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# Spectrally resolved fluorescence lifetime imaging microscope using tunable bandpass filters

Hyeong-Jun Jeong, Jin-Woo Ahn, Dukho Do, and Dae-Gab Gweon

*Department of Mechanical Engineering, KAIST, Daejeon 305-701, South Korea*

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A simple structure of spectral fluorescence lifetime imaging microscope (SLIM) is designed with the use of tunable bandpass filter, a kind of Fabry-perot filter that transmission wavelength is varying according to incident angle of light. Feasibility tests of this angle-tuned bandpass filter (ATBF) are performed and it shows high transmission and constant spectral bandwidth (20 nm) with respect to angle of incidence. Furthermore, using two ATBFs in series, spectral bandwidth can be adjustable down to 4 nm. In this paper, dual ATBFs are implemented to the detection part of fluorescence lifetime imaging microscope (FLIM) system so that we obtained spectrally resolved FLIM images. We compare these SLIM images with an original FLIM image and confirm that the former case provides high accuracy to analyze lifetime distribution as well as high contrast of images. The proposed SLIM microscope with good wavelength selectivity has many opportunities to utilize to other applications such as FLIM-Föster resonant energy transfer and autofluorescence imaging. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4751852>]

## I. INTRODUCTION

Fluorescence is both extremely sensitive and minimally invasive so it can be applied to living cells, tissues, or whole bodies<sup>1</sup> and provide optical molecular contrast, distinguishing different molecular species or variations in bio-specimen.<sup>2</sup> There are some parameters to get microscopic contrast by using fluorescence: intensity, spectrum, lifetime, anisotropy (polarization), or combinations. Fluorescence lifetime, among others, is independent of fluorophore concentration and laser excitation intensity.<sup>1</sup> Also, this quantity is sensitive to many environmental factors such as temperature, pH, viscosity, ionic concentration, oxygen saturation, and influenced by bonds or molecular associations.<sup>2-4</sup> Because of these characteristics, fluorescence lifetime imaging microscope (FLIM) provides high contrast in studies of various diseases, including arthritis, atherosclerosis, and cancer when imaging tissue autofluorescence.<sup>5,6</sup> In particular, the major current application of FLIM in the local fluorophore environment is the detection of Föster resonant energy transfer (FRET) between molecules.<sup>7</sup> When donor and acceptor are separated less than 10 nm, FRET occurs<sup>8</sup> and a lifetime of donor decrease. Thus, FLIM-FRET is useful tool of resolving protein-protein interactions and conformation.

However, multiple fluorophores in specimen give us difficulties to investigate lifetime measurement. This is because emission spectra of many fluorophores are overlapped.<sup>9,10</sup> For example, both flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NADH) can be excited at around 800 nm (two photon excitation), and each emission spectrum also arises nearby another.<sup>11</sup> When we focus on free or bound forms of NADH's lifetime with response to local environment, it is hard to measure exact value because of co-existing intrinsic fluorophores such as FAD. So we need to separate these spectra effectively by using of spectral FLIM (SLIM).

There are several types of SLIM system. The most common method to utilize SLIM is using multichannel photomultiplier tube (PMT) and corresponding time-correlated single-photon counting (TCSPC) unit.<sup>12</sup> It can be used to record spectrally resolved FLIM data in several detector channels simultaneously. But this type of system needs a dispersive unit such as grating or prism to disperse fluorescence and detect that signal at a time. Also, multi-channel PMT has discrete channels so that we cannot select concerning wavelength well. One of other type of SLIM is using several moving slits and PMTs.<sup>13</sup> It requires not only a dispersive unit but also many detection components so that alignment is very difficult and system has bulky size. In this paper, to overcome these problems, we propose a new type of SLIM system and analyze spectrally resolved lifetime data.

## II. MATERIALS AND METHODS

### A. Fabry-Perot thin-film filter

Here, we use a kind of thin film interference filter to resolve fluorescence spectrum. It has a characteristic that transmission wavelength shifts toward shorter when the angle of incidence is increased from 0° (normal incidence) to larger angles.<sup>14</sup> The relation center wavelength to incidence angle is given by

$$\lambda(\theta) = \lambda(0) \sqrt{1 - \frac{\sin^2(\theta)}{n_{eff}^2}}, \quad (1)$$

where  $\theta$  is angle of incidence (AOI) and  $n_{eff}$  is the effective index of refraction of a filter. Especially, multi-cavity Fabry-Perot thin-film filter has been used to tune the spectra over a certain range of wavelengths. We used one kind of this angle-tuned bandpass filter (ATBF), product of Semrock's 550/15 nm Versachrome® of which center wavelength is varying from 550 nm to 486.5 nm when AOI is changing

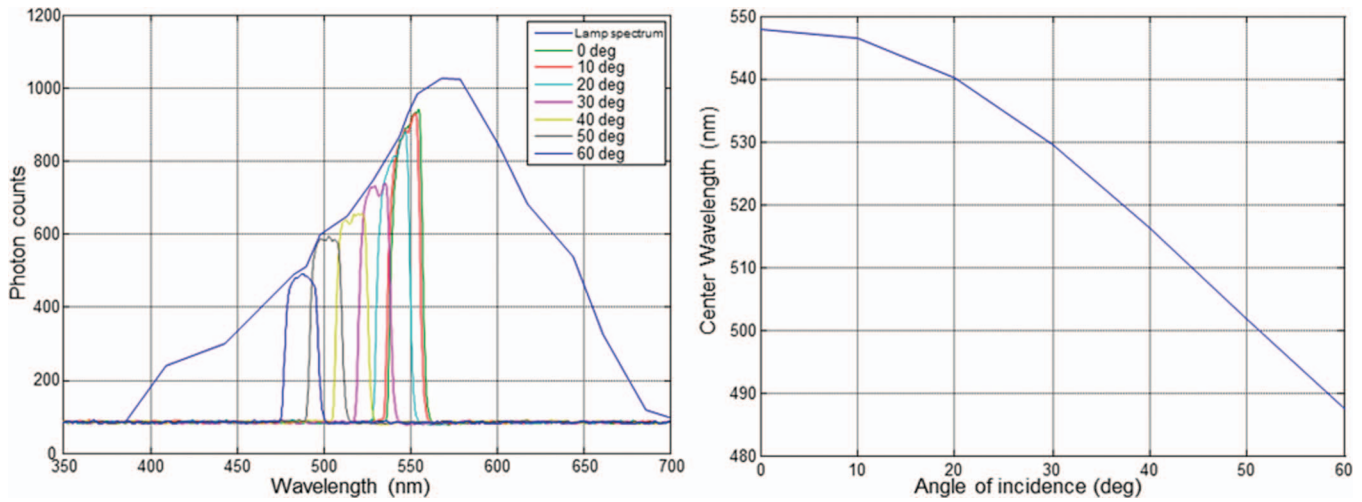


FIG. 1. (Left) A lamp spectrum and transmission characteristics of ATBF as AOI is varying from 0° to 60°. (Right) Shift of center wavelength with increasing AOI.

from 0° to 60° and maintains 20 nm FWHM bandwidth all over the range.

**B. Feasibility test of ATBF**

We first performed feasibility tests of this filter. Testing set up and result is shown Fig. 1.

We use light source as halogen lamp and make it point source by using pinhole. This light becomes parallel light by passing the collecting lens and through the ATBF, spectrum is analyzed by spectrometer. Angle of light incidence on the ATBF is controlled by rotary stage. As a result, we confirm that transmitted light spectra with respect to AOI of ATBF is well coincide to lamp spectrum. This means high transmission characteristic all over the ranges. FWHM bandwidth also maintained about 20 nm entire ranges. If we plot center wavelength to each AOI (bottom right of Fig. 1), it is well correspond to Eq. (1). We have a limitation here: bandwidth of ATBF is fixed, so we cannot select wavelength what we want exactly. To resolve this problem, we propose using two ATBFs in series. If we set an AOI of each ATBF slight differ-

ently, transmission bandwidth will be decreased. So we can control bandwidth by changing relative AOI of each ATBF. This idea is also verified as shown in Fig. 2.

We fixed first ATBF’s AOI as 30° and changed second ATBF’s AOI from 30° to 42° at intervals of 3°. As seen from Fig. 2 and Table I, spectral bandwidth can be reduced minimally to 4 nm. Consequently, it can be said that spectral bandwidth is well adjustable and high spectral resolution is available by using dual ATBFs.

**C. Spectral FLIM system**

Figure 3 shows schematic diagram of a spectral FLIM system using dual ATBFs. A pulselaser (Toptica, FemtoFiber pro NIR) which has 100 fs pulse duration and 780 nm center wavelength with 80 MHz repetition rate is used as the two-photon excitation laser source. The laser beam is collimated to 5 mm and delivered through dichroic mirror to galvano scanner. It is scanned and focused to on a sample through the relay optics and a 20× objective lens (Olympus, NA 0.75). Fluorescence signal from a sample is reflected by a dichroic mirror and is passing through two ATBFs. Center wavelength of fluorescence spectrum is determined by AOI of the first ATBF and spectral bandwidth is adjusted by rotating the second ATBF. A collecting lens is used to compensate lateral shift due to ATBFs. Spectrally resolved fluorescence signal is detected by a PMT (Hamamatsu, H7422) and

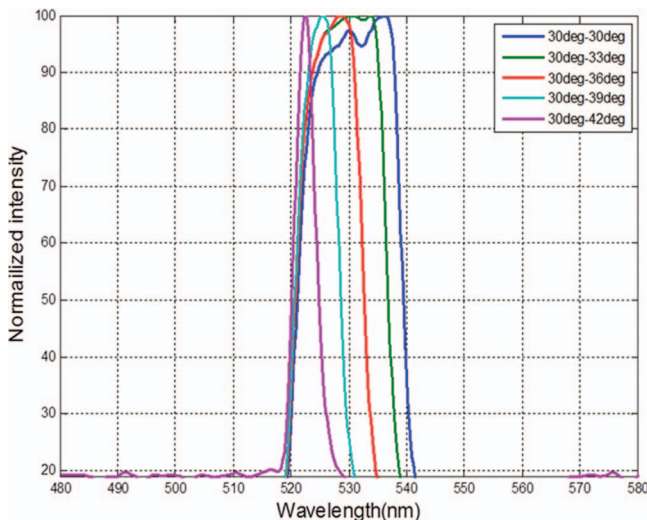


FIG. 2. Spectral bandwidth adjustment with the use of dual ATBFs.

TABLE I. Bandwidth values according to changing relative AOI of dual ATBFs (first one: 30° fixed, second one: increasing from 30° to 42° by 3° step).

Angle (deg)	FWHM (nm)
30–30	17.4
30–33	15.2
30–36	11
30–39	7.2
30–42	4

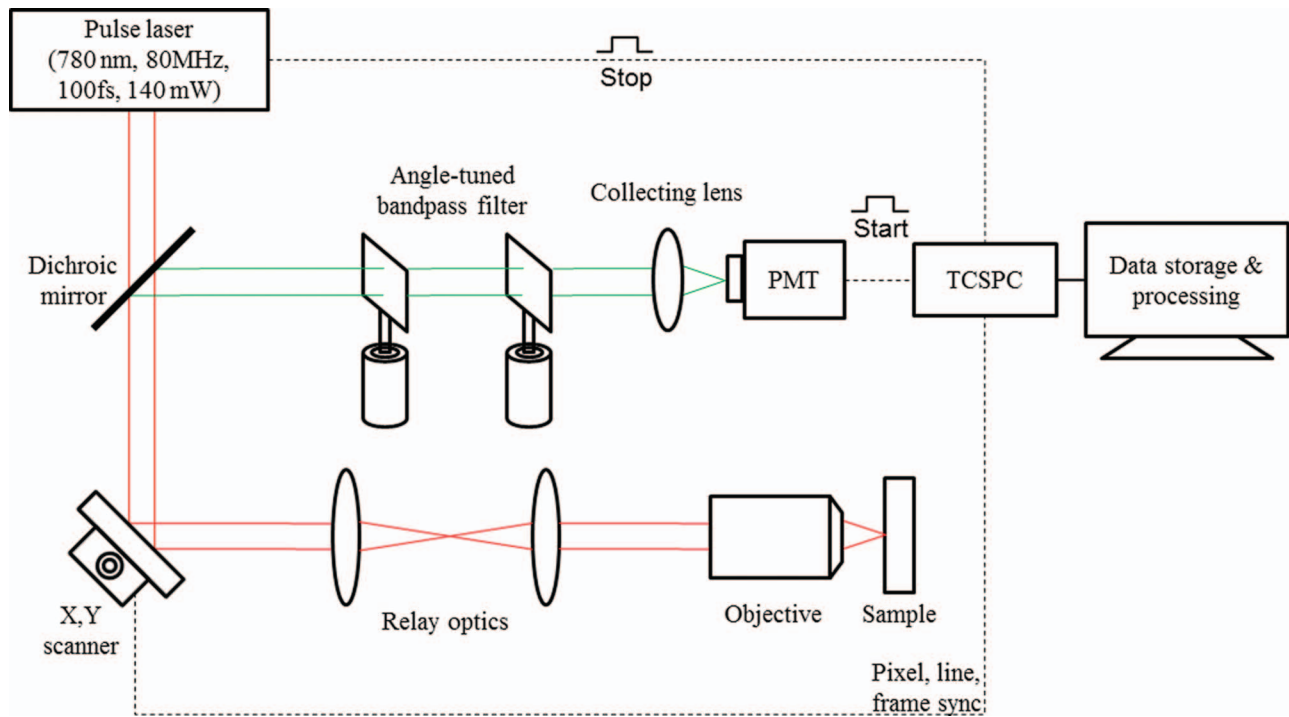


FIG. 3. Schematic diagram of spectral FLIM system.

single photon signals are delivered to TCSPC (Becker&Hickl, SPC-150) to measure fluorescence lifetimes. When a single photon signal from PMT is arrived, time delay is measured until sync signal from pulse laser is arrived. The histogram of this “time delay” determines fluorescence decay curve according to probability distribution. At the same time, pixel, line, frame clock signals from galvano controller enable 2D lifetime distribution storage.

### III. RESULTS AND DISCUSSION

In order to evaluate spectrally resolved FLIM image with the use of dual ATBFs, we used a sample as

lung’s tissue (age 73, female) where nucleus and membranes are dyed by Hoechst 33342 (Ex/Em: 350/461 nm) and AlexaFluor<sup>®</sup>488 (Ex/Em: 499/520 nm), respectively. Before using ATBFs, we inserted an emission filter where center wavelength/bandwidth is 554/221 nm in detection part and got FLIM images.

Figure 4 shows a FLIM image of lung’s tissue and its lifetime distribution. Nuclei and membranes are distinguished by different lifetimes of dyes. According to Ref. 15, each lifetime of Hoechst 33342 and AlexaFluor<sup>®</sup>488 is 2.2 ns and 4.1 ns. We can then expect that two peaks should be appeared in the lifetime distribution because there are just two dyes in the specimen. As seen from Fig. 4(b), however,

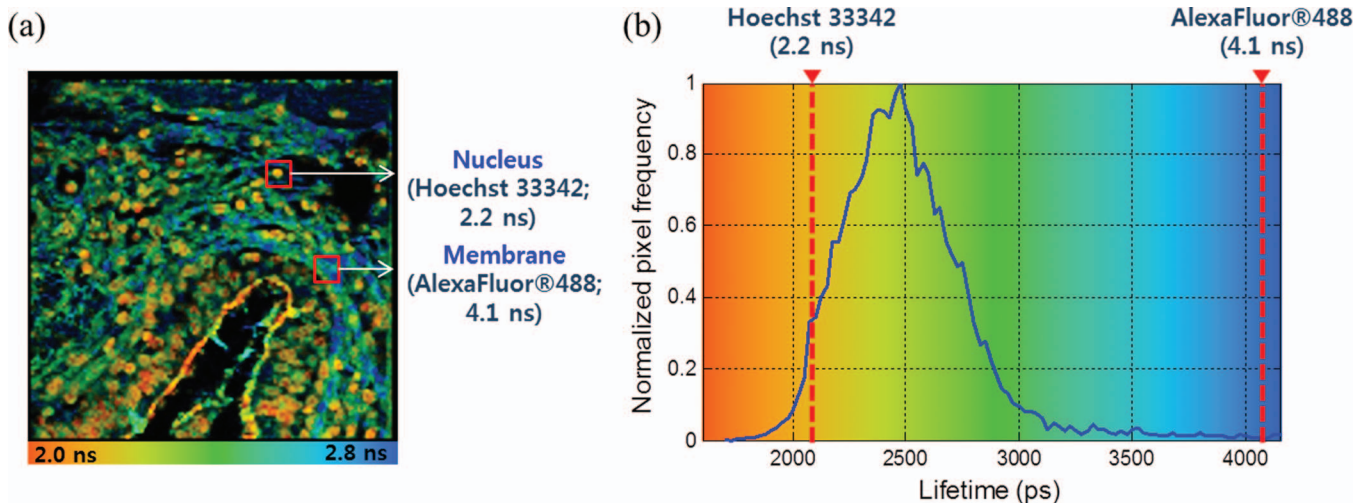


FIG. 4. (a) A FLIM image (FOV:  $200 \times 200 \mu\text{m}^2$ ) of lung’s tissue. Nuclei and membranes are dyed Hoechst 33342 and AlexaFluor<sup>®</sup>488, respectively. Each lifetime is measured 2.2 ns and 2.7 ns, respectively. (b) Lifetime distribution in the image of (a). Reference value of lifetime of each dye is 2.2 ns and 4.1 ns.



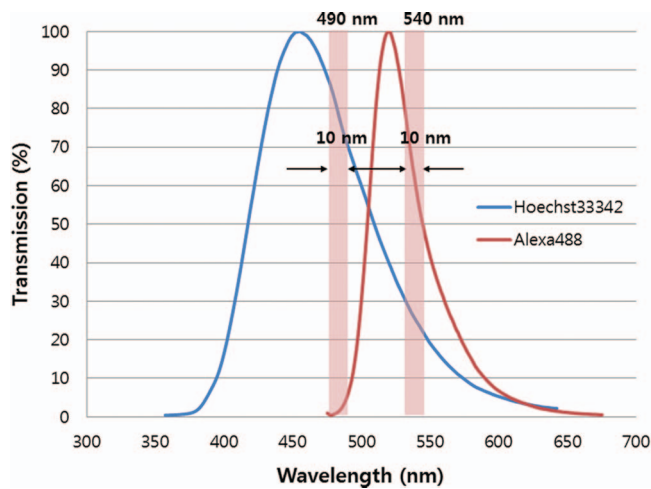


FIG. 5. Emission spectra of Hoechst 33342 and AlexaFluor<sup>®</sup>488.

only one Gaussian distribution is appeared. This means two lifetimes are not distinguished quantitatively. When we inspect some kinds of lesions such as cancer, atherosclerosis, and melanoma, not only morphological analysis of lifetime image is utilized but also quantitative examination, i.e., lifetime distributions is exploited.<sup>16,17</sup> From these perspectives, it is important to investigate individual fluorophore's lifetime distributions separately according to environmental condition. But each of multiple fluorophores in the specimen has own emission spectrum and these are being overlapped. Therefore, we should resolve these data spectrally to get accurate lifetime of each fluorophores.

In this experiment, two fluorescent dyes are used and each emission spectrum is described in Fig. 5. Two emission spectra are close together but we can separate these signals by using dual ATBFs which has narrow spectral bandwidth. So

we inserted dual ATBFs in the detection part of system and adjusted spectral bandwidth to 10 nm. Center wavelength can be varying from 490 nm to 540 nm and we obtained spectrally resolved FLIM images by 10 nm steps.

As we can see from Fig. 6(a), nuclei and membranes are well distinguished by using spectral FLIM imaging. Nuclei's signals are dominant when center wavelength is adjusted to 490 nm and membranes' signals, on the other hands, are remarkable when center wavelength is 540 nm. This phenomenon can also be seen in the lifetime distributions. The most frequent lifetime at 490 nm is 2.18 ns according to Fig. 6(b). This value is well coincide with reference value of Hoechst 33342 (2.2 ns) which is used to dye nuclei. The lifetime of AlexaFluor<sup>®</sup>488 at 540 nm is also well separated and measured peak value is 2.75 ns described as Fig. 6(c). But this value is somewhat different from reference value of AlexaFluor<sup>®</sup>488 (4.1 ns). It seems to be caused by Hoechst 33342's emission spectrum because its peak intensity appears at 461 nm and it is still spread out to 540 nm. In conclusion, we confirmed that two kinds of fluorophores are well separated and accuracy of each lifetime is improved by using spectral FLIM with two ATBFs.

#### IV. CONCLUSION

We have reported on the design and analysis of spectrally resolved FLIM microscope by using tunable bandpass filters. In this paper, a kind of Fabry-perot thin film filter is used as tunable bandpass filter through which transmission wavelength is varying as angle of incidence of light is changing. This characteristic of angle-tuned bandpass filter (ATBF) is verified by feasibility test and this shows high transmission when AOI is increased from 0° to 60°. Moreover, when we use two ATBFs in series, we can adjust spectral bandwidth.

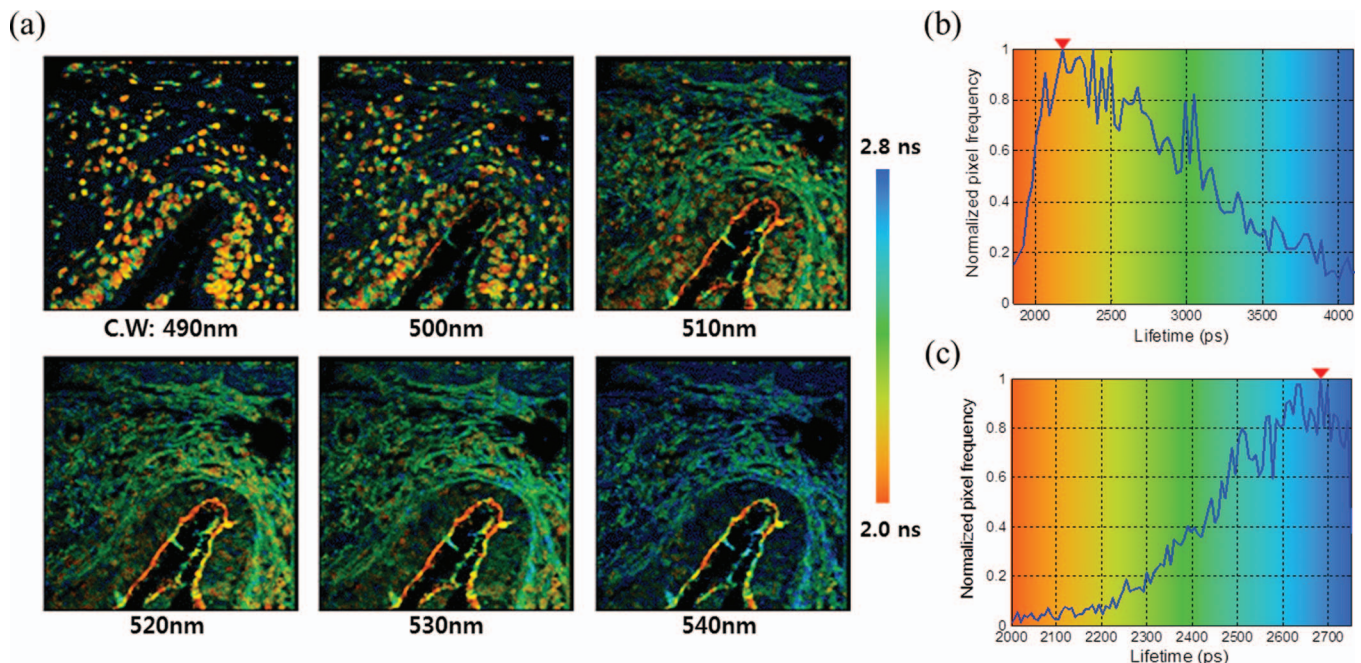


FIG. 6. (a) Spectrally resolved FLIM images (FOV:  $200 \times 200 \mu\text{m}^2$ ) of lung's tissue. Spectral bandwidth is adjusted to 10 nm by dual ATBFs and center wavelength is varying from 490 nm to 540 nm by 10 nm steps. (b) Lifetime distribution at 490 nm (peak value: 2.18 ns) and (c) 540 nm (peak value: 2.75 ns).

When we set AOI of each ATBF slightly different, FWHM of transmitted wavelength is become narrower up to 4 nm. We inserted the dual ATBFs to detection part of the FLIM system and obtained spectrally resolved FLIM images by 10 nm steps with 10 nm spectral bandwidth. In comparison with an original FLIM image, SLIM images provide higher contrast between nuclei and membranes and higher accuracy to measure a lifetime of each dye. This high wavelength selectivity and simple structure of SLIM system can be utilized to get autofluorescence image. Also, it is useful to Förster resonant energy transfer when we want to focus only on donor or acceptor's signal. Because donor and acceptor's emission spectra are usually overlapped together, we should separate these signals and observe a concerned signal. As a result, this can be utilized to FLIM-FRET applications.

### ACKNOWLEDGMENTS

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