Single-shot acquisition of time-resolved fluorescence spectra using a multiple delay optical fiber bundle

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We present a technique for simultaneous acquisition of time-resolved fluorescence spectra using a customized optical fiber bundle with multiple collection fibers of different length. These fibers were aligned at the output plane of a spectrograph corresponding to different wavelengths. Fluorescence signal dispersed by the spectrograph was converted into a pulse train owing to time delays introduced by the length differences between each fiber and subsequently detected by a photomultiplier and digitizer. The performance of the technique was tested with standard fluorescent dyes, and the results are in good agreement with literature values. © 2008 Optical Society of America

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Fluorescence-based spectroscopic techniques have been widely investigated as potential minimally invasive diagnostic modalities such as optical biopsy [1,2]. Fluorescence emission may be analyzed using its intensity, spectral shape, and radiative lifetime. Steady-state fluorescence techniques measure the integrated spectral emission and are extensively used owing to their relatively simple instrumentation. Clinical diagnosis using fluorescence emissions has often been hindered owing to (1) the presence of multiple fluorophores with overlapping spectra, (2) modulation by tissue chromophores, and (3) artifacts caused by excitation-emission geometry variation [2]. Recent studies have shown that, in addition to the intensity and spectral shape, fluorescence lifetimes and their wavelength dependency may provide important diagnostic information [2,3]. Time-resolved fluorescence spectroscopy measures fluorescence decay at multiple wavelengths, where integrated emission spectra may also be calculated. This added dimension of information as along with its intrinsic intensity independence makes time-resolved fluorescence spectroscopy a robust technique for *in vivo* diagnosis.

Fluorescence lifetimes of biological fluorophores are typically in the regime of hundreds of picoseconds to a few nanoseconds. Therefore accurate acquisition and analysis of fluorescence lifetime are nontrivial, especially in clinical applications where acquisition speed is critical. Although modern high-speed detectors and digitizers are capable of handling nano- and picosecond pulses, acquisition of fluorescence lifetime at each wavelength remains mostly a time-consuming task that requires serial scanning in the spectral domain, e.g., ~45 s for a 200 nm time-resolved spectrum [4]. For many clinical procedures, this lengthy acquisition is not practical and has become one of the main obstacles preventing time-resolved techniques from *in vivo* applications. Typical parallel acquisition schemes require not only multiple high-speed detectors but also digitizers, which are prohibitively costly. In this Letter, we present a technique that incorporates a customized fiber bundle consisting of five fibers of different lengths to collect fluorescence pulses at multiple wavelengths. The collected spectrally resolved fluorescence pulses can be temporally separated and then captured by an oscilloscope using a single high-speed photo detector. We have tested the performance of this technique using fluorophores of different fluorescence lifetimes.

Figure 1 shows the schematic of the multiple fiber delay setup. A Nd:YAG laser (PNV, Teem Photonics, Meylan, France) provided excitation pulses at 355 nm (300 ps, FWHM, 15 uJ). A customized bifurcated optical fiber bundle was used to deliver the excitation light to the target and collect emitted fluorescence light, which was coupled into a spectrograph (0.25 m, f/3.9, 1200 l/mm grating blazed at 350 nm, M-74100, Newport-Oriel, Stratford, Conn.). A long-pass filter (WB380, Optima, Tokyo, Japan) was

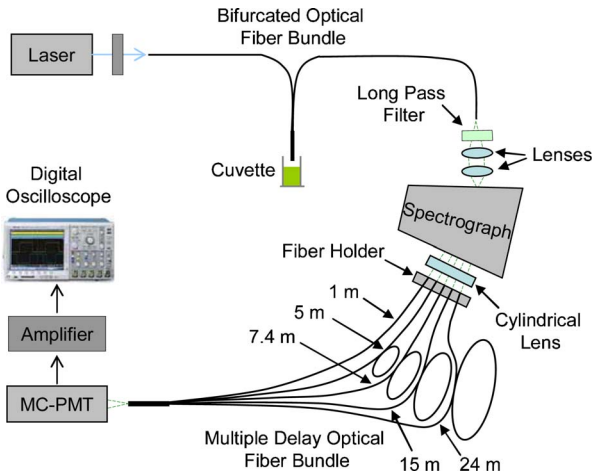

Fig. 1. (Color online) Instrumental setup for a multiple delay optical fiber bundle-based spectrometer.

placed at the entrance slit to block the excitation light from entering the spectrograph. The dispersed fluorescence emissions were coupled into five silica fibers (200 μm core, NA 0.22) using a 25 mm focal length cylindrical lens. The lengths of the five fibers were at 1, 5, 7.4, 15, and 24 m. The proximal ends of these fibers were aligned side by side on a custom-designed holder. The distance between two adjacent fibers corresponded to a wavelength separation of 25 nm. Distally, the five fibers were bound together. The fluorescence pulses delivered by the fibers were detected using a gated microchannel plate photomultiplier tube (MC-PMT, 2 GHz, R5916U-50, Hamamatsu, Iwata-City, Japan). The output signal was amplified using a preamplifier (C5594, Hamamatsu) and digitized by a digital oscilloscope (2.5 GHz, DPO7254, Tektronix, Beaverton, OR).

The time delay between two adjacent fibers was determined with collection of fluorescence from a piece of paper (entrance slit, 1.7 mm; center wavelength, 450 nm). The center wavelength corresponded to the wavelength of the fluorescence pulse collected by the 7.4 m optical fiber. The wavelength of the dispersed fluorescence emission increases in the direction from the 1 m fiber to the 24 m fiber. The captured fluorescence decay pulses are shown in Fig. 2, along with a steady-state fluorescence spectrum of the target collected with a compact spectrometer (OSM-400, Newport Corporation, Irvine, Calif.).

The time delay between two adjacent fibers was measured as the time difference between the peaks of the corresponding decays. For validation, the time delays were also calculated, accounting for the known length differences between adjacent fibers and using a light speed of 2.06×10^8 m/s in pure silica medium ($n=1.458$). The measured time delays are in good agreement with the estimated values (Table 1).

The proposed technique was evaluated with two well-characterized fluorescent dyes: 10^{-4} M fluorescein (Sigma-Aldrich) in ethanol solution with reported lifetime of 6.9 ns [5] and 10^{-5} M

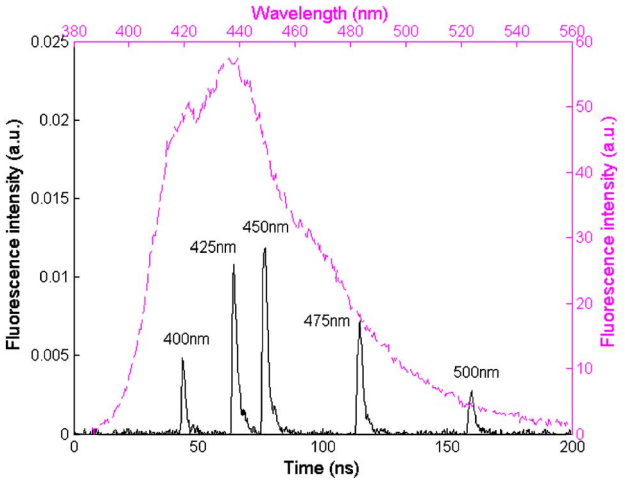

Fig. 2. (Color online) Paper fluorescence decays at five different emission wavelengths (solid lower curve) and its steady-state spectrum (dashed upper curve) measured by a spectrometer.

Table 1. Time Delays between Two Adjacent Optical Fibers

	1–5 m	5–7.4 m	7.4–15 m	15–24 m
Measured delay[a] (ns)	20.7±0.1	12.2±0.2	38.3±0.1	44.8±0.3
Calculated delay (ns)	19.4	11.7	36.9	43.7

[a]Data were averaged over three measurements.

9-cyanoanthracene (9-CA, Sigma-Aldrich) in ethanol solution with emission peak at 445 nm and lifetimes of ~12.58–14.20 ns [4,6]. A mixture solution was also made by adding equal volumes of the 10^{-4} M fluorescein solution and the 10^{-5} M 9-CA solution. For *in vivo* applications, the autofluorescence intensity is much less than the fluorescence dyes used in this proof-of-principle test. Further optimization of the setup would therefore be necessary.

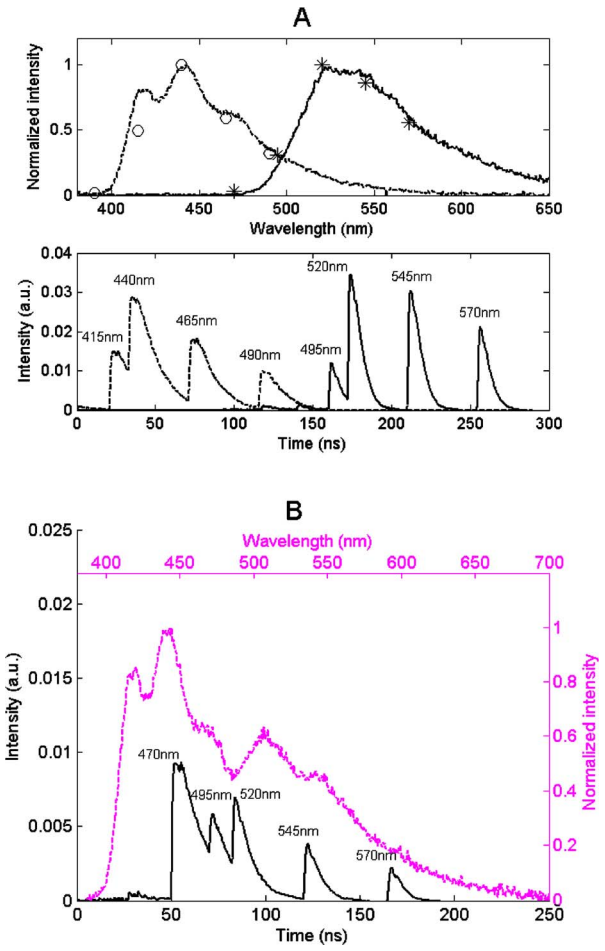

Fig. 3. (Color online) Fluorescence decays of the fluorescence dye solutions at different emission wavelengths. A, Upper panel, normalized steady-state spectra of fluorescein (solid curve) and 9-CA (dashed curve) measured by a spectrometer and normalized time-integrated intensities of the decay pulses at different wavelengths for fluorescein (asterisks) and 9-CA (circles); lower panel decay pulses of the fluorescein and 9-CA at different wavelengths. B, Decay pulses of the mixture solution at different wavelengths (solid lower curve) and normalized steady-state spectrum of the mixture solution (dashed upper curve).

Table 2. Lifetimes[a] (ns) of the Decay Pulses of the Three Solutions

Optical Fiber	1 m	5 m	7.4 m	15 m	24 m
Fluorescein	NA	NA	6.17±0.05	5.97±0.09	5.30±0.08
9-CA	NA	NA	14.47±0.13	13.16±0.14	10.27±0.91
Mixture	14.51±0.28	NA	7.07±0.04	5.55±0.05	5.10±0.18

[a]Data were averaged over three measurements.

To measure the time-resolved fluorescence from the dye solutions, 2 ml of one of the three solutions was added to a quartz cuvette. The distance between the probe tip and the solution surface was about 2 mm. Steady-state fluorescence spectra were also collected using the compact spectrometer. Time-resolved acquisition was conducted with the center wavelength set to 520 and 440 nm for the fluorescein and the 9-CA solution, respectively. For the mixture solution, the center wavelength was set at 520 nm. A total of three fluorescence decay measurements were performed and averaged for each solution (Fig. 3). No photobleaching was observed, and the three decay curves for each of the three solutions were almost identical.

In Fig. 3A, both time-resolved and steady-state fluorescence signals from the fluorescein and 9-CA solutions are plotted. For each fluorophore, there are four visible time-resolved pulses acquired by the 5, 7.4, 15, and 24 m optical fibers. Owing to low emission at 470 nm for fluorescein and 390 nm for 9-CA, the signals collected by the 1 m fiber are almost indistinguishable from background noise. The time-resolved pulses were integrated over time and plotted (as scattered data points) against the steady-state spectra acquired with the compact spectrometer in Fig. 3A. These results demonstrated that the time-integrated spectra from the multiple fiber delay setup are in good agreement with the steady-state measurements and that the spectral intensity profile of the system is well reproducible between measurements during which the grating has been moved and repositioned. Fluorescence lifetimes at different wavelengths were calculated using a multiple exponential deconvolution algorithm [4], and the results are shown in Table 2. The measured lifetimes of both dyes agree well with literature reported values [4–6].

For the mixture solution, the five time-resolved pulses are shown in Fig. 3B along with the steady-state spectrum. The fluorescence lifetimes of the five pulses were calculated and shown in Table 2. The results have demonstrated that the lifetime of the emission at 470 nm was primarily originated from the 9-CA, and the lifetimes at 545 and 570 nm were primarily from the fluorescein.

In this proof-of-principle experiment, fibers with different delays ranging from 11 to 40 ns were used so effects of pulse pile-up may be studied. For complicated delay dynamics, greater time delay is desired [7], i.e., greater fiber length difference is needed. A separate ongoing study is currently examining the effects of pulse pile-up to lifetime estimation. Our initial results have shown that errors due to pulse pile up may be corrected for simple single- and biexponential decays [8].

In summary, we have reported a technique for simultaneous acquisition of time-resolved fluorescence decays at multiple wavelengths with no need of scanning. This technique may find potential optical biopsy application in biology and medicine. The main advantage of this technique is the parallel data acquisition of time-resolved fluorescence spectra so rapid measurements may be done in real time during clinical procedures. Theoretically, time-resolved spectra may be acquired with a single excitation pulse using this technique, while averaging over several pulses was used for data presented here to improve signal-to-noise ratio. Although similar data acquisition speed may be achieved using a gated camera scanning in the time domain [7], a single detector such as a photomultiplier tube offers much superior sensitivity, temporal resolution, and dynamic range. The parallel process enables the use of a single detector and digitizer, making the technique also more economically attractive. The number of possible wavelength channels would be limited by the size of the fibers, the linear dispersion of the emission light, and the required length differences. In practical clinical diagnostic applications, only a limited number of spectroscopic features may be used to achieve the desired sensitivity and specificity. Therefore a limited number of wavelength channels tuned to selected spectral windows may suffice for a specific application.

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