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Spatially resolved micro-absorption spectroscopy with a broadband source and confocal detection

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ABSTRACT

We present a novel approach to measure optical absorption spectra with spatial resolution at the micron scale. The setup combines a continuous white light excitation beam in transmission geometry with a confocal microscope. The spatial resolution is found to be better than 1.4 μm in the lateral and 3.6 μm in the axial direction. Employing multichannel detection the absorption spectrum of hemoglobin in a single red blood cell is measured on the timescale of seconds. Through measurements of the transmitted intensity in solutions in nanoliter quantities we establish that the absorbance varies linearly with concentration. Our setup enables the investigation of spatial variations in the optical density of small samples on the micron scale and can be applied to the study of biological assemblies at the single cell level, in optical diagnostics, and in micro-fluidics.

1. Introduction

The ability to investigate structure and dynamics on a micron scale with non-destructive optical probes is key to studies at the single cell level and applications in micro-fluidics. For visualizing 3-dimensional cell cultures and thick tissue samples non-destructively, imaging techniques that rely on epi-illumination have been extensively used. Optical coherence tomography can image thicker biological specimens at high resolution [1]. This is desirable when 3-d scaffoldings of cell structures are probed that closely resemble the micro-environment of biological tissue. On the other hand, for studies of individual cells optical path lengths in the range of 10 microns are sufficient. Here, confocal microscopy provides enhanced resolution due to elimination of out of focus rays via multi-photon excitation or a spatial filter (pinhole) [2]. A pinhole is located in the conjugate plane of the focal plane (defined by the collection optics) which enables optical sectioning along the axial direction [3]. In widespread use are confocal laser scannning microscopes where a laser beam is scanned across the sample and an image is reconstructed. Generally, contrast agents are required [1]. Fluorescence probes in combination with confocal or other geometries are well established; however they need labeling and are limited by photobleaching and quenching [4]. More recently, gold nano-particles have been used as contrast agents [5] as they are not subject to photobleaching. An approach that does not require exogeneous labels at all for characterizing an unknown material is provided by micro-spectroscopy based on absorption measurements [6,7]. Fourier-transform infrared (FTIR) spectroscopic imaging exploiting vibrational signatures has numerous applications [8]. However, an order of magnitude smaller diffraction limited spot size can be obtained with wavelengths in the visible region. Though light scattering has been used recently as a source of contrast in the visible range of the spectrum [9], standard confocal microscopy so far lacks direct optical absorption profile measurements. The spatial throughput of a microscope is described by the point spread function ('the image of an ideal point object'), and its Fourier transform (the optical transfer function) gives information about the frequency response [10]. The highest spatial frequency that can be recorded is limited by the numerical aperture of the microscope. Extended sources (i.e., lower spatial frequency objects) lose signal strength with defocus because source light rays must originate in the finite field of view of the microscope. The image obtained from each incremental focusing plane contains superimposed information from the planes lying above and below the focusing plane. Thus, the optical transfer function of a wide field microscope is angularly band limited. In the 3-D Fourier spectrum of the point spread function this results in the loss of a biconic regions of frequencies, oriented in the direction of the optical axis [11]. The 'missing cone' [10,11] presents a difficulty for measurements with axial optical sectioning. To provide spatial discrimination in the axial direction for absorption measurements a confocal laser absorption microscope operating at a single wavelength was reported by Sasaki et al. [12]. An excitation laser pulse irradiates the sample so that ground-

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state molecules transit to the excited state, thus creating a spatial distribution of molecules, similarly to what is used in confocal fluorescence. The absorption to higher energy levels is then probed by a monitoring laser beam introduced coaxially. An excited state absorption profile is obtained by scanning the sample. In the laser-induced absorption microscope of Sasaki et al. the monitoring light is transmitted through the same region as the excitation, and the observed transient absorbance which is proportional to the concentration of the excited molecules varies as a function of the axial coordinates. This allows for the depth discrimination capability that is augmented when a pinhole is placed in front of the detector. In general the absorption of the laser beam due to electronic transitions from the ground state is assumed to be negligible, although the attenuation of the propagating light could provide a mechanism for contrast in the axial direction.

We demonstrate a novel approach to obtain ground state absorption spectra with a spatial resolution of better than 1.4 μm in the lateral and 3.6 μm in the axial direction [13]. Our method employs a confocal detection system to probe and spectrally resolve the attenuation of a white light beam in the axial direction. The axial resolution is related to the diameter of the pinhole, which is located at the entrance of the spectrometer. It enables the measurement of absorption spectra of biological assemblies at the single cell level and of small samples with a thickness of few microns. Absorption microscopy offers significant advantages associated with its label free simplicity. Contrast is generated by absorption, which provides a strong signal, yet requires low light intensity for excitation. The overall system cost is significantly lower, as no costly laser sources and coupling optics are required. Confocal absorption microscopy (CAM) is nondestructive and is capable of collecting both spatial and physical information based on light absorption by microscopic structures. Following a description and characterization of the setup, absorption measurements of single red blood cells and solutions in a 50 μm pathlength micro-capillary are presented.

2. Experimental

A schematic diagram of the confocal absorption microscopy method is shown in Fig. 1. Our setup couples a broadband white light excitation source in transmission geometry with a confocal detection system. A conventional tungsten–halogen lamp illuminates the sample through an optical fiber (core diameter 400 μm, NA=0.22). The fiber cylinder is topped with an aperture (e.g. 25 μm diameter) and seated at the center of a slide assembly. The aperture limits the stray light and defines the illuminated are on the sample. The slide assembly has a groove at the center for aligning the micro-capillary with outer diameter of 350 μm and inner bore of 50 μm that is used in solution measurements. The slide is then mounted on 3-D positioning stage allowing scanning the sample through the optical path. Limiting the illuminated area of the object is not sufficient to achieve spatial resolution in the micron range. Therefore the object is sampled with a confocal collection optics by moving the positioning stage.

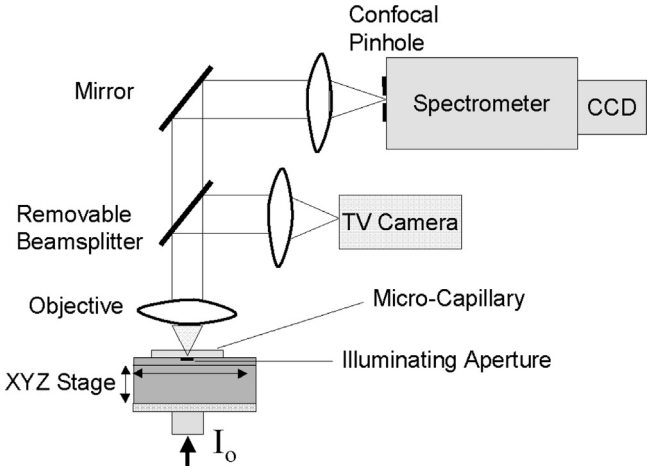

Fig. 1. Schematic of confocal absorption microscopy (CAM) setup.

The transmitted light is collected through a 50x (NA=0.75) dry objective lens, reflected by a mirror and focused through a lens on the confocal hole at the entrance of the spectrometer (LabRam HR800). The spatial resolution is determined by the confocal hole and the objective on the detection side. The collection optics is essentially that of a confocal Raman spectrometer. An image of the sample in the focal plane is formed on the plane of the confocal hole. The (de-) magnification is determined by the optical system and corresponds to 1/70 for the spectrometer used here (LabRam HR 800) with a 50x objective. Thus a 50 μm pinhole has an effective geometric size of 0.71 μm at the sample plane. For high numerical aperture objectives when a monochromatic excitation beam is focused, the diffraction-limited diameter d_o and the depth of focus L can be approximated by $d_o=1.22\lambda/\mathrm{NA}$ and $L=4\lambda/\mathrm{NA}_2$, respectively [2,14]. Typical values for our arrangement are (NA=0.75, $\lambda=500$ nm) $d_o\sim0.8$ μm and $L\sim3.6$ μm.

In the optical beam emerging from the microscope objective, a movable beamsplitter can be inserted for sample observation on a TV camera (Sony CSC790). Spectra are acquired with a back-illuminated CCD detector over a spectral range from 350 to 700 nm at a spectral resolution of 0.5 nm. The instrument is operated in a point scanning mode by moving the sample stage in *x*-, *y*-, and *z*-directions. For each sampling position a complete spectrum is acquired (coverage 300 nm with a 100 grooves/mm grating) in multichannel mode.

3. Results and discussion

In a first test of the homogeneity of illumination an aperture with an opening of 25 μm was centered at the optical axis in front of the sample. The focus in the axial direction was near the plane of the aperture. In addition to the illumination aperture there is a confocal pinhole at the entrance slit of the spectrometer that was set to 40 μm. The aperture on the sample stage was then scanned through the beam along the radial direction with no sample present and the intensity measured. For each position a spectrum over a range from 350 to 680 nm was recorded in multichannel mode. The light source was a fiber coupled tungsten–halogen lamp. The intensity as a function of the lateral position is depicted in Fig. 2 for a wavelength near 420 nm. The measured light intensity inside the aperture varied by less than 7% inside a 20 μm diameter. At the edge the intensity increases from 10% to 90% over a distance of 1 μm. The tests were repeated with apertures having openings of 5 and 10 μm with similar results. We conducted another set of experiments where the size of the illuminating aperture was kept fixed (25 μm) while the confocal hole was varied from 10 to 50 μm. The aperture on the sample stage was moved laterally relative to the confocal spot. The measured light intensity plateaus inside the aperture, but the slope at the boundary becomes steeper with decreasing pinhole size. We found that the resolution (10–90% intensity points) is controlled by the confocal pinhole on the detection side while the aperture limits the illuminated area on the excitation side.

To assess the lateral and axial resolutions in absorption measurements we employ individual red blood cells with diameter of about 6–8 μm as small absorbing objects. Red blood cells were