Schlieren confocal microscopy for phase-relief imaging

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Phase objects are transparent samples with no light absorption. Many biological samples, such as nonstained tissue slices and cell cultures, can be regarded as phase objects. Under conventional microscopes, it is difficult to observe these samples due to the lack of contrast. To overcome this problem, various methods have been developed, e.g., phase contrast microscopy [1], differential interference contrast microscopy [2–4], diffraction phase microscopy [5], and spiral phase contrast microscopy [6]. Most of these technologies need specially designed phase plates, Nomarski prisms, spatial light modulation, or vortex phase modulation to extract the phase information [7]. Alternatively, Schlieren imaging has been reported to generate a phase-relief image, by partially blocking the detection of a wide-field microscope [8–10]. A more complicated form of obstruct phase-relief imaging, termed Hoffman modulation contrast microscopy [11], has also been elaborated. However, a large challenge of combining these phase imaging modalities with confocal laser scanning microscopy (CLSM) [12] lies in the fact that these techniques are designed for wide-field detection, rather than point-by-point scanning mechanism. In this Letter we propose a simple method of phase-relief image detection for CLSM. A fluorescence plate and a beam obstructor are introduced to combine the Schlieren imaging concept with confocal microscopy for phase-sensitive imaging. Its phase imaging mechanism is investigated and a linear relationship between intensity and sample phase gradient is established. Compared with the laser oblique scanning optical microscopy (LOSOM) which we reported earlier [13], the current Schlieren confocal (S-confocal) approach can more easily adapt to the current confocal setups, as it does not require any angle change to the scanning unit to achieve the laser oblique incidence. The setup shown in Fig. 1(a) consists of a standard confocal microscope. To form a S-confocal system, a fluorescence plate is placed behind the specimen. The other key component, a beam obstructor (for Type 1 S-confocal), is inserted in front of the confocal collection lens to partly block the fluorescence beam. The incident laser is focused by an objective lens, and its direction is altered by the phase structure of the specimen, as shown in Fig. 1(b). The laser excites the fluorescence plate to generate fluorescent irradiance to the specimen, as a secondary incoherent illumination light source. The fluorescence travels through the specimen, refracted again, partially blocked by the obstructor, and focused by the collecting lens on the confocal pinhole. The spatially shifted excitation can generate a detectable fluorescence signal, whose change is proportional to the

Fig. 1. Schematic diagram of S-confocal. (a) S-confocal is based on a CLSM, except for a fluorescence plate placed behind the specimen, and a block in front of the fluorescence collection. FM, fluorescent medium; PO, phase object; CP, confocal pinhole; Obj, objective lens; TL, tube lens; SL, scan lens; Obs, obstructor; DM, dichromic mirror; galva, galvonometer. Fluorescence illuminates the whole sample, but only the optical rays that go through the neighborhood of the scanning point could be detected. (b) The phase gradient of the specimen acts as a micro-prism to effectively deflect the incident/fluorescent beams. Here, only the incident laser beam is drawn. (c) Coordinates on the sample scanning stage. O is the laboratory frame, and O' is a moving frame on the sample. The coordination of the scanning point in the sample frame is xo.
phase gradient of the specimen. As can be seen from the theoretical analysis and the experiments, when a small pinhole is used, both excitation and detection can be effectively shifted, resulting in dual modulation; yet, when a relatively large pinhole is used, the phase gradient in the detection focal region is averaged, resulting in a linear modulation sensitive to the local excitation phase gradient.

Without loss of generality, a two-dimensional system with sample scanning is analyzed here for simplicity. First, we investigate the intensity of fluorescence excitation. We use Kirchhoff theory to show that the area illuminated by the laser is determined by the sample phase gradient on the focal point [14]. The excitation system is similar to a typical scanning transmission electron microscope, whose detection intensity on the fluorescence plate plane can be written as [15]

$$U(x_0, x_s) = U_0 \int_{-\infty}^{\infty} h(x_1) t(x_1 + x_s) \exp \frac{ikx_0 x_1}{d} \, dx_1,$$  \hspace{1cm} (1)

where $U_0$ is a constant proportional to the incident light field, $t(x')$ is the transmission rate of the specimen in the specimen coordinate $x'$, $x_1$ is the laboratory coordinate with respect to the scanning point, and $x_s$ represents the scan position in the specimen coordinate. The transmission rate of the specimen can be written as $t(x_1 + x_s) = \exp \{i\phi(x_1 + x_s)\}$, where $\phi(x')$ is the additional phase function on the specimen coordinate $x'$. Since, in confocal systems, the laser is focused on the image point, we expand the phase function into Taylor series, with only two leading orders remaining: $t(x_1 + x_s) = \exp \{i\phi(x_1) + ikx_0 x_1\}$, where $g(x_0)$ is the local phase gradient of the scanning point: $g(x_0) = (1/k)(d\phi(x_0)/dx_0)$. Substituting the expressions $h(x_1)$ and $t(x')$ into Eq. (1), we derive

$$U(x_0, x_s) = U_0 \exp \left( g(x_0) + x_0 / (a/f) \right),$$

which has a straightforward physical interpretation: the local phase gradient acts as a microp prism to refract the light [see Fig. 1(b)]. Since the fluorescence excitation is proportional to incident laser intensity, its intensity can be written as

$$I_f(x_0, x_s) = I_{f0} \exp \left( \frac{g(x_0) + x_0 / (a/f)}{a/f} \right).$$  \hspace{1cm} (2)

Under paraxial approximation, each isotropic excited point has equal contribution to detected intensity; however, since only the optical rays passing through the neighborhood of the scanning point can be detected, the obstruction makes some of the fluorescence points visible to the pinhole. If the refracted angle by sample is $g(x_0) = (1/k)(d\phi(x_0)/dx_0)$, where $x_0$ is the detection point, for a point detection, the detection intensity $I_{det}$ can be derived as the sum of all the fluorescence intensities $I_f$ at position $x_0$:

$$I_{det}(x_0, x_d) = \int_{-\infty}^{\infty} I_f(x_0, x_0) \eta(-g(x_0) d - x_0) \, dx_0,$$  \hspace{1cm} (3)

where $\eta(x) = 1$ if $x > 0$, $\eta(x) = 0$ if $x < 0$, and $x_d$ is the image of $x_0$. For simplicity, we assume the edge of obstruction is on the optical axis so that half of the fluorescence is blocked. Finally, for a finite detector, image intensity is the sum of all the detection points:

$$I_{det}(x_0, x_d) = \int_{-\infty}^{\infty} I_f(x_0, x_0) \eta(-g(x_0) - x_0) \, dx_0.$$  \hspace{1cm} (4)

We have experimentally validated our theory. An inverted microscope (Ti-U, Nikon, Japan) with an objective of $10\times$ ($NA = 0.3$) is employed as the platform to form a home-built CLSM. A 375 nm UV laser (RGB Laser) is used for excitation. A piece of white printing paper or fluorescence plate (Chroma) is used as the fluorescence medium placed behind the specimen. The laser is reflected by a dichroic mirror (Chroma). A pair of galvonometers (Nutfield) is used to realize the raster scanning in CLSM. Then the incidence is focused by the scan lens ($f = 50$ mm), expanded by the tube lens, and collimated by the objective of the microscope. It focuses on the specimen and excites the fluorescence media. The fluorescence then travels through the specimen, de-scanned by the galvonometers, passes the dichroic mirror and filter before being converged by an eyepiece ($f = 22$ mm). There are two $2f$ systems in the optical beam path: one $10\times$ magnification microscope consists of the objective and tube lens; whereas the other $1/2 \times$ microscope consists of the scan lens and the collecting lens. Consequently, 1 Airy unit (with wavelength $\sim 0.4 \mu m$) corresponds to $20 \mu m$ for our system.

First, we show the effect of incident refraction. We used a grating with a period of $40 \mu m$ and height of $1 \mu m$ as the sample. In Figs. 2(b)–2(d), a multimode fiber is used as the confocal pinhole (diameter $200 \mu m$, corresponding to $10$ AU). The UV laser and fluorescence medium shown in Fig. 2(a) are employed as illumination. According to Eq. (5), with such a large pinhole, the detection gradient signal is smoothed and vanishes, and the resultant intensity only depends on the incident gradient. We call this Type 1 S-confocal. The grating image of Type 1 S-confocal is shown in Fig. 2. Figure 2(b) is the S-confocal image without extra block, where the dark stripes are the result of the finite aperture of the lens; (c) is the upside block result, where the rising edges become bright and falling edges become dark; (d) is the downside block result, which shows the opposite
Fig. 2. (a) Laser scanning S-confocal system to illustrate the effect of incident and detection gradients. (b)–(d) show S-confocal image with UV laser incident, fluorescence medium on top and pinhole of 10 AU. The obstruction direction determines the orientation of the shade effect. Image intensity indicates the incident gradient, since the detection gradient is averaged. (e)–(g) are scanning images with Köehler illumination with a 3 AU pinhole, but without laser and fluorescence medium. (h)–(j) show the S-confocal image with UV laser incident, fluorescence medium on top and pinhole of 3 AU. Thereby, the intensity in (h)–(j) is contributed by both the incident gradient in (b)–(d), and the detection gradient in (e)–(g). Left, middle, and right columns are images without obstruction, upside obstruction and downside obstruction, respectively. The image size is 120 μm × 120 μm. (k) Grating profile (green dash and dots), grating gradient (red dashes), and measured gradient (blue solid) along yellow line in Fig. 2(c).

The effect of detection refraction could then be observed in Figs. 2(c)–2(g). These three images are taken from the same block and detection condition as 2(b)–2(d), but with Köehler illumination, as shown in Fig. 2(a). In this case, we used a fiber with a diameter of 60 μm as confocal pinhole, which corresponds to 3 AU. When the sample is illuminated with wide-field illumination, the resulting image is heavily blurred, as the spatial extent of fiber diameter is about the size of the phase edges in the grating sample.

Then, in Figs. 2(h)–2(j), we show that, generally, the final image is the superposition of incident and detection gradients. When a much smaller confocal pinhole is used, with laser and fluorescence medium as illumination, blocking the detection beam can generate a dual-mask imaging result. The confocal pinhole is decided by the fiber with a diameter of 60 μm (3 AU). Since the detection area is now comparable with the excitation area, resulted images have contributions from both Eqs. (2) and (3). Clearly, two different sets of stripes with defects are presented in Figs. 2(h)–2(j), resulted from incident gradients as in Figs. 2(b)–2(d), and detection gradients in Figs. 2(e)–2(g).

Finally, we demonstrate the application of S-confocal in a biological sample. We used a mouse kidney slide (F-24630, Invitrogen) and the 200 μm fiber as confocal pinhole (10 AU). Its S-confocal image, fluorescence image, and merged image of both modalities are shown in Fig. 3. As the nuclei have a slightly higher refractive index over the surrounding tissue [16], these appear as small pits in Fig. 3(a). These pits match well with the blue spots in Figs. 3(b) and 3(c), indicating that S-confocal is very sensitive in detecting the change of refractive index, or the phase gradient. Moreover, S-confocal image mode and fluorescence image mode could be switched conveniently by the top fluoresces medium and block, so that both structure and component information could be obtained from the merged image.

Additionally, since the detected signal is proportional to the overlap between the excitation zone and the detection zone, as shown in Fig. 1(b), we can use a partial

Fig. 3. (a) Fluorescence image of mouse kidney (DAPI stained). (b) Phase-relief image of mouse kidney by S-confocal, and (c) merged image. The image size is 180 μm × 180 μm.

Fig. 4. Type 2 S-confocal image with pinhole of 10 AU and a half-fluorescence plate. (a), (b), and (c) are images with one full fluorescence plate and two half-fluorescence plates with different directions, respectively. The image size is 60 μm × 60 μm.
fluorescent medium, with only the green area being fluorescent, to generate the same effect as Type 1 S-confocal, but without requiring further obstruction. We call it Type 2 S-confocal. Figure 4 shows the phase-relief image of the phase grating with only partial fluorescent medium presented.

In conclusion, we report a simple method of Schlieren modulation to achieve phase edge enhancement, which can be readily adapted to the current confocal setup for phase-relief imaging. It only requires a fluorescent plate behind the specimen, and (in Type 1 S-confocal) a partial beam obstructor before the fluorescence collection lens. The laser can be used for either exciting the fluorescence labeled specimens for standard fluorescent confocal imaging or exciting the fluorescence plate in the S-confocal mode for phase-contrast imaging. Therefore, it combines the multimodality fluorescence/structural phase images within a single system. In S-confocal microscopy, the incident laser is refracted by the local phase gradient, and the resulted partial illuminated fluorescence forms the phase-relief contrast. The final intensity of the image is contributed by both incident and detected phase gradient modulation. A linear relationship between the local phase gradient and the detected intensity is found in both Type 1 and Type 2 S-confocal, implying that this method has the potential for quantitative phase imaging. The measurable phase range is \(-ka/f < d\phi/dx < ka/f\). The sample area illuminated by the laser is about \(\lambda f/2a\), so S-confocal requires the phase difference within the illumination focal spot to be no more than \(\pi\).

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