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DIRECT DETECTION OF MARTIAN MICROORGANISMS BASED ON FLUORESCENCE MICROSCOPY

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ABSTRACT

The direct detection of microorganisms and their traces using optical microscopes is one of the most promising techniques to obtain the decisive evidences for extraterrestrial life. The most significant points of this technique are high sensitivity and spatial information with a resolution of 0.2mm. Besides, information on local environments and microscopic ecology can also be obtained. Many difficulties, however, must be solved to get reliable results. We have started to develop a noble technique based on the fluorescence microscopy with special interest to the detection of microorganisms in extreme environments including Mars. The principle is to detect molecules/subcellular organs which are responsible for the three basic characteristics of life; genetic information, metabolism, and discrimination of self from non-self. We have screened fluorescence probes and found several are applicable. We could detect almost all the microorganisms already identified. Discrimination of viable from dead cells was possible. The terrestrial microfossils, some of the artificial primitive microorganism-like-objects, dried bacteria and polycyclic aromatic hydrocarbons mixed with simulated Martian sand could be detected. We are now designing a compact detection hardware.

INTRODUCTION

Considering the history of life on Earth, the most possible extraterrestrial life is microorganism. However, detection of microorganisms is not easy even on Earth. The reasons are that microorganisms are very small (most of them are less than 0.1 mm) and are often very difficult to distinguish from soil particles or detritus. Besides, general culture techniques are not established. When it comes to extraordinary microorganisms, situation becomes even worse. Considering this situation, we think that the direct detection of microorganisms using fluorescence microscopic techniques is most promising. Detection of microorganisms in natural environments with fluorescence method has been developed these twenty years (Anderson and Westmoreland, 1971; Lundgren, 1981; Mayfield, 1975; Postma and Altemuller, 1990; Ramsay, and Bawden, 1983: Soderstrom, 1977: Soderstrom, 1979: Jepras *et al.* 1995: Bianchi and Giuliano, 1996) . In most cases, adhesive fluorescence probes , such as acridine orange, ethidium bromide, calcofluor white M2R and fluorescent antibodies are used. The fluorescence images were recorded in conventional photographic films. However, these classical methods have many difficulties if we want to obtain reliable, reproducible and quantitative data from soil specimens due to non-specific binding of the probes, weak fluorescence and rapid photobleaching. A characteristic point for the search of extraterrestrial microorganisms resides in the fact that the most possible cases are that the living systems had long been dead and only their traces are remaining, or only pre-biotic systems (primitive cells) are present. The PAHs in the Martian meteorite ALH84001(McKay *et al.* 1996) may be one of the examples. The ideal detection system must be applicable to such cases. Surely, discrimination of biogenic matters from non-biogenic will be much harder than the detection of viable cells. In order to overcome these difficulties, we have developed a new fluorescence method applicable to soil specimen using a combination of fluorescence probes and a highly sensitive solid state camera (Tsuji *et al.*, 1995; Kawasaki *et al.*, 1996a: Kawasaki *et al.*, 1996b). We found that our method can effectively distinguish microorganisms from soil particles. If extraterrestrial life is composed of the same kinds of organic molecules as terrestrial life, this fluorescence microscopic technique can detect most of them. In this paper, I would like to describe our fluorescence microscopic technique.

MATERIALS AND METHODS

Materials

Analogs of FDA were purchased from Molecular Probes Inc., (USA), and other fluorescence probes were purchased from commercial sources. The cellular slime mold, fungi, *Bacillus subtilis* and *Escherichia coli* were cultured as described (Kawasaki *et al.*, 1990: Tsuji *et al.*, 1995: Kawasaki and Tsuji, 1996). Light colored Ando soil (volcanic ash soil) was collected from a forest of white oak (Quercus myrsinaefolia Blume) and dogwood trees (Quercus acutissima Carr) on the campus of Tamagawa University and Kashinokiyama park in Machida City in Tokyo. All soil samples were stored in a dark cold room at 4℃ for a few months prior to use. Simulated Martian sand made after the data of Gooding (1992) was provided from the Obayashi Corporation. Its composition is 83.5% montmorillonite, 1% $CaCO_3$, 13% $CaSO_4 \cdot 2H_2O$, 1% Fe_2O_3. Mixture of polycyclic aromatic hydrocarbons (PAHs) were made with the same composition found in the Martian meteorite ALH84001(McKay *et al.*, 1996): 10% phenanthrene, 15% pyrene, 59% chrysene, 7.8% perylene, 7.8% benzopyrene. Proteinoid microsphere made after Fox and Harada (1960) was a gift from Dr. Yanagawa of Mitsubishi Kasei Institute of Life Sciences. Rocks containing microfossils were gifts from Prof. Akiyama of Shinshu University. Methanogenic bacteria, *Methanosarcina mazei* TMA was provided by Dr. Asakawa of Kyushu National Agricultural Experiment Station.

Instrumental

Fluorescence microscopes used were Zeiss Axiophoto and axiovert 135M equipped with cooled CCD Cameras, CH250 (Photometrics Ltd., USA), Quantics 1400 (Photometrics Ltd., USA) , and MCD1200 (Spectra Source, USA). Obtained data were automatically analyzed by a software IPLab spectrum (Signal Analytics Ltd., USA). Microscopic fluorescence spectra were recorded by a 2-dimensional microscopic fluorescence spectrum analyzer, SD200 (Applied Spectral Imaging Ltd., Israel) . Microscopic infrared absorption spectra were recorded by a FT-IR-8300 spectrometer (Shimadzu Corp.) and an combined apparatus of Micro-20-16 and FT/IR610 (JASCO Co., Japan).

Methods

The cellular slime mold, fungi and bacteria were mixed with the soil and were stained with fluorescence probes. The stained samples were subjected to fluorescence measurements without washing. For FT-IR and fluorescence spectrum measurements, bacteria cultured in liquid media were washed with sodium phosphate buffer (pH7.0, 20mM), and the sediments were dried in a desiccator under reduced pressure (100mmHg). Mixture of PAHs was dissolved in acetone and evaporated into powder. The dried powder was stocked in dark at room temperature. The rocks containing microfossils were cracked and the fleshly exposed surfaces were observed by fluorescence microscopes. All the samples were put in holed slide glasses which bottoms were covered with thin glasses (thickness, 0.12-0.17mm), and the tops were sealed with cover glasses. The exciting light intensity for fluorescence measurements was reduced to the level where photobleaching was less than 10%.

RESULTS

Establish an Experimental Standard for "How Unknown Objects Are Life-Like."

Life can be characterized by three major factors, 1) discriminates self and outer world (cytoplasmic membranes), 3) exhibits metabolic activities (enzymes) , 3) possesses genetic information (nucleic acids) . Each characteristic organelle or molecule is specifically detected by fluorescence probes (Figure 1). We stain unknown objects with three types of the fluorescence probes. If the objects are stained with the three probes, the score is (* * *) and they may possibly be microorganisms, and if

they are stained with only one probe, the score is (*) and they may possibly be detritus or non-biological substances. Thus, we can make an experimental standard for life with fluorescence microscopic techniques. This scoring can be referred to as "Restaurant Michelin Guide Standard". We have checked whether this standard can be realistic. In Figure 2, bacteria, fungi and molds were mixed with soil and the mixture was stained with three types of fluorescence probes. The objects indicated by a star are stained with all three fluorescence probes, while the objects indicated by arrows are stained only with ethidium bromide. Therefore, the formers can be classified as microorganisms and the latters as detritus or soil particles. Actually, the star-marked objects are spores of the cellular slime mold.

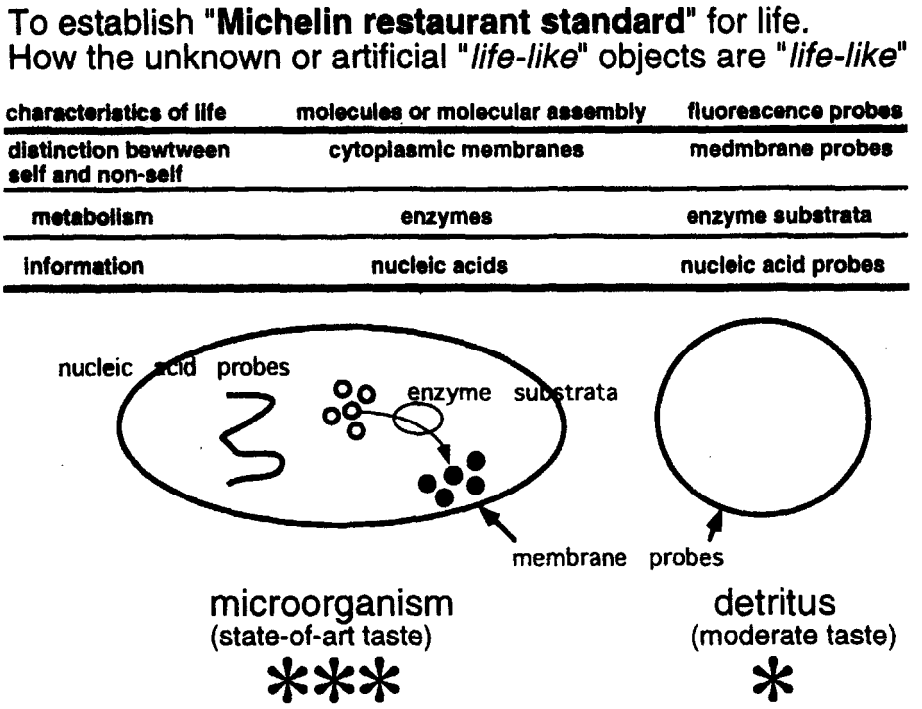

Fig.1. "Standard" for judging how unknown objects are life-like by fluorescence microscopic technique.

Visualization of Soil Microorganisms

Among the three types of fluorescence probes shown in Figure 1, esterase (the most ubiquitous enzyme and is widely distributed except viruses) substrata is unique in the sense that it specifically detects viable cells (Thomas *et al.*, 1979; Tsuji *et al.*, 1995). Unprocessed soil was stained with one of the fluorescent esterase substrata, an analog of fluorescein diacetate. Result is shown in Figure 3. It is almost impossible to identify from the conventional DIC image where viable soil microorganisms are. On the other hand, the microorganisms are clearly observed in the fluorescence image. When the soil was pre-heat treated (100℃ 15min), no objects with green fluorescence were observed (data not shown). This assures the feasibility of the fluorescent esterase substrata.

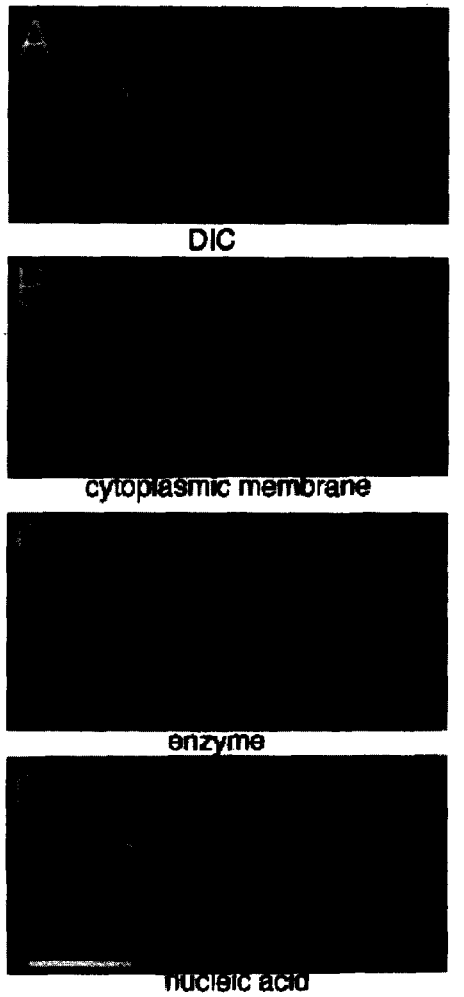

Fig.2. Triple staining microscopic images of the mixture of microorganisms and soil.

Cultured Escherichia coli, the cellular slime mold and fungi were mixed with soil and stained with 1-anilinonaphtalene 8-sulfonate (membrane probe), fluorescein daiaceta analog (enzyme probe) and ethidium bromide (nucleic acid probe).
A: differential interference contrast (DIC) image, B: fluorescence image (membrane probe), C: fluorescence image (enzyme probe), D: fluorescence image(nucleic acid probe).
Objects marked by → seem to be detritus because they are not detectable in B and C. On the other hand, objects marked by ★ can be recognized as microorganisms because they are fluorescent in B, C and D.
Actually, they are spores of the cellular slime mold.

scale: 20 μm

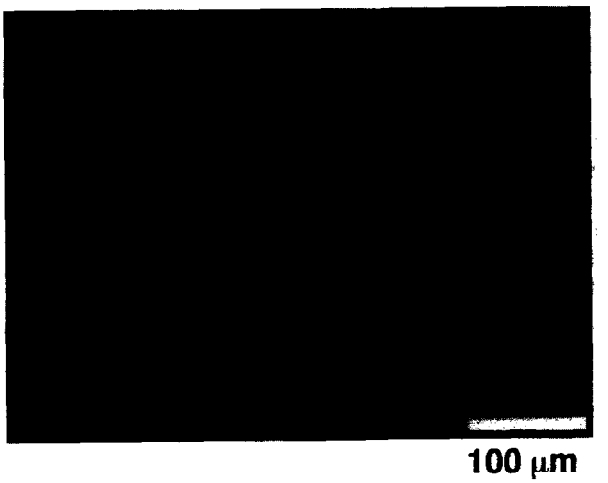

Fig.3. Detection of viable microorganisms in soil.

Soil was stained with an fluorescence esterase substratum, an analog of fluorescein diacetate. The objects with green fluorescence are viable microorganisms (excited at 490 nm). The fiberous objects are possibly fungi. Note that background is very low.

Detection of Bacteria with Infrared Absorption Spectroscopy

Although the fluorescence microscopys is very useful for detecting microorganisms, combination with other methods based on different principles will greatly decrease the ambiguity of obtained results. Infrared absorption microscopy is one of the most promising candidtes. We studied whether colonies of bacteria canbe detected by a fourier-transform infrared microscope (FT-IR microscope). Figure 4 shows a clear infrared absortion spectrum from dried *Bacillus subtilis*. Absortions at 3,300, 2,960, 1,650, 1,540, 1,230 and 1,075 /cm are characteristic to bacteria we studied and can be good biomarkers. Although the spatial resolution of FT-IR microscope is several tens times lower than fluorescence microscope, we beliebe combined and/or simultaneous measurements of fluorescence and infrared absorption will give highly decisive results than single measurements. Organic matters can also be detected by raman scattring microscope. Unfortunately, however,we found that in some specimens, such as some bacteria, rice straw, and the most of the stained specimens, fluorescence from these specimens interfered raman spectra severely by raising background(Data not shown).

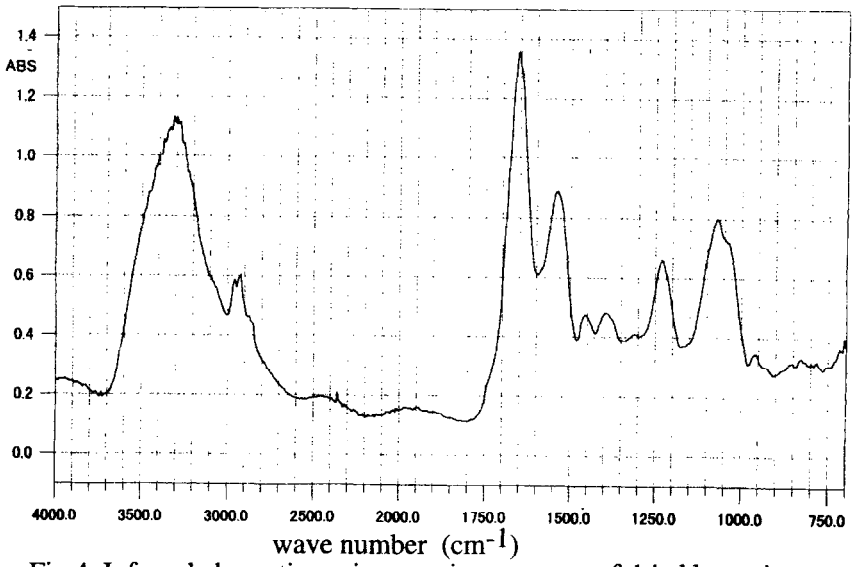

Fig.4. Infrared absorption microscopic spectrum of dried bacteria.

Bacillus subtilis 168 trpC2 was dried in a deciccator under reduced pressure. Its infrared absorption spectrum was measured with a field mask of 10X10 μm^2. Note that very sharp characteristic absorptions were obtained. Most of the absorption peaks were common to bacteria irrespective of species and can be good biomarkers.

Detection of PAHs

McKay *et al.* (1996) reported that PAHs were found inside the Martian meteorite ALH84001. They also found tiny microorganism-like objects (0.1mm in length). However, they could not conclude whether these microorganism-like objects are composed of PAHs because of the limited spatial resolution of their mass spectrometer (50 μm). Since the major components of PAHs found in ALH84001 are all fluorescent, they can be easily detected by the fluorescence microscopic technique. We investigated whether these trace amount of PAHs can be detected in our system and also directly find the correlation between PAHs and the microorganism-like objects. For the model case, we mixed PAHs with the simulated Martian sand. The result is shown in Figure 5. From the DIC image, it is impossible to find

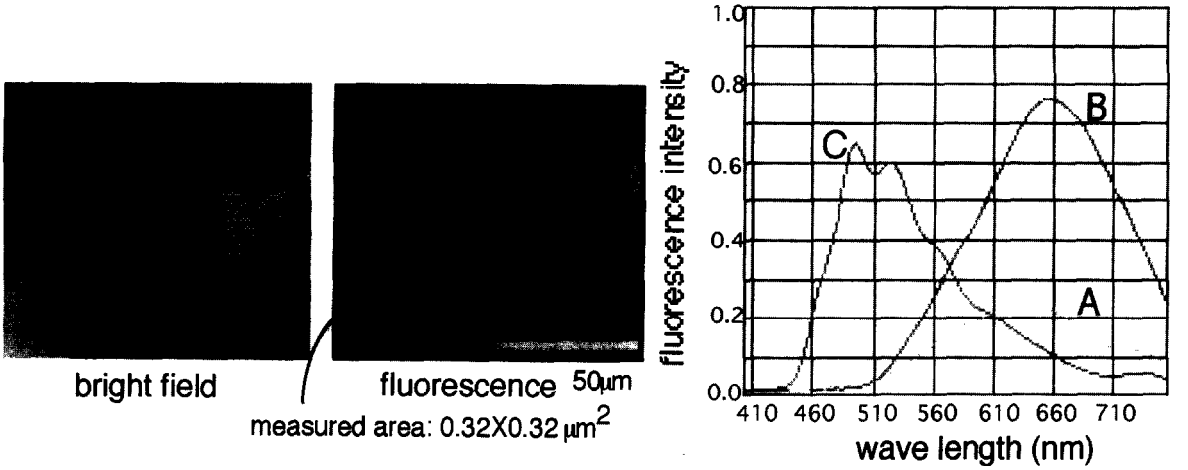

Fig.5. Fluorescence images and spectra of PAHs mixed with the simulated Martian sand

In the fluorescence image, three bright spots (A, B, C) were observed. Their fluorescence spectra were shown in the right figure. From these spectra, it is known that A and B are rich in perylene and C is rich in pyrene or benzopyrene.

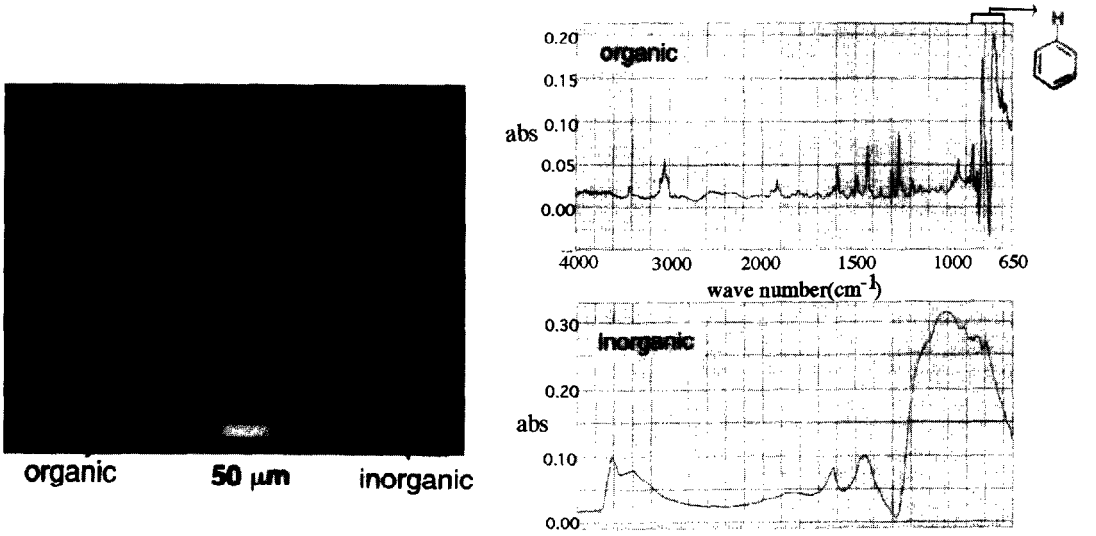

Fig.6. FT-IR microscopic image and spectra of PAHs mixed with the simulated Martian sand.

Left figure is a bright field image of the mixture which are shown as dark objects. Red squares are regions where infrared absorption spectra were measured. The characteristic sharp absorption bands of PAHs are observed at the wave number of 850-700cm^{-1} (See the upper spectrum of the right figure.).

In the fluorescence image, three bright spots are observed. By comparing these fluorescence spectra with those of PAHs, we concluded that these bright spots are PAHs, A and B rich in perylene, C rich in pyrene or benzopyrene.We further tried to detect PAHs with a different method. In Figure 6, the result of FT-IR microscope measurements is shown. Again in the bright field image, discrimination of PAHs and sand is impossible. However, the infrared absorption spectra of the two regions are different. From the comparison of the standard PAHs spectra, we could know that the central region is rich in PAHs while the leftmost is not. When PAHs are very dilute in samples, we have to scan wide area before we encounter PAH aggregates. If we make a combined microscope of fluorescence and FT-IR, we can easily and confidently find PAHs even if they are buried in inorganic matters.

Rocks Containing Microfossils.

We also studied the capability of detecting organic matters in the fossils. The result is shown in Figure 7. We see many fluorescent particles on the fleshly cleaved surface of the rock (A,B,C). The fluorescence spectra from the spot C is very similar to those of PAHs. Also it is not enough to conclude, we think this rock is rich in PAHs, probably, phenanthrene or chrysene.

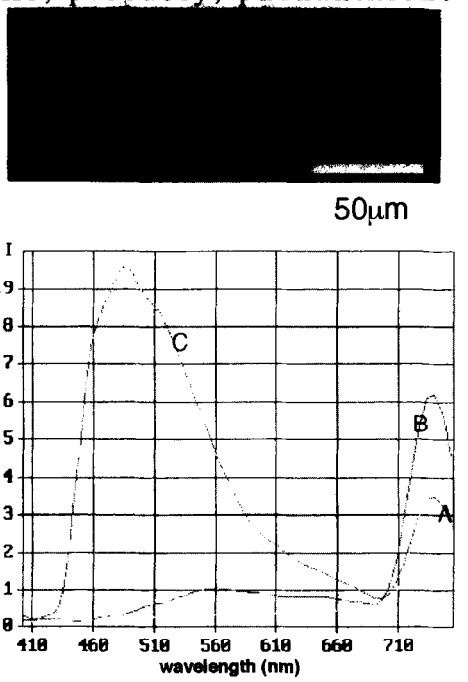

Fig.7. Fluorescence image and spectra of the cleavage surface of the rock containing microfossils (From East Greenland, 700-750 mega years old).

Detection of Pre-biotic Cells-Like Objects.

Even today, life may be under development in some planets, or it is freezed at this stage especially on Mars. We finally tried to detect these pre-biotic cell-like objects. The result is shown in Figure 8 on the proteinoid microsphere. In the DIC image, spherules of the proteinoid are observed. In correspondence with these objects, the green fluorescence of the cleavage products of FDA analogs are seen. This finding indicates that the artificially synthesized microspheres have enzymatic activity and they can be easily detected by the present method.

Design of Optical Fiber-Assisted Fluorescence Microscope.

On Mars, the surface environment is so harsh for microorganisms and their traces to remain. Subsurface exploration, therefore, is inevitable. For this purpose, we are designing the optical-fiber-assisted fluorescence microscope which can work automatically on Mars. The head of the fiber will attached to the tip of a drilling machine and explore the subsurface region *it situ*. The prototype model (conventional size model) could detect PAHs easily. We have just started to miniaturize the apparatus.

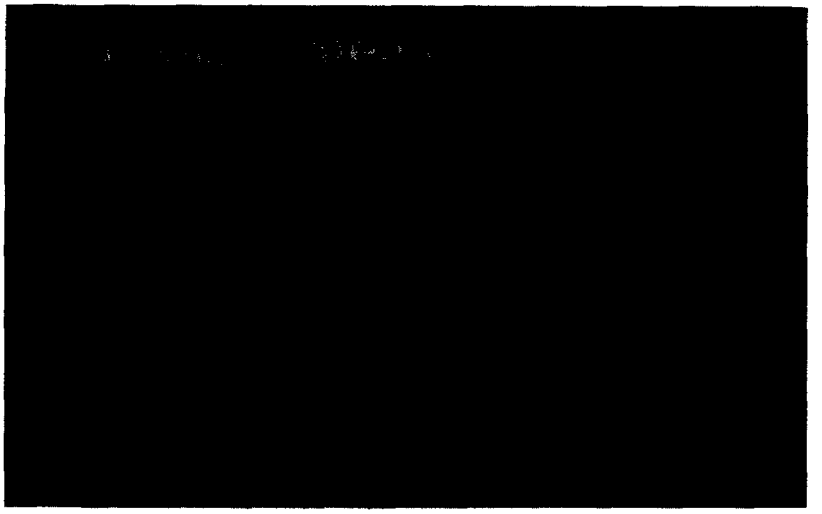

Fig.8. Detection of proteinoid microspheres by a fluorescence esterase substratum.

Note that every spherule is fluorescent. This result assures that even some type of pre-biotic cells can be detected by the present method. (excited at 486nm)

DISCUSSION

We introduced the feasibility of the fluorescence oriented optical microscopic techniques to detect terrestrial as well as extraterrestrial microorganisms and their traces. Detection of microorganisms in natural environments especially in soil has been very difficult. Detection of trace amount of organic matters in soil are even more difficult. Fluorescence image is the first step to find these targets. Fluorescence spectrum is the second. Of course, there still is some distance for the complete evidence. The fluorescence microscopic technique has a enough sensitivity to detect fluorescent objects with less than one hundredth of the volume of typical bacteria. The problem is selectivity. Non-biological matters often emit fluorescence and interfere the specific detection. We propose the introduction of FT-IR and also atomic force microscopic techniques to the fluorescence microscope to reduce the distance. The effectiveness of FT-IR microscope was shown in the present work. For the detection of organic matters, Raman scattering spectroscopy can also be used. However, we found that as far as fluorescent objects are concerned as introduced in the present study, Raman spectrum was severly distorted by the fluorescence. If proper fluorescence subtraction software is developed, Raman sacattering spectroscopy will be effective and can be combined with fluorescence microscopy . We are further planning to involve mass spectrometer into this combined microscope. The resultant super-complex microscope will detect incredibly small amount of microorganisms or their traces with high selectivity. If the putative extraterrestrial life has completely different properties from ours, some fluorescence probes introduced are non-effective. However, as far as it is composed of hydrocarbons, many probes , staining and detection techniques we have introduced here can successfully be applied.

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