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ABSTRACT

A spectrometer has been developed for the Phoibos confocal scanning laser microscope (CSLM). With this spectrometer, spectral information from a single point, or a user defined region, within the microscope specimen, can be recorded. The spectrometer is based on an integrated spectrometer module, manufactured by Carl Zeiss, Germany. The module takes its light input signal through a fibre with an entrance diameter of 0.5 mm. Integrated in the spectrometer module are dispersing optics, based on a grating, as well as preamplifier electronics. A regulated cooling unit keeps the detector at -4°C, thereby allowing longer integration times. The spectral resolution, defined as the minimum distance between two peaks (Rayleigh criterion) is approximately 10 nm. The entrance of the optical fibre is employed as a pinhole. With different magnifications in the optical path leading the light to the spectrometer, the entrance can either be employed as a pinhole of the same size as the one used during conventional confocal scanning, i.e. the 3-D spatial resolution will be retained, or the light throughput can be increased at the expense of optical resolution.

With the described equipment, studies of rodent lung and liver specimens containing porphyrins have been made. Organs from animals injected with δ-amino levulinic acid (ALA), a precursor to protoporphyrin IX and haem in the haem cycle, have been studied. Spectroscopic detection is necessary in order to separate the porphyrin signal from other fluorescent components in the specimen.

1. INTRODUCTION

PDT, Photodynamic therapy is a method under current evaluation for treatment of malignant tumours[1,2]. The method involves excitation of a photosensitizer given to the patient being treated. The photosensitizer absorbs incoming light and starts a photochemical reaction, leading to cell necrosis. Due to a selective accumulation of the photosensitizers in the malignant tissue, only the tumour cells are affected by this treatment. Several porphyrins are used in PDT. These have the ability of accumulating in tumours, as well as transferring incident light energy to oxygen molecules, which are thereby transferred to an excited electronic singlet state. Oxygen in this state is cytotoxic. For PDT, excitation is often performed at a wavelength of approximately 635 nm, as this wavelength combines good absorption by the porphyrin molecules with deep tissue penetration. The aim of our studies is to understand the properties of some porphyrins, which make them suitable for PDT. A number of photosensitizers are under evaluation. In this study we investigated δ-amino levulinic acid (ALA), which is a precursor to haem in the haem cycle. In the mitochondria of the cells it will be transformed to protoporphyrin IX (PpIX). The latter one is the photochemically active substance.

1.1. Existing material

In order to distinguish the porphyrin signal from other fluorescent signals in the specimen, a spectrometer in conjunction with a confocal scanning laser microscope was used. Such an instrument, previously built at the department, and used for studies of porphyrin-containing specimens[3,4], had a too low signal-to-noise ration, and too low sensitivity to allow studies of sub-cellular structures. This spectrometer used a prism as a dispersing element and a Reticon diode array with 256 elements to detect light. The diode array combined with an evaluation board provided by the manufacturer gave a signal-to-noise ratio of only 40:1 at high light intensity levels, and even lower at low light intensity levels. Another problem with the setup was, that the entrance aperture of the spectrometer, a pin hole with a diameter of 50 μm, also was used to discriminate light from out-of-focus planes, i.e. as the normal pin hole in a confocal microscope. When the optics of the microscope scanner were realigned, the entrance aperture of the spectrometer also had to be moved, thereby making a complete realignment and recalibration of the spectrometer necessary.

2. MATERIALS AND METHODS

In order to increase the signal-to-noise ratio, and thereby gain in sensitivity, a new spectrometer was integrated in the Phoibos CSLM. A mirror allows light from the scanned specimen to go either to a photo-multiplier tube (PMT) detector, or to the spectrometer. During conventional confocal scanning, a pin hole with a diameter of 50 μm ensures confocal imaging. Spectrometer readings are performed with the entrance aperture of the spectrometer acting as a pin hole. The spectrometer, an integrated module manufactured by Carl Zeiss, Germany, includes a complete spectrometer, connected to a diode array detector producing a sequential analogue output signal, with a voltage proportional to the intensity incident at the wavelength interval corresponding to the element. The spectrometer module (fig. 1) takes its light input through a bundle of optical fibres. This bundle, consisting of 30 fibres with a core diameter of 70 μm, is arranged in such a manner, that the entrance aperture is circular, with a diameter of 0.5 mm, while the distal end, connecting to the dispersing optics, is linear, 2.5 mm x 70 μm, thereby forming an entrance slit to the spectrometer. The dispersing element in the spectrometer is a combined imaging system and grating, blazed for approximately λ=340 nm. The light is detected by a Hamamatsu S3904-256 diode array with 256 elements, dimension 25 x 2500 μm. The spectrometer is designed for a spectral range of 300 - 1150 nm, with a dispersion of approximately 3.3 nm per pixel and a resolution of 10 nm (Rayleigh criterion)[5]. In order to reduce the dark current, the spectrometer module was cooled with a Peltier cooling element. The cooling device kept the module at a temperature of -4°C, thereby reducing the dark current to approximately 1/25 of the value obtained at normal operating temperature (≈ +30°C).
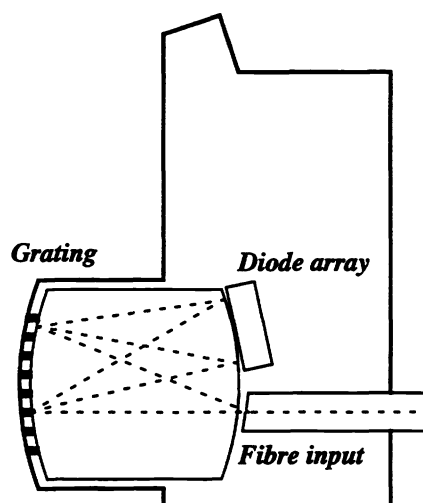

Fig 1. *The Zeiss MMS 1 spectrometer module, size 1:1.*

An achromatic lens couples the light from the microscope into the spectrometer (fig. 2). Two different lenses, with magnifications of 5x and 10x respectively can be used. The 10x lens yields the same confocal properties as conventional confocal readings, while the 5x lens provides more light, but at the expense of optical sectioning.

2.1. Alignment and calibration

After alignment of the CSLM, a mirror was inserted into the ray path, thereby deflecting the scanned light onto the spectrometer fibre input. This arrangement allows realignment of the microscope without recalibration of the spectrometer, as only the fibre, and not its distal end had to be adjusted. Coefficients for calculating the wavelength corresponding to each of the 256 detector elements were provided by the manufacturer. A sensitivity calibration was performed by recording light from a standard of spectral irradiance, an Oriel 200 W quartz halogen lamp. The light was recorded through the microscope optics, thereby compensating for wavelength dependent transmission of the CSLM optics. The wavelength calibration was verified by recordings of several spectral lines within the intended operating wavelength range, i.e. 400 - 800 nm. The accuracy was found to be better than 3 nm, depending on the method used to calculate the centre location of the spectral peak. In fig. 3, a reading of the 488 nm Ar^{+} line is shown. The reading verifies the spectral resolution of 10 nm (Rayleigh criterion) stated by the manufacturer.
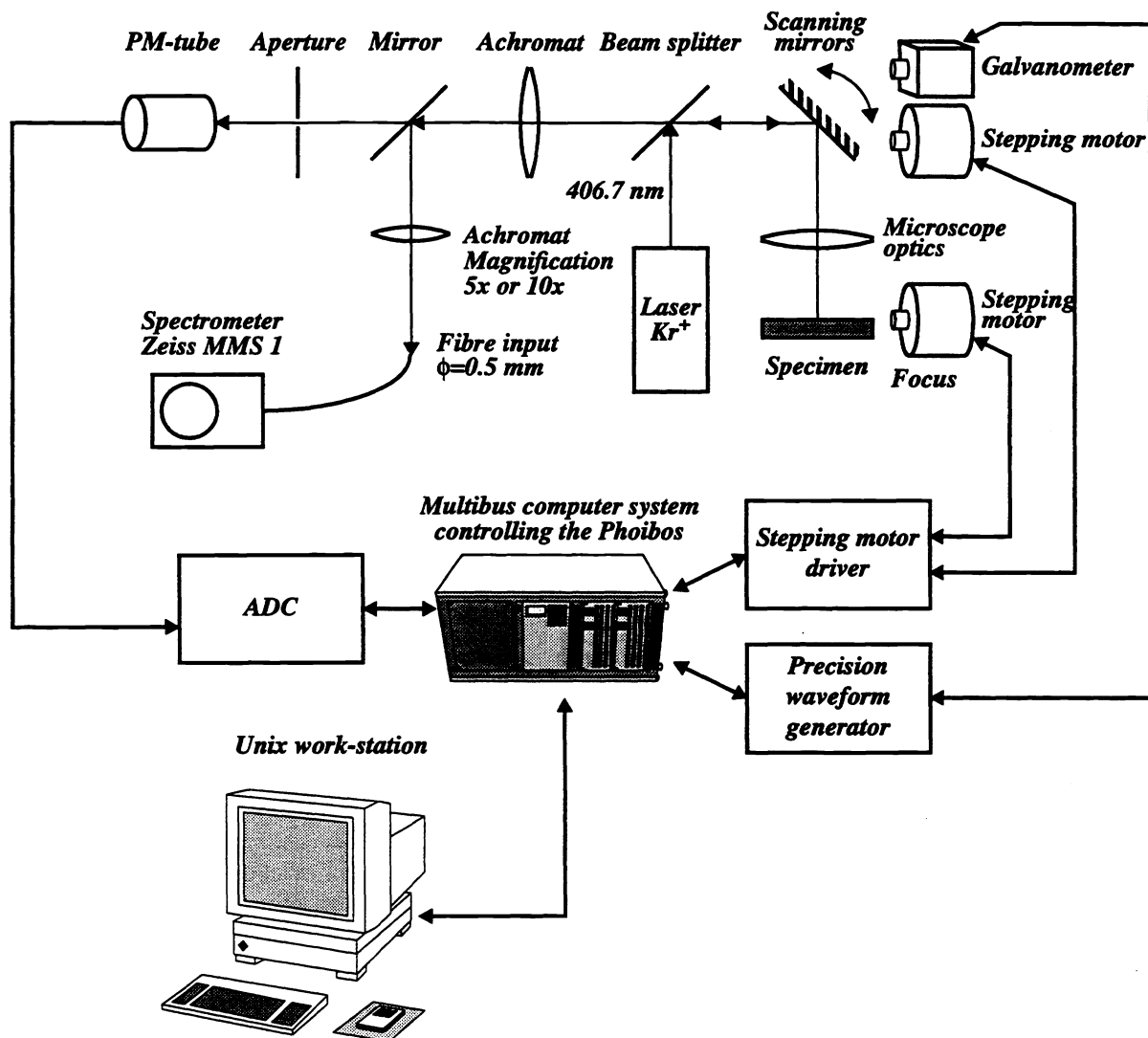
Fig 2. *The Phoibos CSLM equipped with a precision waveform generator, allowing flexible scanning modes, and e.g. statical positioning of the recording point.*

The signal-to-noise ratio of the spectrometer system was determined by making 100 consecutive readings of a specimen consisting of Lucifer Yellow dye, 0.1 mg/ml dilution, 1.5 s spectrometer integration time. Excitation was provided with a Kr^+ laser operating at $\lambda=406.7$ nm and giving 100 mW of power. Comparing the mean value for the detector element with the highest output signal to its standard deviation gave a signal-to-noise ratio of better than 1000:1. It should be noted, that this includes noise from all parts of the system, the laser, the microscope optics/mechanics, and the spectrometer.
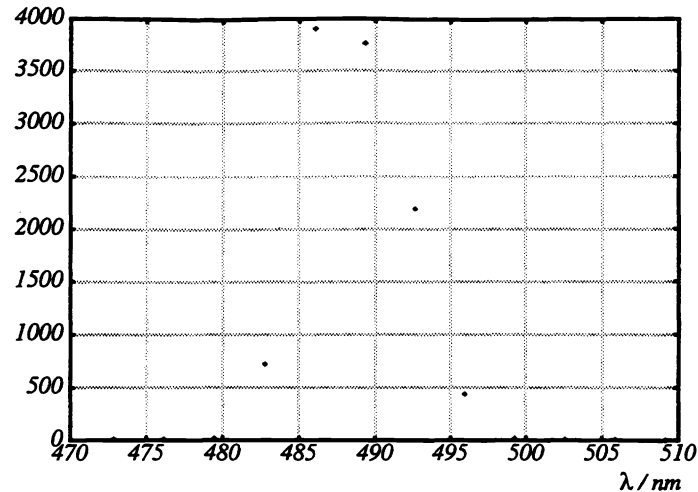
Fig 3. *Spectrometer reading of the 488 nm Ar$^+$ line. The spectrometer has a FWHM of approximately 10 nm.*

2.2. Scanning

The Phoibos CSLM scans images by using two scanning mirrors, a fast, galvanometer-mounted mirror scanning along lines, and a slow, stepping-motor controlled mirror advancing to the next scan line each time the fast mirror retracts. In the original design, the fast mirror was guided by a fixed sawtooth waveform at a frequency of 50 Hz[6]. In order to allow scanning to be performed in a more flexible manner, the sawtooth ramp generator was later exchanged against a digitally controlled precision waveform generator[7]. This generator, which also records the read back position signal from the galvanometer scanner, makes it possible not only to scan specimens in the conventional manner, but also to e.g. statically position the recording point at any user selected position within the specimen (fig. 2). The accuracy of the positioning method has been verified to be better than 1 pixel at an image size of 256 x 256 pixels. For spectral recordings of a specimen, an overview image of the specimen was scanned at an image size of 256 x 256 pixels. A rectangular region of interest was then interactively indicated. During the spectrometer integration time, the microscope recording point was moved over this region.

2.3. Specimens

In the studies, samples of rodent liver were investigated. The rats were injected with ALA, and after about three hours the animals were sacrificed and the organs removed. After that, the samples were temporarily placed in isopentane with carbonic ice. For storage they were held frozen at a temperature of -18°C. No further staining and fixation procedures were adopted. Before microscopic investigation, the specimens were cut in slices approximately 0.5 mm thick.

3. RESULTS

Recordings were performed with a Zeiss Planapo 10x/0.32 objective. The specimens were first examined ocularly in fluorescence mode in the microscope. Illumination was provided by light from a mercury lamp with a 405 nm filter in the illumination path. At this ocular examination, larger areas with intensities different from the surroundings could be distinguished, but no cellular structure was seen. At higher magnifications, the situation was similar. Confocal scanning revealed basically the same images as the ones seen in fluorescence mode, although the images, for obvious reasons, were sharper. Spectral recordings were made at different locations. In fig. 4 a recording made over one of the brighter parts in the specimen is shown.
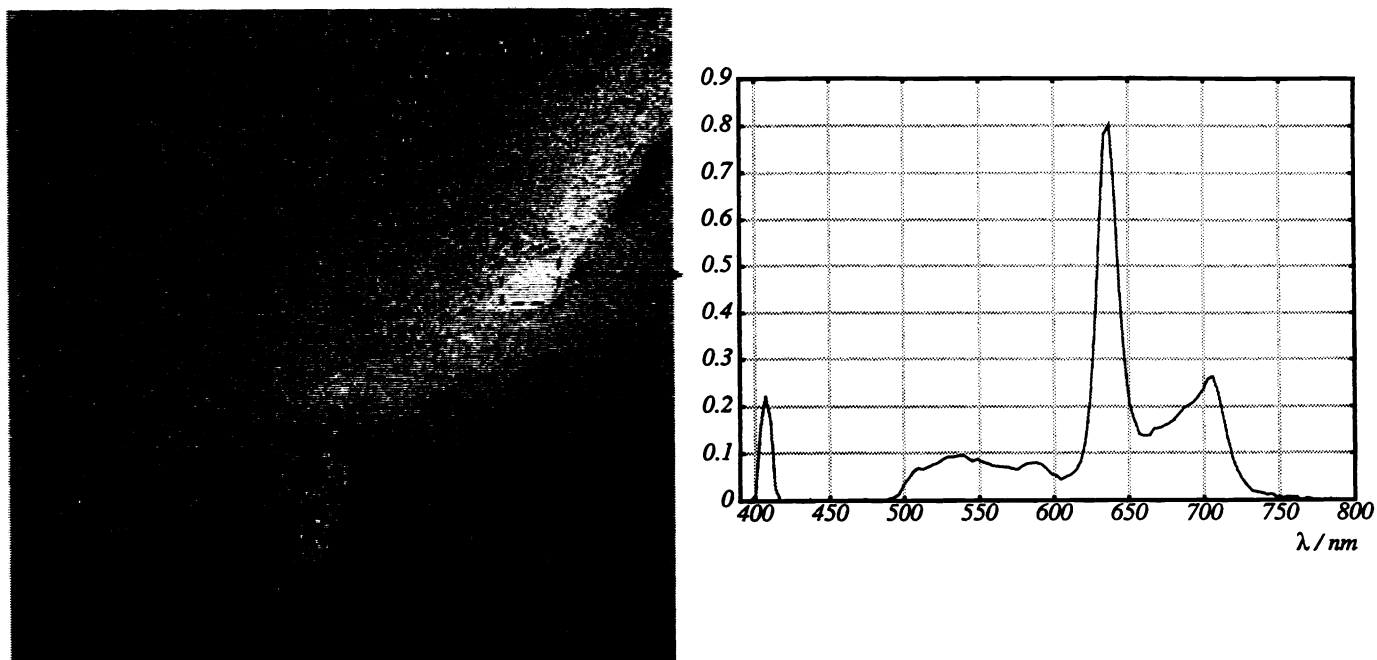
Fig 4. *Porphyrin-containing rodent liver specimen. The spectrum recorded over the identified region shows the characteristic porphyrin dual peaks at 630 nm and 700 nm. A reflection in the microscope optics of the exciting light, 406.7 nm, can also be distinguished. A 500 nm beam splitter was used for the recording.*

A problem encountered with the specimens was, that they were subject to bleaching as well as aging. While the bleaching was rather moderate, a specimen could be scanned at least a few times before any significant changes in the intensity could be distinguished, aging could be observed quite easily. In fig. 5 a recording of a specimen held at room temperature 24 hours before examination is shown. The spectral curve has changed significantly (compared with fig. 4), a new spectral peak at 590 nm can be observed, while the peak at 700 nm has decreased. Comparing spectra recorded over brighter regions to spectra recorded over darker regions reveals, that the spectral shapes did not change.
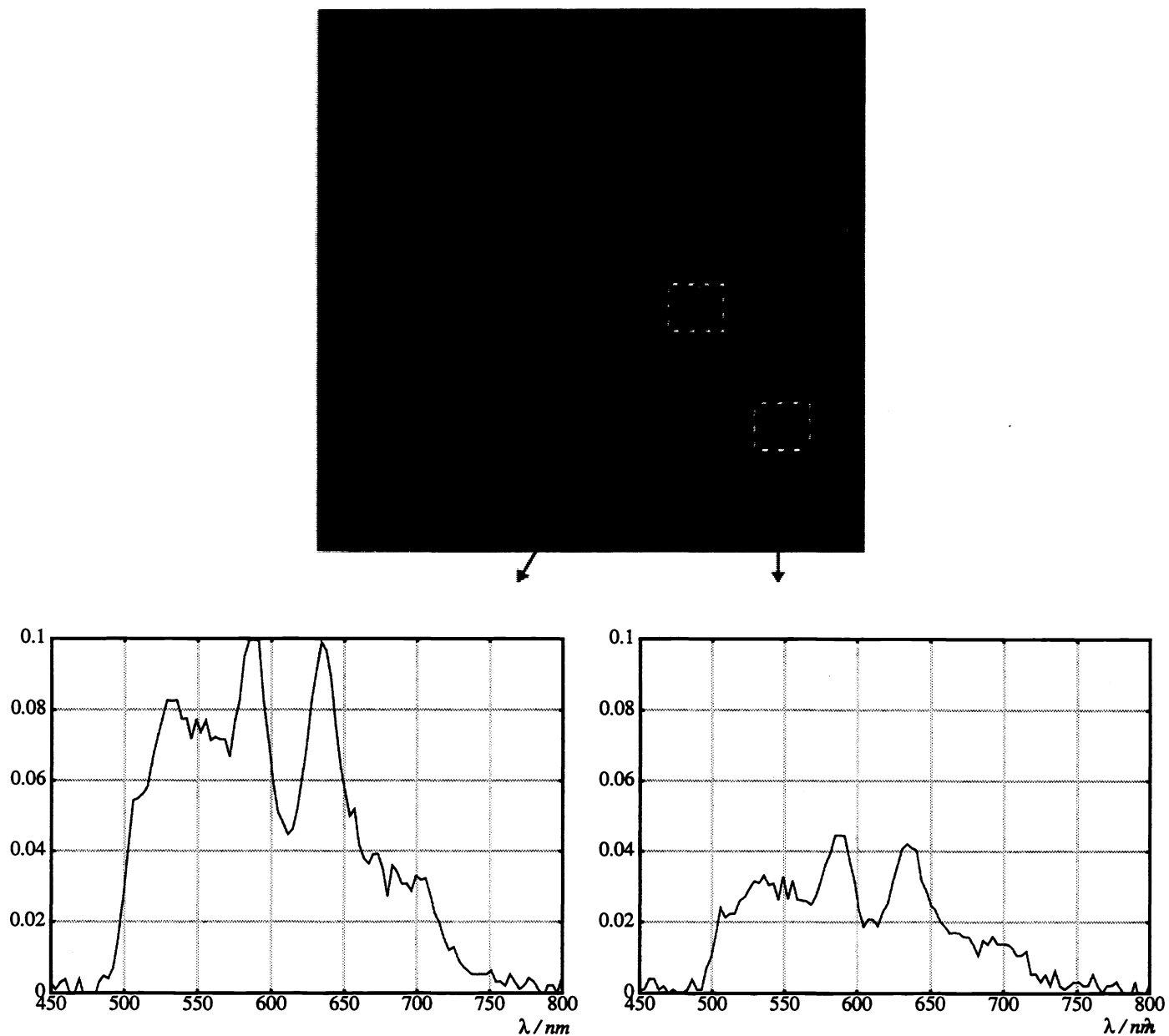
Fig 5. *Spectra of porphyrin-containing rodent liver specimens, which were held at room temperature 24 hours before the recordings were made. The spectral peak at 700 nm (fig. 4) has been reduced, and a new peak at 590 nm has appeared. Two regions were identified in the scanned overview image. The recorded spectra differ only in their amplitudes, not in their shapes.*

4. CONCLUSIONS

In the specimens used in the study, no cells could be distinguished, neither in confocal, nor in fluorescence mode. The reasons for the lack of cellular structure are to be examined. One possible reason may be the freezing process, which could destroy the cells, and let the porphyrins be distributed in a uniform manner in the specimen. The equipment, consisting of a microscope allowing fluorescence and confocal microscopy, as well as spectrally resolved confocal microscopy, has been tested and found to be satisfactory. With sufficiently bright specimens, the spectrometer would yield signal-to-noise ratios of 1000:1. Detector cooling allows integrations times up to 120 s.

5. REFERENCES

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