in the mixing zone where the fluorescent complex forms. The applied electric field, which gives rise to the ion current for feedback control of the SICM, drives fluo-3 out of and calcium into the pipet. Together with the fast diffusion of the molecules, this provides a constant supply of fluo-3 to the tip region. Hence, the method of light generation is experimentally simple, and the constant supply of reagents to the pipet tip eliminates problems of photobleaching. In addition, the brightness of the source is straightforwardly controllable through regulating the voltage applied to the pipet.

In this article, we characterize the diffusion and photophysics of this novel chemical light source to be able to optimize its intensity and size. We then demonstrate its potential use for optical imaging with scanning ion conductance distance control of the probe−sample distance by recording an optical image of a model sample in liquid.

EXPERIMENTAL SECTION

The experiment (Figure 1b) is built around an inverted microscope (Eclipse TE 200, Nikon Corporation, Tokyo, Japan). Light (160 μW) from the 488-nm emission line of an Ar^+ laser (model 5400, Ion Laser Technology, Salt Lake City, UT) was focused by an oil immersion objective (100×, NA = 1.3 for the characterization experiment or 50×, NA = 0.95 for the imaging) through a glass dish (WillCo, Wells B. V., Amsterdam) 5 μm deep into the solution. The pipets were pulled from borosilicate glass capillaries with outer and inner diameter 1.0 mm and 0.58 mm respectively, using a laser-based micropipet puller (model P-2000, Sutter Instrument Co., San Rafael, CA). The tip diameters, measured by scanning electron microscopy were in the range 240−280 nm with a central hole of 90−130 nm (data not shown). The pipets were backfilled with 250 mM NaCl and 2.5 μM of the fluorescent calcium indicator fluo-3 (Catalog no. F-3715, Molecular Probes Europe BV, Poort Gebouw, The Netherlands) and inserted into a solution containing 250 mM NaCl and 4.4 mM $CaCl_2$. A piezoelectric *xyz* stage with a scan range of 40 μm (Tritor 38, Piezosystem Jena GmbH, Jena, Germany) allowed the tip to be precisely positioned over the laser spot. A voltage was applied using a dc power supply or a pulse generator between two Ag/AgCl electrodes, one in the bath and one inside the pipet. Fluorescence with an emission maximum of 526 nm was monitored by a CCD camera with a long-pass filter placed in front of the CCD to reject the scattered laser beam.

For the fluorescent correlation measurements, a 100 μm pinhole, a filter (535AF45 and 06515 Omega Optical, Brattleboro, VT) and an avalanche photodiode (SPCM-AQR-141, EG&G, Canada) connected to a multichannel scalar photon-counting card were used (for details, see ref 15). For these experiments, the pipets were gently bent by 90°in a flame (see Figure 1c).

For the simultaneous optical and SICM experiment, the sample was scanned by distance modulation control[4] using a second *xyz* stage (P-517.3CL, modified for 20-μm *z* resolution, Physik Instrumente (PI) GmbH & Co, Waldbronn, Germany). The ion current recorded by a sensitive homemade amplifier was used as a feedback signal (for details, see ref 7). In this case, a photomultiplier (Photon Technology Surbiton, England) collected the optical signal that passed through a pair of adjustable slits and a long-pass filter (BA520, Nikon Corporation, Tokyo, Japan).

RESULTS AND DISCUSSION

A microscope image of the fluorescent light source, when a potential of −600mV is applied to the electrode inside the pipet, is shown in Figure 1d. The bent pipet was illuminated as shown in Figure 1c, resulting in an elliptical shaped spot. The Ca^{2+} inside the bath diffuses into the pipet and binds to the fluo-3 diffusing out to form the fluorescent complex. The light source is confined by the tip diameter in two dimensions and by the laser spot in the third dimension along the axis. In the geometry used for the combined optical and SICM experiments, this results in a light source of 100−150-nm diameter, but an effective depth of at least one micrometer. The latter is determined by the laser profile and the collection efficiency function of the confocal microscope.[16]

Next, we measured the light intensity as a function of the applied potential by slowly ramping the voltage from +0.2 V to

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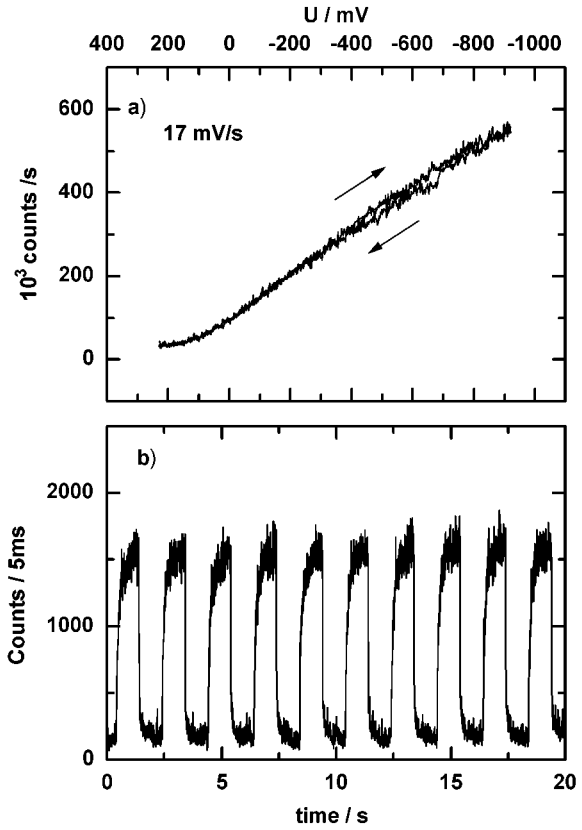

Figure 2. (a) Intensity of the light source as a function of applied voltage. (b) Intensity of the light source when the voltage is switched from 100 to −1000 mV at 0.5 Hz.

−0.9 V and back to +0.2 V, as shown in Figure 2a. An almost linear increase in intensity was found when a negative potential was applied to the electrode inside the pipet; however, only a low background signal was measured for potentials more positive than 0.2 V. Switching the potential between +0.1 V and −1.0 V every second results in reversible switching on and off of the light source. The intensity changes by a factor of 10 (Figure 2b), promising sufficient contrast for imaging. The system shows characteristic exponential time behavior with rise and decay times of 80 ms and 9 ms, respectively.

To learn more about the diffusion of the fluo-3−Ca^{2+} complex inside the nanopipet, we performed confocal fluorescence correlation spectroscopy (FCS) measurements. They are based on measuring fluorescence intensity fluctuations in order to obtain information about molecular diffusion.[17,18] Assuming a Gaussian laser intensity profile for the lateral direction and an infinite axial extension, the measured autocorrelation curves were fitted to the two-dimensional diffusion model,[19]

$$G(t) = \frac{A}{1 + t/t_d}$$

where the autocorrelation amplitude A is inversely proportional to the number of fluorescent molecules and t_d is the halftime of

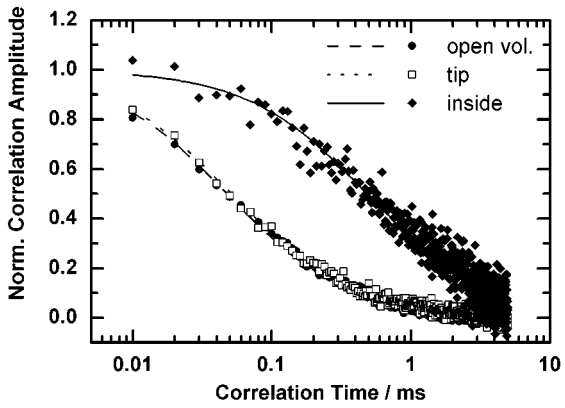

Figure 3. Autocorrelation curves at −600 mV in the open volume (circles), the tip region (open squares), and inside the pipet (diamonds). Data is normalized to the autocorrelation amplitude from fits (shown as lines) using the two-dimensional diffusion model. The dynamics in the tip region of the pipet can mainly be described by fast diffusion.

$G(t)$. It is related to the diffusion coefficient D by

$$t_d = \frac{r_0^2}{4D}$$

with r_0 being the distance where the Gaussian laser distribution drops to $1/e^2$.

First, we considered the case of free diffusion in the open volume using an aqueous solution of 100 nM fluo-3 and 2.5 mM $CaCl_2$ (see Figure 3). The diffusion time t_d was found to be 47 μs. This results in a diffusion constant of 34×10^{-7} cm^2 s^{-1}, which is comparable to 46×10^{-7} cm^2 s^{-1} for fluorescein in aqueous solution.[20] When the laser power is increased from 160 to 940 μW, the countrate decreases from 500 to 300 kHz, but the autocorrelation amplitude and the diffusion time do not change significantly (data not shown). This shows that irreversible photobleaching does not occur on the time scale of the measurement. The molecules might cross to the triplet state, where they are likely to be trapped for microseconds to milliseconds, as is observed with fluorescein.[21]

Also shown in Figure 3 are the results for the bent pipet filled with 4.4 μM fluo-3 and the laser focused at the tip. The data could still be fitted by the two-dimensional diffusion model, but it was not possible to fit it using a combined flow and diffusion model.[22] The observed halftime was 51 μs, very close to the open volume value. This suggests that the process is dominated by the fast diffusion of the fluo-3−Ca^{2+} complex, rather than the flow through the tip.

The situation changes when focusing inside the pipet at a distance several micrometers from the tip. The diffusion time increases to 490 μs (see Figure 3). In the case of Cy5−dCTP and for a larger pipet diameter of 0.3−0.7 μm, Zander et al.[23] reported

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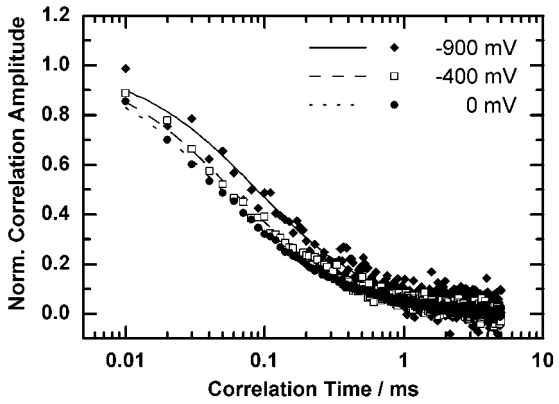

Figure 4. Autocorrelation curves of the tip region for applied voltages of 0, −400, and −900 mV, normalized to the autocorrelation amplitude from fits (shown as lines). The diffusion halftime increases from 51 to 87 μs, while the autocorrelation amplitude decreases from 0.6 to 0.032 ms, reflecting the increase in the number of fluorescent molecules.

a diffusion time of 2 ms and a flow time in the range of 0.4−6 ms, dependent on the applied electric field. Our results show a similar longer diffusion time due to the confined environment of the molecule or adsorption onto the glass walls of the pipet. When the applied electrode potential is changed, we expect a change in the number of molecules inside the tip from our potential−intensity measurements. The autocorrelation amplitude was found to decrease by a factor of 20 when changing the voltage from 0 to −900 mV. This reflects a 20-fold increase in the number of fluo-3−Ca^{2+} complexes inside the confocal volume; however, the diffusion halftime also increases from 51 to 87 μs (see Figure 4). This effect may be due to a voltage-dependent partial blockage of the tip, and further work is required to investigate this observation.

The autocorrelation measurements show that the dynamics in the tip region of the pipet can mainly be described by fast diffusion. On leaving the tip, the fluorescent complexes diffuse away rapidly so that they no longer contribute to the light source. Molecules that cross to the excited triplet state also cannot contribute to light emission.

Having characterized the light source, we tested its performance in optical imaging with ion conductance distance control. A test sample was prepared by evaporating 100-nm aluminum onto a glass cover slip over a random distribution of 1-μm polymer microspheres (Duke Scientific, Palo Alto, CA). The beads were partially removed by ultrasonic sound, leaving 1-μm holes in the Al film. The regions with two neighboring holes provide submicrometer structures for testing our light source. Simultaneous topographic and optical images were recorded with an uncoated pipet of ∼150-nm inner diameter. The ion−current feedback of the SICM provides a constant tip−sample distance during the scan so that the imaging is performed in a constant distance mode, with the tip held 75 nm away from the surface. The upper image in Figure 5 shows the topography, and the higher features of the evaporated Al are visible as bright structures, whereas dark areas are due to removed Al. The optical image shows the same Al-coated areas as dark features. A linescan near two neighboring holes (arrow) shows a submicrometer dip in the topographic image and a feature of higher intensity and similar size in the optical image. This shows that imaging with the fluo-3 light source is possible.

The optical resolution obtained in the experiments is estimated as 400 nm. It should depend on the inner tip diameter of ∼150 nm, the diffusion of the fluor-3−Ca^{2+} complex away from the tip, and a contribution due to the depth of the light source. The light originating from the tip is at a subwavelength distance from the sample surface; therefore, we can expect a near-field contribution to the image. The additional far-field contribution from inside the pipet could be removed by the use of large amplitude modulation of the pipet−sample distance and the use of lock-in amplification detection. Coating of the outside of the pipet with metal is also possible to further confine the light source, although this will reduce the topographic resolution. To break the diffraction limit of far-field optical imaging, the magnitude of the diffusion contribution to the light source has to be minimized. Using the steady-state concentration profile derived for ultramicroelectrodes in the diffusion-limited case,[24] we can estimate the full width at half maximum (fwhm) of the concentration profile. For

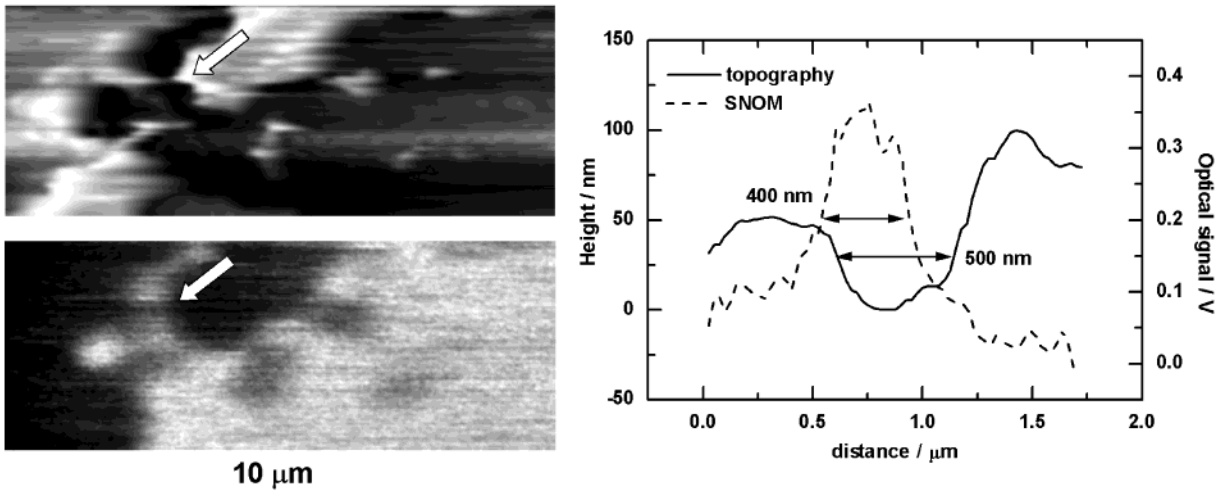

Figure 5. Topographic (upper left side) and optical (lower left side) image of a test sample with evaporated Al structures, using a random distribution of 1-μm beads. A line-wise background subtraction was used to enhance features in the topographic image. Arrows indicate where the linescans (right side) of the topographic and optical image were taken.

a 150-nm pipet held 75 nm from the surface, we predict a fwhm of 340 nm, which is close to the experimentally determined optical resolution.This suggests diffusion is the dominant factor decreasing the optical resolution in our experiments. Reducing the tip diameter to 50 nm should decrease the fwhm to 120 nm, with the potential of breaking the diffraction limit with our novel light source. The tip diameter can be reduced to 10 nm using quartz micropipets, and SICM images have been obtained with 50-nm resolution[3] showing the feasibility of scanning with finer pipets. In principle, when using smaller diameter pipets, the light intensity can be maintained by the use of a higher concentration of fluo-3 in the pipet and a higher applied voltage. Since SICM control works reliably on living cells, the application to biological specimens should be straightforward. This work is in progress.

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CONCLUSIONS

This works demonstrates a promising new light source for optical imaging that is compatible with SICM control for imaging of biological samples in liquid. It also presents a new general principle for the development of novel light sources by using the micropipet as a local reservoir for the controlled delivery of one or more reagents to the pipet tip region to produce a new fluorogenic or light emitting chemical species.

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