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Abstract. Fluorescence holographic microscope (FINCHSCOPE) is a motionless fluorescence holographic imaging technique based on Fresnel incoherent correlation holography (FINCH) that shows promise in reconstructing three-dimensional fluorescence images of biological specimens with three holograms. We report a developing two-step phase-shifting method that reduces the required number of holograms from three to two. Using this method, we resolved microscopic fluorescent beads that were three-dimensionally distributed at different depths with two interferograms captured by a CCD camera. The method enables the FINCHSCOPE to work in conjunction with the frame-straddling technique and significantly enhance imaging speed. [DOI: 10.1117/1.JBO.19.6.060503]

Keywords: Fresnel incoherent correlation holography; fluorescence holographic microscope; two-step phase-shifting interferometry; spatial light modulator.

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Holographic technique has evolved rapidly in recent years, mainly because of increasing demands in recording three-dimensional (3-D) structures of an object.[1,2] Because conventional holography requires a coherent light source to create holograms, and fluorescence is incoherent, application of holography in 3-D imaging of fluorescent objects has been limited. Fresnel incoherent correlation holography (FINCH) has recently been introduced to record 3-D information from incoherent sources.[3–8] By employing a spatial light modulator (SLM) that splits an incident object beam into two beams, the FINCH system produces self-interference for each object point and incoherently superimposes them to create a Fresnel hologram. The method has been used to design a fluorescence holographic microscope, FINCHSCOPE,[5] which records high-resolution 3-D fluorescence images of biological specimens without the need for scanning.

The FINCHSCOPE employs three-step phase-shifting interferometry (PSI) to eliminate the twin image and the bias term that result from each single hologram.[9] Consequently, three holograms with different phase-shifting factors are required, the phase modulation on the screen of the SLM must refresh upon each acquisition, and the recording speed is limited. In this study, we developed a two-step PSI-based FINCHSCOPE that requires only two holograms for reconstruction of a microscopic fluorescent object. An electron-multiplying charged-coupled-device (EMCCD) camera, working in frame-transfer mode, was utilized to record the holograms. The two exposures and the frame transfers were controlled by two specific sets of parallel-register clock sequences to match the refresh rate of the SLM (for full digital variable resistor [DVR] settings, 0.03 to 4.19 V, that lead to 2π modulation at 1064-nm wavelength, the rise time is 16 ms and the fall time is 21 ms; for the DVR settings that lead to 2π modulation at 633 nm, 1.48 to 2.87 V, the rise time is 23 ms and the fall time is 54 ms), enabling rapid acquisition (~10 Hz) of 3-D microscopic images.

Figure 1 is a schematic of our experimental setup, which is similar to the FINCHSCOPE developed by Rosen and Brooker.[5] The fluorescent sample is excited by a mercury arc lamp with an excitation filter that selects wavelengths centered at 475 nm for excitation. The objective collects fluorescence centered at 508 nm (Olympus, Tokyo, 10×, 0.30 NA), and it passes through a dichroic mirror and an emission filter. It is then reflected at 12 deg from a phase-only SLM (PLUTO NIR2, 1080×1920 pixels) and projected onto an EMCCD (Andor iXon+, 14 bit, 1024×1024, 13 μm^2).

To study the effectiveness of the two-step phase-shifting approach, we fabricated a fluorescent sample, as shown in Fig. 1(b), we added fluorescent polymer microspheres (Duke Scientific Corp., California, 35 to 8, 48-μm diameter, excitation maxima 468 nm, emission maxima 508 nm) to a liquid mixture of a poly-dimethylsiloxane (PDMS) base and a curing agent (ratio 10:1). Once the PDMS mixture was cured, the fluorescent beads were randomly distributed at various depths of the PDMS membrane.

The key element of the PSI-based FINCHSCOPE is the SLM. Several strategies for modulating an SLM to produce the FINCH interference have been reported, including the plane wave plus spherical wave method,[3] the dual-spherical wave method,[7] and the polarization method.[8] Our two-step PSI technique is applicable to the FINCHSCOPE with any of these SLM modulation strategies. Here, we use the plane wave plus spherical wave method as an example to describe how we reconstructed a 3-D fluorescent object with only two holograms. As described by Rosen and Brooker,[3] mathematically the desired reflection function of the SLM can be expressed as

$$R(x,y) = \frac{1}{2} + \frac{1}{2} Q\left(-\frac{1}{f_d}\right) \exp(i\theta), \tag{1}$$

where $Q(\bullet)$ is a quadratic operator such that $Q(-1/f_d) = \exp[-i\pi/(\lambda f_d)(x^2 + y^2)])]$, representing a Fresnel lens with a focal length of f_d relative to a wavelength λ; the constant phase term, 1/2, denotes a plane wave modulation; and θ is the phase-shifting factor. The phase map of the SLM modulation function is shown in Fig. 1(a).

To theoretically explain the working process of the two-step phase-shifting FINCHSCOPE, we assume a 3-D fluorescent object $p(x_p, y_p, z_p)$ located at the working distance, f_0, of the objective lens, as shown in Fig. 1. The hologram recorded by the CCD camera, according to Rosen and Brooker,[3] can be written as

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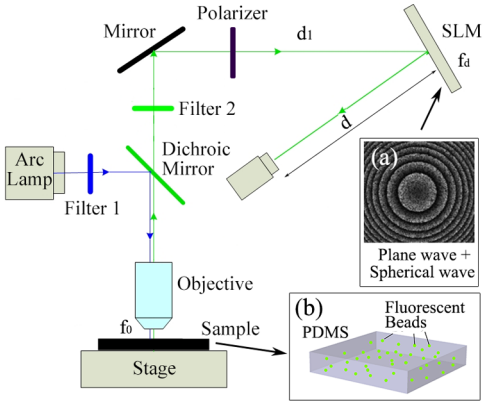

Fig. 1 Schematic of the experimental setup: (a) Phase map loaded on the spatial light modulator (SLM) where half of randomly selected pixels display a constant phase and the remaining pixels display a quadratic phase; (b) fluorescent sample fabricated by curing fluorescent beads in a PDMS membrane.

$$H(x,y)=A_0(D+\exp(i\theta)\int\int\int p(x_p,y_p,z_p)\times\exp\left\{\frac{i\pi}{\lambda z_r}[(x-x_r)^2+(y-y_r)^2]\right\}\mathrm{d}x_p\mathrm{d}y_p\mathrm{d}z_p+\exp(-i\theta)\int\int\int p(x_p,y_p,z_p)\times\exp\left\{\frac{-i\pi}{\lambda z_r}[(x-x_r)^2+(y-y_r)^2]\right\}\mathrm{d}x_p\mathrm{d}y_p\mathrm{d}z_p),\quad(2)$$

where A_0 is a constant and can be neglected in the reconstruction process, D is the sum of the intensities of the two SLM-split beams at the CCD plane, (x_r, y_r) represents the lateral location in the reconstruction coordinates, and z_r is the reconstruction distance related to the object's depth information. Equation (2) can be broken down into three parts, a bias term and two correlation terms, which carry the virtual and real images of the 3-D object. Here, we achieve the two-step technique by approximately estimating the bias term in the hologram using a two-dimensional (2-D) low-pass filter in the frequency domain.[10,11] The low-pass filtering method can be performed with the following three steps:[12]

(a) Calculate the discrete Fourier transform, $F(u, v)$, of the recorded hologram, $H(x, y)$;

(b) Multiply $F(u, v)$ by a low-pass filter function, $P(u, v)$;

(c) Calculate the inverse Fourier transform of the result in (b); the result is an estimation of the bias term.

In our study, we utilized a Hanning filter to retrieve the low-frequency bias in a hologram. The Hanning filter function is represented as[10]

$$P(u,v)=0.25\left[1-\cos\left(\frac{2\pi u}{K}\right)\right]\left[1-\cos\left(\frac{2\pi v}{L}\right)\right]\quad 0\le u\le K-1, 0\le v\le L-1,\quad(3)$$

where K and L are non-negative integers and denote the window size of the filter. In our case, the window was set to fit the size of the CCD image plane, 1024×1024 pixels. Upon retrieval by the Hanning filter, the bias term can be subtracted from the recorded holograms, and thus we have

$$H'(x,y)=\exp(i\theta)\int\int\int p(x_p,y_p,z_p)\times\exp\left\{\frac{i\pi}{\lambda z_r}[(x-x_r)^2+(y-y_r)^2]\right\}\mathrm{d}x_p\mathrm{d}y_p\mathrm{d}z_p+\exp(-i\theta)\int\int\int p(x_p,y_p,z_p)\times\exp\left\{\frac{-i\pi}{\lambda z_r}[(x-x_r)^2+(y-y_r)^2]\right\}\mathrm{d}x_p\mathrm{d}y_p\mathrm{d}z_p.\quad(4)$$

For simplification, we use $B(x, y)$ and $C(x, y)$ to represent the two integral parts in Eq. (4). Then, Eq. (4) can be rewritten as

$$H'(x,y)=\exp(i\theta)B(x,y)+\exp(-i\theta)C(x,y).\quad(5)$$

For the proposed two-step PSI-based FINCHSCOPE, two holograms with different phase-shifting factors, θ_1 and θ_2, are required. θ_1 can be arbitrarily selected, while θ_2 must be a $\pi/2$ shifting from θ_1, that is $\theta_2=\pi/2+\theta_1$. After applying the Hanning filter on both holograms, $H_1(x, y)$ and $H_2(x, y)$, and subtracting the bias components from them, we can then create a Fresnel hologram using the two holograms according to the following equation:

$$\begin{aligned}H_F(x,y)&=H_1'(x,y)+iH_2'(x,y)\\&=\exp(i\theta_1)B(x,y)+\exp(-i\theta_1)C(x,y)\\&\quad+i\left\{\exp\left[i\left(\theta_1+\frac{\pi}{2}\right)\right]B(x,y)+\exp\left[-i\left(\theta_1+\frac{\pi}{2}\right)\right]C(x,y)\right\}\\&=2(\cos\theta_1-i\sin\theta_1)C(x,y),\end{aligned}\quad(6)$$

where $2(\cos\theta_1 - i\sin\theta_1)$ is a neglectable constant, and thus we have

$$H_F(x,y)\cong C(x,y)=\int\int\int p(x_p,y_p,z_p)\times\exp\left\{\frac{i\pi}{\lambda z_r}[(x-x_r)^2+(y-y_r)^2]\right\}\mathrm{d}x_p\mathrm{d}y_p\mathrm{d}z_p.\quad(7)$$

Consequently, a 3-D image of the object can be reconstructed by

$$p'(x_p,y_p,z_p)=H_F(x,y)*\exp\left[\pm\frac{i\pi}{\lambda z_r}(x^2-y^2)\right],\quad(8)$$

where the asterisk denotes a 2-D convolution.

In our experiment, the two holograms were captured with phase-shifting factors of 0 and $\pi/2$, respectively. Two corresponding bias-free holograms were calculated using the described Hanning filter with a window of 1024×1024 pixels such that the noise-like bias background was suppressed, and the correlation component was well retained in the holograms (results are not shown here). With the proposed two-step PSI, we reconstructed the image of a 3-D fluorescent sample. The reconstructions are shown in Fig. 2. The first image, Fig. 2(a), is a regular fluorescence image showing the map of the fluorescent beads in 2-D. The other three images were reconstructed with

different Fresnel diffraction distances; they display focused views of various fluorescent beads at different depths. In each image, a red arrow points to a specific bead in focus. These results demonstrated the 3-D information acquisition capability of the two-step PSI-based FINCHSCOPE.

Compared to the original FINCHSCOPE, the two-step phase-shifting method has a faster imaging rate because only two holograms are required. Figure 3(a) is a schematic of the clock sequence that was utilized to synchronize the phase refreshment on the SLM and the CCD exposure. It is possible that our method could double the imaging speed of the FINCHSCOPE.

The SLMs with a super-fast refresh rate (500 Hz or above, e.g., boulder nonlinear systems XY Series SLM) are commercially available. Thus, the 3-D imaging speed of the FINCHSCOPE would be limited not by the SLM's refresh rate but by the camera's readout time between successive frames. In the case of two-step PSI, it is possible to employ the frame-straddling technique to achieve very short separation time (e.g., submicroseconds) between two consecutive exposures with a frame transfer camera. The working principle of frame-straddling[13] is illustrated in Fig. 3(b). The gap between two camera frames is denoted by Δt, and the interval of two excitation pulses by Δt_1. The double-pulse illuminations occurred at the end of the first frame and the beginning of the second frame, respectively. When an excitation flash lamp and a frame transfer camera are precisely synchronized, the frame-straddling technique enables $\Delta t_1 \cong \Delta t$; that is, the time separation of recording two holograms could be reduced to hundreds of nanoseconds. Obviously, this technique is not applicable to three successive captures and thus not to three-step PSI. The total time required to record three successive holograms, Δt_2, is at least equal to a full frame duration plus two interframe gaps, which are typically in milliseconds. As a consequence, two-step PSI could be more than 1000 times faster than three-step PSI.

In conclusion, we have developed a two-step phase-shifting FINCHSCOPE that requires only two holograms to reconstruct a 3-D fluorescent sample. The two-step phase-shifting strategy could be applied to other FINCH imaging systems at both the microscales and the macroscales. The strategy could be used in observation of dynamic 3-D processes with incoherent light sources, such as fluorescence. It should also be noted that

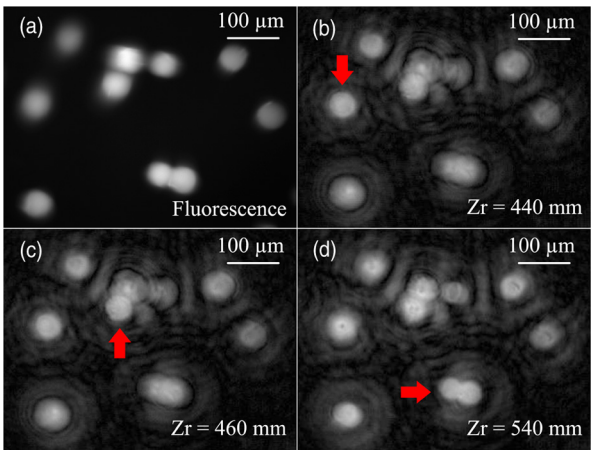

Fig. 2 Two-step phase-shifting reconstruction results: (a) regular fluorescence image under microscope; (b)–(d) reconstructed images with different diffraction distances, zr. Red arrows indicate locations of different fluorescent beads in focus.

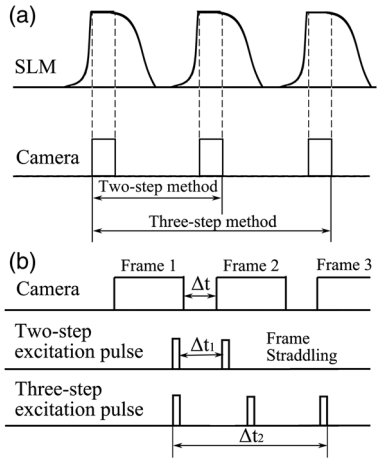

Fig. 3 (a) Optimized synchronization of clocks of SLM and CCD; (b) schematic of frame-straddling technique.

the two-step PSI method involves digital-signal processing operations, low-pass filtering, and low-frequency subtraction. Although the Hanning filter has been shown to be ideal for two-step PSI, low-pass filters always result in slightly degraded 3-D reconstruction. The two-step phase-shifting method greatly increases imaging speed, but slightly decreases image quality compared with the three-step phase-shifting method.

Acknowledgments

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