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Sub-Attomole Molecule Detection in a Single Biological Cell *in-vitro* by Thermal Lens Microscopy

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We have developed a novel thermal lens microscopy coupled with an optical microscope, and presently applied it to the ultratrace molecule detection in a single biological cell (mouse hybridoma) *in-vitro*. The determination results obtained for individual cells were calibrated by the average values determined by absorption spectrophotometry. The determination limit of the thermal lens microscope, defined as twice the standard deviation of the calibration curve, was 37.8 amol/cell, which was more than one order smaller than that of a fluorescence microscope under our experimental conditions. This superiority should come from escaping light scattering by a cell membrane and by cytoplasm, which is inevitable in fluorescence spectrometry. The absolute determination limit of the thermal lens microscopy was calculated down to sub-attomole level. In addition, the method is more widely applicable, even to non-fluorescent samples without chemical preparation, and therefore, the thermal lens microscope has proved to be very useful in directly quantifying ultratrace chemical species in a single biological cell *in-vitro*.

Keywords Thermal lens microscope, fluorescence microscope, single biological cell, *in-vitro*, determination limit, sub-attomole

A small amount of chemical species in advanced materials or biological samples often plays a vital role in realization or impediment of their functions and characteristics. Thus the determination of its quantity and spatial distribution has been regarded as of major importance for a full understanding of the samples. Trace analysis of chemical species in a biological cell, for example enzyme, immunological substances, physiological activators, or Ca^{2+}, is especially recognized as a challenging task and success will make a great contribution to biological and medical sciences and technologies.[1-3]

Microscopic techniques are being extensively investigated recently.[4] Elemental analysis with electrons or ions as an excitation source is a well-established method and its spatial resolution now exceeds the nanometer region. As it needs high vacuum environment, however, it is applicable to only a limited kinds of samples. X-Ray fluorescent spectrometry is another elemental analysis technique without vacuum, but an X-ray microbeam is not available unless a synchrotron radiation source is used. Though scanning probe microscopes have attracted considerable attention lately, their applicable size is unsuitable for biological cells. On the other hand, optical microspectroscopic methods are also making rapid progress in their spatial resolution and sensitivity due to the development of microscopic techniques and powerful light sources such as lasers. Among them, laser induced fluorometry (LIF) is one of the most highly sensitive methods and the confocal laser fluorescence microscopy has the highest spatial resolution. There are many successful applications reported, but its only shortcoming is that the sample is not always intact, because non-fluorescent samples must be derivatized with fluorescent reagents for analysis.

Photothermal spectroscopy based on the thermal detection is in principle applicable to any kinds of samples. It has many attractive features from an analytical point of view; these include high sensitivity, nondestructiveness, noncontact, *in-vivo*, and *in-situ*. Some novel photothermal spectroscopies have been continuously developed[5] and applied to microscopy.[6] Among the many kinds of photothermal detection techniques, the photothermal deflection method (mirage technique)[7-9] has proved to be highly sensitive, particularly to microparticles;[10] this has been ascribed to the temperature gradient being enhanced by the sample curvature.[11] It was successfully applied to the measurement of a single human white blood corpuscle (5 – 15 μm in diameter).[12] As the signal amplitude, however, strongly depends on the optical alignment[13], it is difficult to measure a sample less than 100 μm in size with a good reproducibility and it is almost impossible to measure any particular microscopic area of interest in the sample.

So we have conceived the coaxial excitation and probe beams configuration for photothermal measurements under an optical microscope, and verified that signal generation mechanism follows the thermal lens

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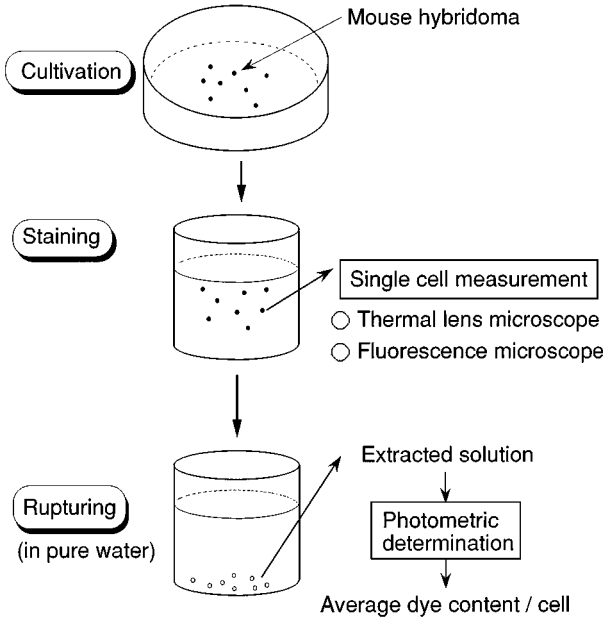

Fig. 1 A schematic diagram of sample preparation and experimental procedure.

effect.[14-16] Such thermal lens microscopy enables us to measure the particular microscopic sample or the particular microscopic area of the sample as long as it is visible under the optical microscope. Recently it was successfully applied to quantification of zeptomol drug concentration in a renal tubule of a fixed kidney.[17] In this research we determined the absolute amount of chemical species contained in a single biological cell and investigated its superior quantitative ability in detail by comparing with the fluorescence microscopy results.

Experimental

Sample preparation and experimental procedure

Sample preparation and experimental procedure are briefly shown in Fig. 1. The biological cells used were cultivated mouse hybridoma, the diameters of which ranged from 10 to 20 μm. The sample was centrifuged and then washed with phosphate-buffered saline (PBS). After this procedure was repeated twice, ethanol was added. Then the sample cells were stained by the fluorescent dye, fluorescein-5-isothiocyanate (FITC), which has an absorption maximum around 490 nm combined with protein. After warming for about 10 min and centrifugation, bovine serum albumin (BSA) was added. Six kinds of sample cells different in their dye contents were prepared by changing the concentration of the fluorescent dye solution. The cells of each sample were measured one by one both with the thermal lens microscope and with the fluorescence microscope for comparison. A fluorescence yield of FITC (0.65) is somewhat advantageous to the fluorescence microscope.

Table 1 Comparision of the obtained results

	Thermal lens microscope	Fluorescence microscope
Wavelength (nm)	488	480±20
Excitation pewer (mW)	20	20
Focus size (μm)	2	7.5
Detection length (μm)	~2.3	≦15.8
Limit of determination (amol/cell) (2σ for the determination lines)	37.8	434
Coefficients of variance (%)	39.6	40.0 (for beyond 500 amol/cell)

To determine the average absolute amount of dye in a single cell, a known number of cells were dispersed in pure water and ruptured by the osmotic pressure. The extracted solution was quantified by the absorption photometric determination. The average dye content was calculated as the total dye amount divided by the number of cells included.

Apparatus

The experimental setup of the thermal lens microscope is the same as that used in the previous studies[15,16] and only a brief description is presented here. The excitation beam was an argon laser with an emission line of 488 nm and its intensity was modulated by a mechanical chopper. The modulation frequency was 320 Hz and the output power was 200 mW. The probe beam was a 1-mW He-Ne laser with an emission line of 633 nm and it was made coaxial with the excitation beam by a dichroic mirror. Both the excitation and probe beams were passed through the body tube of the optical microscope for photographing and tightly focused by an objective lens onto the sample. The transmitted beams were collected by the other objective and filtered. Only the probe beam intensity was monitored by the photodiode. The signal from the photodiode was pre-amplified and then synchronously amplified with a two-phase lock-in amplifier (NF Model LI-575).

The absorption measurement for determination of average dye content was made with Hitachi U-1100 spectrophotometer. And the fluorescence microscope for comparative study was Hitachi U-6000. The 480 nm line of the 100 W high-pressure mercury lamp was used as an excitation beam. The other experimental conditions are listed in Table 1.

Results and Discussion

The schematic enlargement of the single cell measurement by the thermal lens microscope is shown in Fig. 2. The culture medium containing the sample cells was placed between slide and cover glasses in the same

way as in usual optical microscope observations. It has been already proved that the measurement can be practicable even in such a sample environment.[16] The cell to be measured was freely and reproducibly selected by direct observation from the eyepiece. As is reported elsewhere in details, the probe beam intensity profile is modulated by the thermal lens generated by the excitation beam in the cell.

About 10 cells from each of the 6 samples different in their dye content were measured individually by the thermal lens microscope. The diameter of the 55 cells measured was 15.8±1.8 μm. It was experimentally shown that the signal intensity of the thermal lens microscope is independent of the cell diameter in this size region. The results are plotted in Fig. 3, together with the results of the fluorescence microscope. It is clearly seen in the results obtained by thermal lens microscope that the signal intensity is roughly proportional to the dye content. Also in the results by the fluorescence microscope a little dependency of the signal intensity on the dye content is found. The data points, however, are scattered, especially in the low dye content region of the fluorescence microscope. The quantitative results derived from the data are summarized in Table 1, together with the experimental conditions. The determination limits for the cell measurements were calculated as twice the standard deviation from the calibration curve. The determination limit of the thermal lens microscope is 37.8 amol/cell and over 10 times smaller than that of the fluorescence microscope under these experimental conditions. In the fluorescence microscope the bandpassed light of 460 – 500 nm was used for excitation; its power was calculated to be about 20 mW. On the other hand, the throughput of the thermal lens microscope was less than 10% and so the virtual optical power for excitation was estimated to be less than 20 mW. Therefore, the excitation condition of both microscopies was considered to be roughly the same. The detection volume in the thermal lens microscopy, however, was mainly limited to the confocal length (~2.3 mm), while all the analytes in the excitation beam path were excited in the fluorescence microscope. The larger dispersions were observed in the low dye content region of Fig. 3b, which deteriorated the determination limit for the fluorescence microscope.

We gained insight into the source of the large dispersions. The coefficients of variance (CV) for the six samples measured by the thermal lens microscope were constant (39.6%). The CV value of the thermal lens method itself was not so bad for the FITC standard solution and the repeatability was also excellent. In the fluorescence microscope, the CV values of the 2 samples in the dye content more than 500 amol/cell were

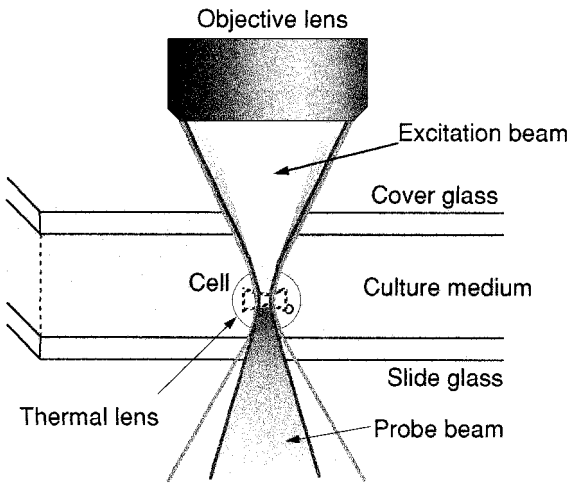

Fig. 2 An enlargement illustrating the measurement of a single biological cell and its principle.

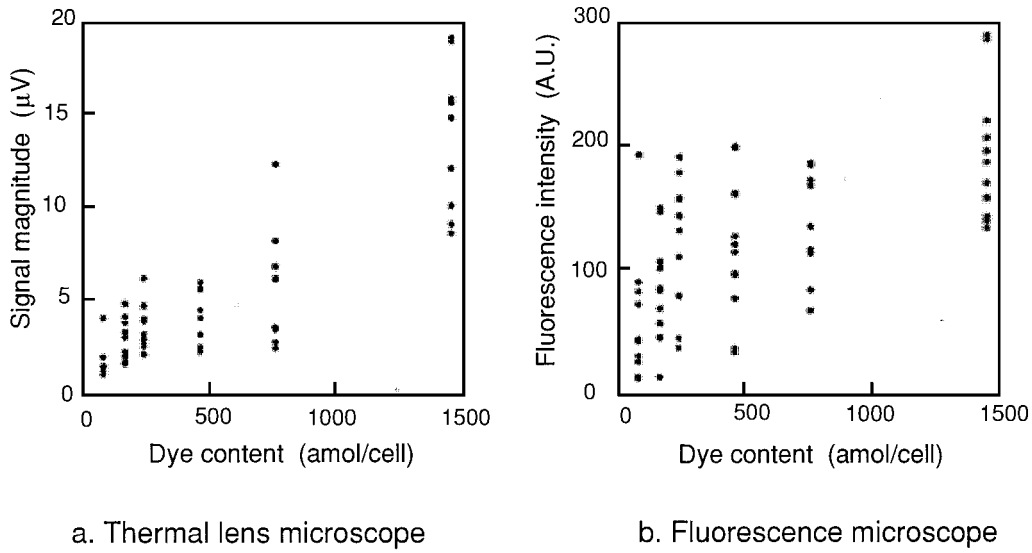

Fig. 3 Dependency of the signals on the dye content in a single cell: (a) thermal lens microscope and (b) fluorescence microscope.

40% and almost the same as those of the thermal lens microscope, while those of the 4 samples in the dye content less than 500 amol/cell were far beyond 40%. Therefore the dispersion of 40% common to both methods was considered not due to the measurement, but due to the different amounts of dye absorbed in a cell. So it equally contributed to both methods and gave no explanation to the dispersions beyond 40% in the low dye content region of the fluorescence microscopy. Another factor inherent in the fluorescence microscopy was strongly suggested. When the light is incident on the cell, the cell membrane and cytoplasm strongly scatter the light. A cell is well known as a strong light scattering material. The excitation beam is first scattered by these scattering processes, which affect both methods equally. In the fluorescence microscope, however, the fluorescence to be detected is also scattered by the cytoplasm and the cell membrane. It seriously perturbs the weak fluorescence in particular. On the other hand, in the thermal lens microscope the thermal lens itself is not sensitive to the scattering effect, which is a common characteristic of the photothermal techniques. This is probably the reason why the large scatterings are observed especially in the low dye content, that is low signal intensity, regions for the fluorescence microscope. This effect is inherent in the fluorescence measurements and inevitable in principle with all ingenious techniques in practice.

The determination limit in the absolute amount was estimated for the thermal lens microscope. The detection volume is assumed to be limited to the focal volume of the excitation beam, where the thermal lens is generated and the probe beam is modulated. The focal volume (2 μm in diameter and 2.3 μm in thickness) was 7.2 fl and the minimum absolute amount determined was thus calculated to be 0.13 amol. In addition, it should be noted that the signal intensity of the thermal lens microscopy does not depend on the sample thickness, but on the focal volume only, as long as the thermal lens is generated in the sample. This is often significantly advantageous to quantification, and the analyses of heterogeneous samples are made possible.

In conclusion, the thermal lens microscope has proved to be very promising in quantification of ultratrace chemical species in a single biological cell *invitro*. There is no need of derivatization by a fluorescence reagent and so the use of ultraviolet laser, for example, makes it possible to quantify chemical species produced in a cell directly. A single molecule detection with the thermal lens microscope and its theoretical analysis are now under way.

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