High-contrast epi-fluorescence wide-field imaging of biological cells using integrating-bucket method

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1. Introduction

The use of fluorescence for biological imaging is a simple but very powerful method for visualizing protein distribution within cellular organelles. Fluorescence-contrast imaging has greatly aided biologists in understanding the complex biological processes involved in protein interaction dynamics, which is important for cell proliferation [1]. For cellular imaging, synthetic fluorescent dyes have been mainly used to enhance fluorescence contrast and identify the region of interest within a sample. However, there are several aspects of synthetic dye implementation that require improvement, such as low uptake efficiency, which restricts the contrast in fluorescence imaging. Photobleaching of fluorophores also hinders the long-term study of labeled cells. The staining rate can be increased by increasing the dye concentration, but this may adversely affect the cell viability owing to possible toxicity of the fluorophores [2,3]. Alternatively, the optical power of the excitation laser could be increased; however, this may worsen the photobleaching problem of the dyes [4].

The fluorescence imaging technique that uses a lower dose of fluorescent dye and/or lower excitation laser power is highly desirable for the long-term study of single cells. Hence, the use or development of a fluorescence signal acquisition method that has a high signal-to-background ratio (SBR) is essential. To this end, several digital lock-in techniques, which can extract a periodical signal from noisy measurements, have been proposed for charge-coupled device (CCD)-based fluorescence microscope systems. Fisher et al. extracted the fluorescence signal in the Fourier domain using the fast Fourier transform (FFT) with a series of phase-locked fluorescence signals [5]. Marriott et al. applied a typical cross-correlation analysis to the modulated fluorescence signals, from which the threshold correlation coefficients were extracted and subsequently used to map the fluorescence image [6]. Despite their high imaging quality, these lock-in methods commonly require acquisition of the time-varying fluorescence intensity signal over several modulation cycles and the pixel-by-pixel processing of dataset; thus, an image acquisition time of a few minutes is required to obtain a background-free fluorescence image. This time-consuming approach may hamper the cell viability owing to the long-term exposure to a strong excitation light [7].

In this paper, we propose a high-speed and high-contrast fluorescence imaging technique that uses the integrating-bucket method, which is useful for the long-term study of biological cells. By inducing a sinusoidal intensity modulation to the excitation light source and by simply capturing the CCD image for each quarter of a modulation period, only the fluorescence emission signal modulated by the source could be extracted. This homodyne
optical lock-in approach to epi-fluorescence microscopy enables the extraction of a low-noise fluorescence map within a sub-second time duration.

2. The four-bucket method

The four-bucket method is a type of digital lock-in detection technique, which extracts only the periodically modulated signal from very noisy measurements. It rejects the signals having frequencies other than the modulated one and selectively determines the amplitude and phase of the signal modulated at a fixed frequency. Thus, the method offers the possibility to enhance the SBR of a fluorescence imaging system by reducing the background level originating from ambient light and/or the dark current of the camera. The fluorescence emission in a sample can be modulated by simply modulating the intensity of the excitation light and synchronously detected by a CCD camera. In general, since the fluorescence emission occurs within nanoseconds after the absorption of excitation light, only the fluorescence signals excited with a known frequency can be identified.

Fig. 1 shows the schematic diagram of the proposing four-bucket technique. Four CCD frames are captured during one modulation period of the excitation light. The modulated fluorescence intensity, detected on a pixel \((x,y)\) of the CCD camera, can be expressed as

\[
I(x, y, t) = I_0(x, y) + \Delta I(x, y) \sin(2\pi ft + \phi_0),
\]

where \(I_0\) is the intensity of the unmodulated signal, \(\Delta I\) is the amplitude of the modulated fluorescence signal, \(f\) is the modulation frequency, and \(\phi_0\) is the overall delay between the modulated signal and the camera trigger. Then, the four frames captured by the camera at a frequency of \(4f\) are expressed as

\[
E_1 = \frac{1}{4f}I_0c + \frac{\Delta I}{2\pi f} \cos\phi_0 + \frac{\Delta I}{2\pi f} \sin\phi_0
\]

\[
E_2 = \frac{1}{4f}I_0c + \frac{\Delta I}{2\pi f} \cos\phi_0 - \frac{\Delta I}{2\pi f} \sin\phi_0
\]

\[
E_3 = \frac{1}{4f}I_0c - \frac{\Delta I}{2\pi f} \cos\phi_0 + \frac{\Delta I}{2\pi f} \sin\phi_0
\]

\[
E_4 = \frac{1}{4f}I_0c - \frac{\Delta I}{2\pi f} \cos\phi_0 + \frac{\Delta I}{2\pi f} \sin\phi_0
\]

From these four frames, the amplitude of the modulated fluorescence signal can be simply extracted as

\[
\Delta I(x, y) = \frac{4f}{\sqrt{2}} \sqrt{(E_1 - E_2)^2 + (E_2 - E_4)^2}
\]

It is noted that when the overall delay \(\phi_0\) is negligible, the 4 equations (Eqs. (2)–(5)) are degenerated into two, which means taking only on and off states are enough. However, to cope with general cases we assume delay \(\phi_0\), which is not negligible. The camera trigger can have a delay from the excitation, for example.

3. Experiments

The experimental set-up was similar to that of a typical epi-fluorescence microscope, as shown in Fig. 2. A 3 mW 405 nm laser diode (OZ-1000, OZ Optics Ltd.) was used as the excitation light source, and its intensity was voltage-controlled by a function generator (AFG3022B, Tektronix). The light from the laser was focused with a lens (L1) at the back focal plane of a water-immersion microscope objective (UNPFLN, 10× magnification, numerical aperture (NA) 0.3, Olympus) via a dichroic mirror (DMLP425R, reflection/transmission wavelength: 405/450 nm, Thorlabs, Inc.). The laser illuminated the sample through the objective with an average incident power of 365 \(\mu W\). The fluorescence signal emitted from the sample was directed to the dichroic mirror and was captured by a scientific CCD camera (COOL-1300, 512 x 640 pixels, 12 bits, VDS Vosskuhler GmbH) through the tube lens (L2). A 450 nm bandpass filter (MF460, Thorlabs, Inc.) was

![Fig. 1. Diagram of the image acquisition process. The numbers denote the time sequence of the frames acquired by the CCD camera. One image is calculated from four frames.](image1)

![Fig. 2. A schematic of the epi-fluorescence microscope with a homodyne lock-in configuration with EF: emission filter; L1-2: lenses; DM: dichroic mirror; CCD: charge-coupled device; MO: microscope objective (water-immersion, 10× magnification, NA 0.3); S: excitation laser source (405 nm laser diode).](image2)
used in front of the CCD camera to filter residual excitation light. The excitation light intensity was sinusoidally modulated with a 4 Hz frequency. The camera was synchronized and triggered at each quarter of the modulation period. Thus, the time-varying fluorescence emission was consecutively captured by the CCD camera operating at a frequency of 16 Hz.

To validate the proposed imaging method, we imaged fluorescent polystyrene beads (10–14 μm in diameter, excitation wavelength of 400 nm, emission wavelength of 450 nm, Spherotech, Inc.), which were sparsely dispersed in a water chamber to mimic cells in culture. First, we measured and compared two fluorescence images as shown in Fig. 3. Fig. 3(a) shows the image obtained by using a conventional emission filter (MF460-60, Thorlabs, Inc.) of a 60 nm spectral bandwidth, and Fig. 3(b) shows the image obtained with both the emission filter and the proposing digital lock-in filter. The line intensity profiles are

**Fig. 3.** Epi-fluorescence images of the fluorescent beads obtained with (a) a conventional method and (b) with the four-bucket method. The inset in each figure shows a close-up view of a single fluorescent bead. (c) Co-plotted fluorescence intensity profiles extracted from the middle of both inset images. The white bar indicates a scale of 150 μm.

**Fig. 4.** (a) Fluorescent bead images with spectral filtering and the four-bucket method. (b) Comparison of the SBR of the images obtained with the above methods. (c) Temporal noise equivalent signals at each excitation laser intensity: the STD of the fluorescent bead intensities measured with the four-bucket method, calculated from ten repetitions of the fluorescence intensity measurements for each excitation laser intensity.
compared in Fig. 3(c); the digital lock-in filtered image exhibits better image contrast than the conventional fluorescence image. Enlarged views of a single bead located at the same position (insets) visually clarify the contrast enhancement. The intensity profiles extracted from the middle of both insets are plotted together in Fig. 3(c), illustrating that the signal peak in Fig. 3(b) is much sharper than the one in Fig. 3(a).

Subsequently, the SBR obtained with the two different methods was investigated with respect to the excitation light intensity. To alter the overall intensity of the excitation light on the sample, five neutral-density (ND) filters (NE-A, Thorlabs, Inc.) with optical transmission rates of 32%, 25%, 10%, 5%, and 1% were inserted between the excitation laser and the dichroic mirror. The SBR was defined as the ratio of the maximum signal level for a single bead to the average of the background of the fluorescence image. The intensity at a given CCD pixel was saturated when the 32% ND filter was inserted.

Fig. 4(a) shows the fluorescence images taken with different ND filter transmission rates. In Fig. 4(b), we see that the SBRs obtained with the four-bucket method were about 4–10 dB higher than those achieved with the conventional fluorescence imaging method. To assess the imaging reliability, we calculated the temporal noise equivalent signals. We made ten fluorescence intensity measurements for each excitation laser intensity and calculated the standard deviation (STD) of the intensities, which was averaged over a 4 x 4 pixel area in a single bead region. The result, shown in Fig. 4(c), indicates that the magnitude of the fluorescence intensity fluctuations was smaller than 20 counts for transmission rates between 32% and 5%.

To verify the performance of this method for cellular imaging, MCF 7 (breast cancer) cells stained with different concentrations of fluorescent dye were prepared and imaged. The 10 x objective was replaced with a 40 x objective (LUMPLFLN, NA 0.8, Olympus) for single-cell observation. The labeled fluorophores in the cells absorbed 405 nm light and emitted 670 nm light. The conventional images exhibited a large difference in the background between cells stained with 0.5 μM and 2 μM, while the four-bucket images stained with 0.5 μM and 2 μM showed similar contrast, as shown in Fig. 5. Fig. 5(e) shows the normalized line intensity profiles taken along the white lines in Fig. 5(c) and (d), demonstrating that the edge in the four-bucket image was much sharper than in the conventional image. Moreover, there is appreciable background suppression in the four-bucket image. Fig. 5(f) shows the normalized line intensity profiles taken along the white dashed lines in Fig. 5(b) and (c). The four-bucket image of the cells stained with 0.5 μM concentration showed a higher contrast than the conventional image of the cells with 2 μM concentration. Even though both samples were not exactly the same, we could say that

![Conventional method image vs four-bucket image](image-url)
the fluorescent imaging with the four-bucket method gave a sharper image than the conventional one; thus, it needs lower dose of fluorescent dye in general. More thorough qualitative analysis is under preparation.

Finally, we prepared and imaged adhesive FaDu cells (hypopharyngeal carcinoma cell lines) stained with a photosensitizer. The conventional epi-fluorescence image (Fig. 6(a)) shows a high background comparable with that of the four-bucket method (Fig. 6(b)). We see that the background was appreciably suppressed with the four-bucket method while preserving the fluorescence signals from the cells, permitting cleaner and finer visualization of the cell morphology. Furthermore, the random noise in Fig. 6(b) could be appreciably reduced by averaging 10 and 50 images, as shown in Fig. 6(c) and (d), respectively.

4. Discussion

Experimentally, we have demonstrated that the four-bucket fluorescence imaging method could improve the SBR of the epi-fluorescence imaging microscopy mainly by reducing the background noise. The SBR improvement capability of the four-bucket method can be effective for the long-term study of live cells. The sinusoidal modulation of the excitation light could also be beneficial in this respect, as the four-bucket method requires only half amount of illumination light of that used in the conventional method; therefore, the photobleaching is reduced [8].

Wide-field fluorescence microscopy provides the advantage of a simple configuration, which consists of a spectral filter set, i.e., a dichroic beam splitter, an excitation filter, and an emission filter. Typically, in conventional fluorescence microscopy, the emission filter is used to reject ambient light to increase the SBR of the system. Use of the narrow bandpass filter reduces SBR of the fluorescence microscopy as shown in the first experiment (Fig. 3). In single-color fluorescence imaging, the long pass filter can be employed as the emission filter to acquire fluorescence emission, but it cannot transmit all of the light with a transmission rate of 100%. On the other hand, fluorescence imaging with the four-bucket method can provide both background suppression and enhancement of the fluorescence signal by obtaining all light emitted from a fluorophore. Thus, the four-bucket method can be another option for the high-contrast epi-fluorescence microscopy.

A lock-in detection technique, for fluorescence signals, based on the FFT method has previously been employed [5,6]. Although this FFT method can give a higher SBR than that obtained with the four-bucket method, the FFT method requires dozens of seconds to display the background-free image and dozens of CCD frames are typically required. In contrast, the four-bucket method requires only 0.25 s to extract and display one fluorescence image with low background; this performance is also superior to that of existing optical lock-in detection techniques [6]. Furthermore, the four-bucket method does not necessitate large data storage capacity and sophisticated data processing procedures.

5. Conclusions

We have demonstrated epi-fluorescence microscopy, which is suitable for imaging of biological cells, based on the four-bucket wide-field method. To reduce the background level of the epi-fluorescence imaging, the excitation beam was intensity modulated with a fixed frequency. The excited fluorescence signal was captured with a CCD by integration during each quarter of the modulation period. With four CCD frames, one fluorescence image could be successfully reconstructed. Fluorescent imaging with the four-bucket technique showed better SBR than conventional fluorescence microscopy; thus, the four-bucket method requires a lower dose of fluorescent dye or lower excitation laser power to achieve images with enough contrast. The proposed homodyne optical lock-in technique reduced the fluorescence background induced by ambient light and increased the signal contrast by up to 10 dB compared with conventional epi-fluorescence microscopy. Based on the experimental results, we believe that the use of the four-bucket method in epi-fluorescence microscopy could be advantageous for real-time imaging of live cells. Of course, the imaging rate can be increased appreciably by using a high-speed
CCD camera.

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