Subtractive imaging in confocal scanning microscopy using a CCD camera as a detector

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We report a scheme for the detector system of confocal microscopes in which the pinhole and a large-area detector are substituted by a CCD camera. The numerical integration of the intensities acquired by the active pixels emulates the signal passing through the pinhole. We demonstrate the imaging capability and the optical sectioning of the system. Subtractive-imaging confocal microscopy can be implemented in a simple manner, providing superresolution and improving optical sectioning.

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Confocal laser-scanning microscopy (CLSM) has been demonstrated as a powerful technique in three-dimensional (3D) specimen analysis and has many useful applications [1–4]. In the standard configuration, the sample is illuminated with a coherent beam focused through a microscope objective (MO). The light reflected by the sample (or emitted in the case of fluorescence) is collected by the same MO and focused on to the image plane. A pinhole is placed in this plane, blocking most of the light coming from sections of the specimen that are out of focus. Commonly, the detection is performed using a large-area detector placed behind the pinhole, for instance a photomultiplier tube (PMT), which integrates the light arriving on its area into a signal. This signal contains information about a single point of the specimen. To obtain the 3D image of the specimen, a 3D scanning process is necessary. The scanning can be performed in various ways. For example, transverse scanning can be performed by deflecting the beam by means of galvanometric mirrors. For each step of the scanning, the PMT returns a signal value, and the image is synthesized in a computer. Because CLSM provides optical sectioning, an axial scanning of the sample produces a stack of sections that can be combined in a 3D representation of the sample.

PMTs are used in CLSM because of their highly linear response, their low response time, and also their large dynamic range. For many years, no good alternative to PMTs in CLSM was available.

The development of CCD cameras, however, has permitted the achievement of 16 bits of dynamic range and frame rates up to 500 f/s. In addition, the pixel size has been reduced to a few microns. These improvements allow our proposal, which includes the replacement of the system composed of the pinhole and the PMT (or three PMTs and a set of filters for three-channel detection) with a CCD sensor only. The CCD sensor is placed in the pinhole plane (see Fig. 1). Then the same signal as is conventionally acquired with the pinholed PMT can be emulated by a numerical integration of the signal detected by few pixels of the CCD. Different authors have suggested similar schemes for CLSM with detector arrays [5–7]. Detector arrays have also been used in combination with pinholed detection [8]. In any case, the use of detector arrays provides extra information about the sample, which can be extracted in a postprocessing procedure to improve resolution of the system. In the second stage of this research, and with the aim of demonstrating the advantage of using the CCD, we demonstrate the possibility of performing subtractive-imaging superresolution techniques.

Detection is performed by selecting the active pixels of the CCD, creating a synthetic pinhole. The numerical integration within this area provides the signal at each pixel of the confocal image. In practice, the synthetic pinhole that one builds with camera pixels can be a square or a pixelated circle. Note that some commercial confocal systems use a dynamic square pinhole created by two adjustable slits.

The use of the CCD for the confocal detection has many advantages over conventional detection. One advantage is that we can visualize simultaneously the illumination spot and a bright-field image. This can be done by illuminating the sample with an extended white-light source in addition to the laser illumination. This simultaneous visualization permits an easy localization of the region of interest in the sample and also selection of

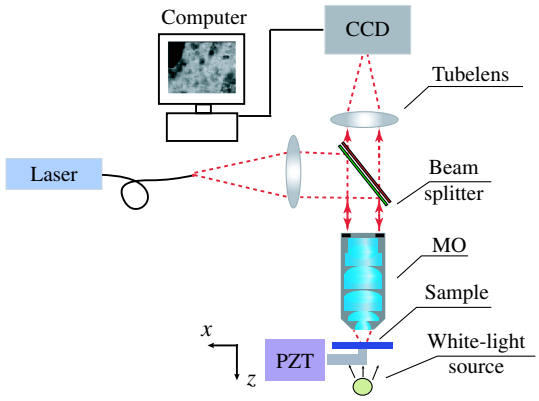

Fig. 1. (Color online) Schematic of the proposed CLSM.

the synthetic-pinhole size. Second, it permits the easy active alignment of the detection arm, which is a critical issue in confocal imaging [9]. Another advantage is that our proposal is a low-cost implementation of a confocal scanning system (especially for the case of three chromatic detection channels) inasmuch as it eliminates the set of pinholes (or the dynamic pinhole) and the PMT. The simple addition of a scanning stage would permit the use of a wide-field microscope for confocal microscopy. An additional, but not less important, advantage is that the use of the CCD permits the easy implementation of subtractive-imaging procedures for the improvement of 3D resolution [10–12].

But we should not forget that typical PMTs have more dynamic range than high-quality CCDs. In some applications, a very low signal has to be detected, as occurs in the case of fluorescence microscopy, and the signal cannot be detected by a standard CCD camera. In the same way, the speed response of PMTs is a few nanoseconds for the whole detector, whereas the response of high-speed CCD sensors is more than 20 ns per pixel. This can be a bottleneck when scanning speed is important. The fast development of CCD cameras, however, suggests that these disadvantages will be overcome in the future.

To prove the utility of our proposal, we performed a series of proof-of-concept experiments in which we used a common CCD camera, whose speed response is far from that of high-speed cameras. In our experimental setup, the light exiting from a fiber coupled to a He–Ne laser (632.8 nm) is collimated through a lens with focal length of 200 mm. A beam splitter directed the collimated beam to a $100\times$ dry NA $= 0.9$ MO. The light reflected by the sample is collected by the same MO. Then a tube lens of 200 mm focal length conjugates the plane of the focal spot with the plane of the matrix sensor. The CCD used has 1600×1200 pixels of 4.4 μm size. The scanning was performed by moving the sample with a PiezoSystem stage (PZT) that permits 3D displacements of 80 μm in steps of 0.1 μm for each direction. The camera frame rate was 12 f/s for the whole detector. To have an idea of the acquisition time, for a synthetic pinhole formed by 12×12 pixels (which corresponds to $\sim$50 μm, a frequently used pinhole size), the frame rate is increased up to 421 f/s. Our speed limitation is on the PZT, however, which scans 50 points per second. The acquisition software was implemented in Labview©. With this software, we have free control of the camera settings (exposure time, gain, number of active pixels, and their position). Then, we can visualize the laser spot reflected by the sample before the acquisition and therefore choose the synthetic-pinhole position and size. At this point, we select also the gain and exposure time to avoid saturation of the pixels. After selecting the scanning zone and the step, the sample is scanned synchronously with the acquisition of the emulated signal.

To demonstrate the optical sectioning capability of the system, which is intrinsic to CLSM, and also to select the optimum synthetic-pinhole size, a flat mirror was used as the sample in our first experiment. Then we obtained the classically known $V(z)$ function [4], i.e., the signals collected when scanning the flat mirror along the axial direction for different synthetic-pinholes sizes, see Fig. 2(a). The size of the pinhole plays a very important

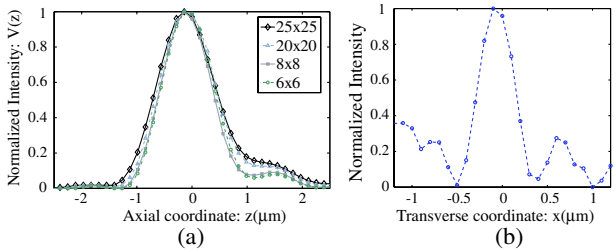

Fig. 2. (Color online) (a) Axial response to a flat mirror for different pinhole sizes (labeled in number of pixels). (b) Profile of a microsphere image of 0.1 μm radius.

role in confocal imaging [13,14]. From these curves, it can be seen that the optimal pinhole size, in terms of the optical sectioning, is around 8×8 pixels (about 35.2 μm). The asymmetry in these profiles is caused by a small amount of spherical aberration.

To have an estimation of the optimum pinhole size, we could have calculated its theoretical value simply by [1]

$$\Delta_{\text{pinhole}} = \frac{0.5\lambda}{NA}M, \tag{1}$$

where λ is the wavelength of the collected light and M is the magnifying factor of the MO. For the MO used in this experiment, $\Delta_{\text{pinhole}} = 35.1$ μm, which matches the experimental value. On the other hand, the axial resolution of a CSLM using the Rayleigh criterion is [1]

$$r_{\text{axial}} = \frac{1.4\lambda}{NA^2}. \tag{2}$$

This formula provides an axial resolution limit of 1.09 μm for the MO used in our experiment. This value could be a good estimation for the FWHM of $V(z)$). With the aim of estimating the transverse resolution of the system, we performed the confocal image of a reflecting microsphere with 0.1 μm of radius. The profile of the spot created in the confocal image is shown in Fig. 2(b). As can be seen, the distance between the first zeros of the impulse response is $\sim$0.7 μm, as is expected for this MO.

For the second experiment, we used a 1951 USAF Hi-Resolution target in negative (clear pattern and a chrome background) as the sample to verify the imaging capability of the system. In Fig. 3(a), we show a confocal image obtained with a synthetic pinhole of 8 pixels. To have a proof of the depth discrimination, the sample was tilted. In Fig. 3(b), we show the confocal image obtained with our system. Note how the out-of-focus light is rejected.

Fig. 3. 1951 USAF Hi-Resolution target. (a) Confocal image acquired with our system. A titled test target is shown in (b) for the confocal images and in (c) for bright-field images.

In Fig. 3(c), we show the bright-field image of the tilted object.

An immediate application of our system is referred to as a procedure that increases both the lateral resolution and the optical sectioning capability in CLSM. We refer to subtractive-imaging confocal microscopy [10]. The basic idea is to subtract, with the appropriate scale factor γ, two confocal images that were acquired with an open and closed pinhole, respectively. The subtracted intensity is obtained by

$$I_{\text{sub}} = I_{\text{closed}} - \gamma I_{\text{open}}. \tag{3}$$

The scale factor is selected in such a way that the weight of the open-pinhole image intensity, I_{open}, is one-half of the one acquired with the closed pinhole, I_{closed}.

In the usual realization, two pinholes with different sizes are needed for this subtractive-imaging procedure. To do this in parallel, two collection arms are required in the conventional confocal setup. In our system, however, both signals can be extracted from the same synthetic-pinhole information. Basically, the signal is acquired using a synthetic pinhole composed of several active pixels, $N_1 \times N_1$, from which we can obtain I_{open}. At the same time, by applying a mask that crops the original size to a smaller number of pixels, $N_2 \times N_2$, we also can acquire the signal I_{closed}. Finally, both signals are combined to produce I_{sub}. By using this method, the subtractive confocal image can be displayed at the acquisition time, while being sure of perfect alignment between both acquired images and without the need to incorporate extra elements into the system.

In Fig. 4, we show an example of the subtractive imaging performed by our system. In this capture, we used the same MO as before, and the sample used is onion skin imaged in reflection confocal illumination mode. To appreciate the resolution improvement, the scanning step was 0.2 μm, which is beyond the lateral resolution limit for this MO. The open synthetic pinhole was created by 60×60 camera pixels, whereas the closed pinhole was formed by applying a mask of 8×8 pixels over the first one. As it can be seen, a good improvement in the image quality is achieved with the subtractive procedure. This visual improvement is not only due to a slight raise of the lateral resolution [10] but mainly also to the fact that the subtractive image has higher optical sectioning capability.

In our last experiment, we evaluate the optical sectioning capability of the subtractive-imaging confocal microscope by measuring $V(z)$, see Fig. 5(a). As can be seen,

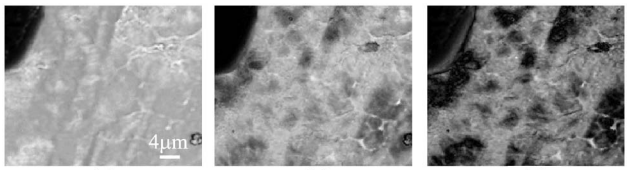

Fig. 4. Confocal images of an onion skin cell taken with (a) open pinhole and (b) closed pinhole. In (c) the subtractive image obtained from the open pinhole and closed pinhole images is shown.

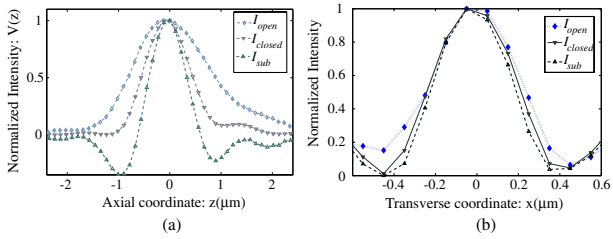

Fig. 5. (Color online) (a) Axial response to a flat mirror and (b) microsphere-image profile in the cases of open pinhole, closed pinhole, and the resulting response with the subtraction of both intensities.

the subtractive axial response for $\gamma = 0.21$ is narrower than the other two, which means that it has better axial resolution. By measuring the FWHM of I_{closed} and I_{sub}, we find an improvement of 19% in the axial resolution. As expected from the theoretical calculations, the subtractive signals present negative values in the sidelobes. Their height depends on the pinhole sizes, the MO used, and the value of γ [10]. In Fig. 5(b), we show the corresponding profiles of the reflecting microsphere image. It is shown that the subtractive in-plane resolution is slightly sharper than the case of closed synthetic pinhole.

Summarizing, we have presented an alternative manner in which to acquire confocal images by using a CCD camera as the detection system. We have demonstrated the functionality of the system, providing confocal images with its inherent optical sectioning capability. Then we have shown the applicability of this confocal microscope to optimize techniques in which images taken with different pinhole sizes are involved. As an example, we have implemented the subtractive imaging technique in the system, permitting the reconstruction of the subtractive image during the acquisition itself. Preliminary results of this research were presented at the 10th Workshop in Information Optics (WIO 2011).

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