CCD-Based Confocal Laser-Microscope System For Spectroscopic Investigation And Direct Imaging

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A Laser-induced confocal microscope system with the CCD-camera detection suitable both for spectroscopic investigation and for direct imaging is described. In combination with a spatial pinhole, depth discrimination was obtained. A method based on the reflected light from a quartz plate was proposed to detect the capability and a vertical resolution of 2.7 µm in terms of the full width at half maximum was achieved. The spatially-registered and temporal fluorescence spectra can be acquired by this system. Without using monochromator, direct real time fluorescence imaging with temporal time as short as 0.5 ms can be obtained. The potential applications of the confocal microscopy to air-liquid interface studies are discussed.

Keywords confocal microscope, CCD camera, imaging, fluorescence spectra, air-liquid interface
and focused to the sample by the microscope objective. Detection light was collected by the same objective. After filtered by the same beam splitter and passing the inserting pinhole, the light was led to the slit of a monochromator (Thermo Jurrell Ash Monospec 18 or Chromex 250is) by reflective mirrors and focus lenses. The pinhole was mounted on a x-y-z positioner. Spectra were then captured by the CCD camera (Santa Barbara Instrumentation Group ST-6 or Hamamatsu PMA-100). The sensitivity of fluorescence microscopy is much enhanced by using Charge-coupled device (CCD) detector. By adjusting the microscope stage manually or motor-controllably in x-y-z three dimensions, spatial-registered spectra can be acquired. When the CCD camera head was directly mounted on the top of the microscope without using monochromator, direct imaging can be obtained. In such case, the physical pinhole was removed and instead, the rows or columns of pixels on the CCD serve as a pinhole effectively. With the high performance of the PMA-100 intensified CCD camera, temporally resolved information down to 0.5 ms can be obtained.

A method, which we call reflection-light method, was used to detect the depth resolution of our setup. A quartz plate (1/4 \( \lambda \), flat) was put on the microscope sample stage to reflect a small portion of the attenuated laser light into detection system. Neutral filter was necessary for the reflection-light method. Chemicals used in experiments were all of analytical grade or above. 5,10,15,20-tetraphenyl-21H,23H-porphine (TPP) was purchased from Aldrich and 2,3,7,8,12,13,17,18-octaethyl-21H,23H-porphine (OEP) from Wako Pure Chemical Industries. A small amount of OEP or TPP dissolved in benzene was pipetted onto the water surface of a quartz cuvette with the area of 19.6 cm². The organic solvent was evaporated rapidly and the molecules remained at air-water interface. Because it is insoluble in water, OEP or TPP only stays at air-water interface.

**Results and discussion**

Laser light can be focused to a submicron beam waist in the radial dimensions, but there is no confinement along the beam axis (z-axis). Fig. 2(a) explains the depth discrimination along the beam axis of the confocal microscope. Light from B or C is not focused on the pinhole, and its majority does not pass through the pinhole. Thus only the light originating from the probe position A can be detected. When using the reflection-light method to detect the depth resolution, one should be careful not to damage the CCD camera by inducing the strong laser light into it. Depth resolution depends on pinhole size and the numerical aperture (N.A.). In a certain range, the ability of depth discrimination increases with the reducing pinhole size and high N.A. number. We tested three kinds of objective (N.A. 0.25 / x10, 0.4 / x20, 0.65 / x40) and several pinholes ranging from 25 \( \mu \)m to 1000 \( \mu \)m. The best result in depth resolution was obtained by the combination of a 25- \( \mu \)m pinhole and a 40x Objective with 0.65 N.A.. According
to the definition of the full width at half maximum (FWHWM), resolution of 2.7 µm was obtained (Fig. 2(b)). The resolution does not depend on the monochromator slit width (10 - 100 µm). However, if the optics are not all well-arranged or precisely-positioned (which may often occur for a normal operator), the monochromator slit could function as a second spatial-filter.

In order to illustrate the performance and potential applications of this setup, several experiments were performed. Air-liquid interface is one of the most important systems for surface chemistry. An instrument which could be used to discover the surficial spectroscopic imaging information sensitive to submonolayer at a microscopic spatial and time scale would be very useful. Therefore, we took some examples related with air-liquid interface in this work.

For air-liquid interface studies, we could not use a coverslip on the sample. Thus the evaporation of liquid and the change of interface absolute position are unavoidable. Using our confocal microscope capable of the depth discrimination, the signal change due to the vertical position change of surface can be detected easily. The evaporation speed increased with temperature dramatically. When the room temperature reached 30 degree, the evaporation speed is ca. 2 µm per minute for a cuvette with 19.6 cm² surface area. Therefore, one experiment should be carried out within a short time. However, this should be traded off with the sensitivity and the stability of measurements.

Fig. 3 shows the spectra of rhodamine B as 10-µm increments of z-axis for the microscopic sample stage. At each point, a spectrum was taken with an integration time of 120 s. A 25-µm pinhole and a 40x objective with 0.65 N.A. were used in these experiments. The SBIG CCD camera was used to acquire spectra. A sharp decrease in signal intensity from curve (4) to (3) corresponds to the air edge of rhodamine B solution. The signal of curve (5) is slightly more intense than that of (4). Whether such decrease is due to the adsorption of rhodamine B molecules to the surface or due to the optical index change is under investigation. The change of spectra demonstrates the performance of the instrument and provides some interfacial characters of the investigated samples.

The fluorescence spectroscopic signal coming from molecules with low surface density at the air-water interface is very weak. The high sensitivity of the intensified CCD makes it possible to finish measurements in a short time to avoid the problem of evaporation. The spread OEP samples at air-water interface were checked. OEP molecules can not be solved in water. Making use of the constructed system with the ability of depth discrimination, it was easily demonstrated that there were not fluorescent molecules coming from the bulk. By moving the sample stage up, the laser beam focus at the bulk position of water and no fluorescence signal was observed. The fluorescence spectrum from OEP at water surface (19 pmol/cm²) was obtained as shown in Fig. 4. The integration time was 5 s for this spectrum.

![Fig. 3 Fluorescence spectra of rhodamine B measured as the interface of air-liquid is translated axially through the laser focus at 10-µm increments. The position 0 is arbitrary and above the surface. Integration time: 2 min. Pinhole: 25 µm.](image)

![Fig. 4 The fluorescence spectrum from OEP at water surface.](image)
For different applications, different objectives may be selected. Objectives with higher N.A. could provide higher depth resolution. However, the shorter working distance accompanying with higher N.A. makes some investigations difficult such as the comparison of the signal between from surface and from the bulk. An interesting phenomena was found out that when the distance between the edge of a objective is within 0.2 mm, the water may adhere to the objective suddenly even though there was a small interval initially between them. This may due to the attraction force between water and the objective or the mechanical instability resulting from outer vibration. To obtain the proper surface spectrum, careful manipulation of the apparatus is required.

Spatially and temporally resolved imaging can be obtained by the setup. It provides a potential tool to visualize the molecule distribution at a microscopic scale. The temporal time for an image could be as short as 0.5 ms, which makes the trace of molecule movement possible. Fig. 5 is a real time fluorescence image of TPP at air-water interface. Like OEP, TPP is insoluble in water and only stays at water surface.

![Fluorescence image](image.jpg)

Fig. 5 Fluorescence image of TPP at water surface (4.6 pmol/cm²).

The lateral random movement due to micro-brownion motion results in molecule collision. The bright spots in the image would represent the molecule clusters. Continuous change of the images measured in succession were also visualized. From the microscopic viewpoint, the distribution of molecules at water surface is not homogeneous and subject to change. Visualizing such molecular behavior can be achieved by the constructed microscope system.

The versatile CCD-based confocal microscope system would find many applications in the investigation of air-liquid interface. Although we are interested in employing it in this field, the apparatus can also be a powerful tool for studies in other fields.

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References: