Whole spectrum fluorescence detection with ultrafast white light excitation

Jing Yong Ye[1,2*], Charles J. Divin[1], James R. Baker, Jr.[2] and Theodore B. Norris[1,2]

[1]*Center for Ultrafast Optical Science, University of Michigan, 2200 Bonisteel Blvd., Ann Arbor, MI 48109-2099*
[2]*Michigan Nanotechnology Institute for Medicine and the Biological Sciences, University of Michigan, Ann Arbor, MI 48109-0648*
**E-mail: jyye@umich.edu*

Abstract: We have developed a new detection mechanism for ultrabroadband multicolor fluorescence detection using an ultrafast supercontinuum white light source without spectral filtering to simultaneously excite different fluorophores. A nonlinear photonic crystal fiber was utilized in conjunction with a femtosecond laser to generate the supercontinuum. A time-resolved detector was tested to detect the whole spectrum fluorescence while gating out the excitation white light in the time domain.

OCIS codes: (120.4640) Optical instruments; (170.6280) Spectroscopy, fluorescence and luminescence; (170.7160) Ultrafast technology; (300.2530) Fluorescence, laser-induced; (300.6500) Spectroscopy, time-resolved.

References and links

1. R. Y. Tsien, "Building and breeding molecules to spy on cells and tumors," FEBS Letters **579**, 927–932 (2005).
2. K. M. Marks, G. P. Nolan, "Chemical labeling strategies for cell biology," Nature Methods **3**, 591-596 (2006).
3. A. Brodeur and S. L. Chin, "Ultrafast white-light continuum generation and self-focusing in transparent condensed media," J. Opt. Soc. Am. B **16**, 637-650 (1999).
4. A.L. Gaeta, "Nonlinear propagation and continuum generation in microstructured optical fibers," Opt. Lett. **27**, 924-926 (2002).
5. A. V. Husakou and J. Herrmann, "Supercontinuum generation, four-wave mixing, and fission of higher-order solitons in photonic-crystal fibers," J. Opt. Soc. Am. B **19**, 2171-2182 (2002).
6. H. N. Paulsen, K. M. Hilligse, J. Thgersen, S. R. Keiding, and J. J. Larsen, "Coherent anti-Stokes Raman scattering microscopy with a photonic crystal fiber based light source," Opt. Lett. **28**, 1123-1125 (2003).
7. G. McConnell, "Confocal laser scanning fluorescence microscopy with a visible continuum source," Opt. Express **12**, 2844-2850 (2004).
8. D. M. Grant, D. S. Elson, D. Schimpf, C. Dunsby, J. Requejo-Isidro, E. Auksorius, I. Munro, M. A. A. Neil, P. M. W. French, E. Nye, and G. Stamp, "Optically sectioned fluorescence lifetime imaging using a Nipkow disk microscope and a tunable ultrafast continuum excitation source," Opt. Lett. **30**, 3353-3356 (2005).
9. Alfano RR, and Shapiro SL, "Emission in the region 4000 to 7000 Å via four-photon coupling in glass," Phys. Rev. Lett. **24**, 584–587 (1970).
10. Unruh JR, Gokulrangan G, Wilson GS, Johnson CK, "Fluorescence Properties of Fluorescein, Tetramethylrhodamine and Texas Red Linked to a DNA Aptamer." J. Photochem. Photobio. **81**, 3, 682–690 (2005)

1. Introduction

Fluorescence measurements are an invaluable tool for a wide range of applications in basic science and clinical research. Despite the broad applications of fluorescence measurements and a long history of development, the basic detection scheme remains the same for most

#84077 - $15.00 USD Received 12 Jun 2007; revised 24 Jul 2007; accepted 25 Jul 2007; published 2 Aug 2007

applications. One of the basic requirements for any fluorescence detection system is to separate fluorescence signals from the excitation light. Spectral filters are generally used to screen out scattered excitation light, while allowing fluorescence emission from a sample to pass to the detector. However, if the Stokes shift of the fluorophore is not sufficiently large, some fluorescence signal has to be sacrificed even when a sharp filter is used to completely block the excitation stray light. Also, because each specific dye absorbs only over a relatively narrow spectral range, a single excitation wavelength can excite only a limited number of fluorophores. These drawbacks of conventional fluorescence measurements have not only reduced the detection speed and sensitivity, but also limited the selection of detectable fluorescent markers. On the other hand, there is an increasing demand for staining biological samples with multiple biomarkers with a wide range of absorption and emission spectra [1,2]. Simultaneous detection of multicolor biomarkers enables one to monitor different physical and biological cellular functions and processes in a complicated biological system. To meet the demand of multicolor fluorescence detection, conventional systems have been expanded to include multiple excitation sources and detection channels. The optical configurations in those systems then become very complicated and further improvements become difficult and costly. In this letter, we report the development of a new approach to ultrabroadband fluorescence detection. In contrast to the conventional approach of filtering scattered excitation light in the frequency domain, we utilize time-resolved detection to gate out scattered pump light in the time domain. By doing so, we can use a single broadband supercontinuum source to simultaneously excite all kinds of fluorophores, and collect the entire spectrum ranging from visible to near infrared. This scheme eliminates the need for multiple band pass filters and dichroic mirrors, thus significantly simplifying the optical configuration.

2. Experimental method

We utilized the supercontinuum white light generated from an ultrafast pulsed laser (Coherent, Mira 900) as the excitation source. We coupled 70 mW of 50-fs pulses at 800 nm and 76-MHz repetition rate into a 1-m long nonlinear photonic crystal fiber (PCF, from Crystal Fiber) having a zero-dispersion wavelength of 750 nm. Through nonlinear interactions including self-phase modulation, self-steepening, stimulated Raman scattering and four-wave mixing [3-5], a broad sub-picosecond continuum is produced extending down to 460 nm, as shown in Fig. 1. Although the spectrum shows some structure, the spectrum is continuous, enabling the excitation of any fluorophore with absorption from 460 nm to the near infrared. The average spectral density between 460 nm and 780 nm is 130 μW/nm and is sufficient to enable efficient excitation of fluorescence. The high repetition rate of this supercontinuum source enables one to detect weak signals using synchronous detection (discussed below), and to monitor rapidly changing fluorescence signals such as one has in a flow cytometer. The source is also simple and very compact.

#84077 - $15.00 USD Received 12 Jun 2007; revised 24 Jul 2007; accepted 25 Jul 2007; published 2 Aug 2007

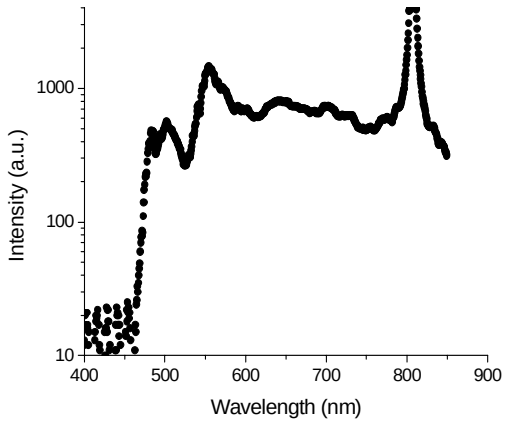

Fig. 1. Spectrum of the supercontinuum generated from a nonlinear PCF seeded by 50-fs pulses from a Ti:Sapphire oscillator.

The full spectrum of the supercontinuum was focused into a sample cuvette containing a mixture of different dyes (Fig. 2). It should be noted here that the whole-spectrum supercontinuum excitation used here is clearly different from previous studies [6-8] in which a narrow band pass filter was used to select a certain wavelength range from the supercontinuum for fluorescence excitation. The fluorescence detection in those studies was carried out using the same approach as the conventional one, where an emission filter was used to block the excitation stray light while allowing fluorescence signals to pass through. In contrast, here we use the whole spectrum of the supercontinuum without spectral filtering in order to excite multiple dyes in a wide wavelength range all at once.

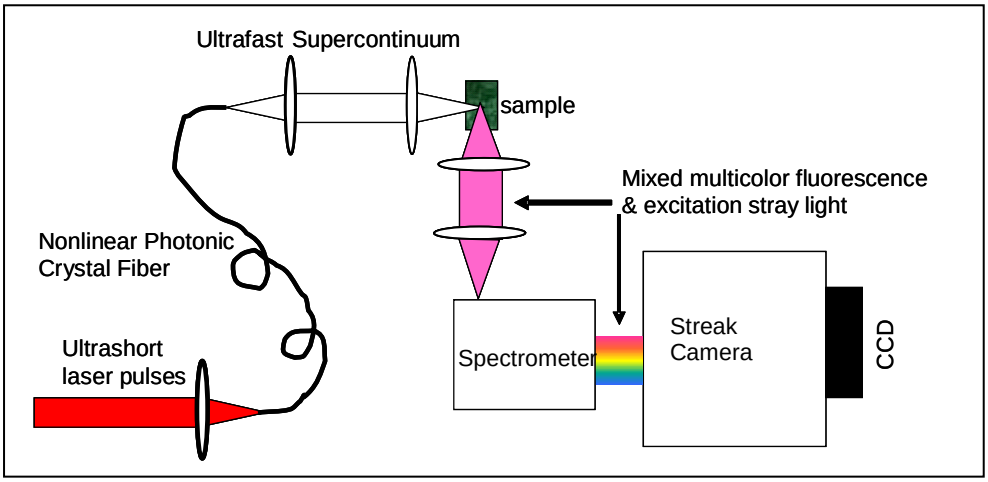

Fig. 2. Schematic diagram of the whole-spectrum fluorescence detection system.

An achromatic lens was used to collect the light from the sample and a second achromatic lens was used to focus the signals into a spectrometer. The light collected includes both scattered excitation light and fluorescence. The output from the spectrometer consists of the two overlapped spectra of the fluorescence and the supercontinuum stray light. Because the excitation and fluorescence are spectrally overlapped, it is necessary to separate the two signals in the time domain, using a time-resolved detector. This is straightforward to do, since

#84077 - $15.00 USD Received 12 Jun 2007; revised 24 Jul 2007; accepted 25 Jul 2007; published 2 Aug 2007

the duration of the scattered excitation light is less than 10 ps, whereas typical fluorescence lifetimes are on the order of hundreds of picoseconds to tens of nanoseconds.

The key requirements of the time-resolved detector are first, that it has sufficient time resolution to temporally separate the scattered pump light from the fluorescence. Thus it need not resolve the excitation, but merely resolve the fluorescence lifetime and thus enable the scattered excitation to be gated out. Second, the detector must have a very high contrast, so that in cases where the scattered excitation may be strong (e.g. in tissues or cell cultures), and the fluorescence weak. Third, an array detector is desired, so that the different fluorophores may be spectrally distinguished.

Our approach to separating the scattered excitation from the fluorescence was to use a streak camera (Hamamatsu, M1955). The camera was used in synchroscan mode, in which the electron beam in the camera is swept over the detector screen synchronously with the excitation pulse train at 76 MHz. Since the streak trace is synchronized with the laser pulse, it is straightforward to gate out the scattered pump light by adjusting the delay (phase) of the streak camera sweep voltage relative to the laser such that the scattered pump falls off the detection screen (i.e. is out of the detection time window), while the fluorescence falls within the time window. This approach enables extremely high rejection of scattered pump light, since that signal never hits the detection screen. The streak camera operated in this mode offers extremely high rejection of scattered pump – orders of magnitude higher than the actual detection dynamic range – while maintaining high fluorescence detection sensitivity. In our implementation, the output of the streak camera was detected either with a cooled CCD (Princeton Instruments TEA/CCD-512-TKE), or a photon-counting photomultiplier tube (Hamamatsu H7421-40) coupled to a multichannel scaler (Stanford Research SR430). The CCD was used to record the time-resolved fluorescence spectrum, while the photomultiplier was used to detect rapidly changing signals such as that from flowing beads (discussed below).

3. Results and discussion

3.1. Excitation sources

As discussed in the introduction, traditional fluorescence-based systems rely on spectral separation of scattered excitation and fluorescence signals. Lasers are often used as excitation sources due to their high brightness, focusability, and narrow excitation linewidth (facilitating spectral filtering). The excitation of multiple fluorophores requires multiple excitation sources, resulting in an increasingly complicated system of multiple lasers, dichroic mirrors, and sets of filters. An alternative to laser excitation is an incoherent broadband source such as a xenon arc lamp. Broadband sources allow one to select different excitation wavelengths, but they are typically extended sources which are difficult to collimate and focus. In addition, high quality excitation filters have to be used to select specific excitation wavelength ranges while blocking the other wavelengths to prevent the scattered excitation light from overwhelming the fluorescence signal. Thus, the full spectrum cannot be used simultaneously for excitation. The supercontinuum excitation source used in our experiments combines the advantages of the previous two excitation schemes while addressing the limitations of each. The supercontinuum has the high spatial coherence typical of fiber sources, while retaining the broad bandwidth necessary to excite a wide variety of fluorophores. Furthermore, the supercontinuum excitation also has the advantage of being an ultrashort pulse in the time domain, which allows one to temporally separate the excitation from the fluorescence, eliminating the need for excitation filters and enabling simultaneous whole-spectrum excitation.

#84077 - $15.00 USD Received 12 Jun 2007; revised 24 Jul 2007; accepted 25 Jul 2007; published 2 Aug 2007

3.2. Multicolor Fluorescence Detection

In order to verify that the high-repetition-rate supercontinuum source has sufficient intensity to excite fluorescence efficiently, we focused the supercontinuum into a cuvette containing a test sample consisting of a mixture of two different dye molecules, 6-Carboxytetramethylrhodamine (6-TAMRA) and Deep Red. 6-TAMRA is a widely used fluorophore for automated DNA sequencing and capillary electrophoresis. It has an absorption maximum around 547 nm and emission peak around 573 nm. Deep Red is a mitochondria staining dye, which has absorption maximum of 640 nm and emission peak around 662 nm. Despite the widely separated absorption spectra, these two dyes were both efficiently excited by the supercontinuum light source. The fluorescence was collected and temporally filtered using the streak camera system described above. Since the streak trace time delay was adjusted to screen out the scattered pump light, the detected signal consists of only the fluorescence from the two dyes. The temporally integrated signal is shown in Fig. 4(a), and the fluorescence decay curves are shown in Fig. 4(b). Extension to a large number of dyes is trivially accomplished, the only limitations being the ability to spectrally distinguish separate dyes and the lack of excitation below 460 nm.

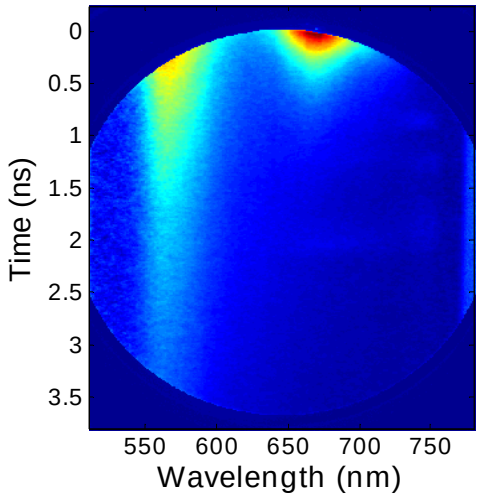

Fig. 3. Time-resolved fluorescence spectra of two different dye molecules, 6-TAMRA and Deep Red. The fluorescence emission peaks of 6-TAMRA and Deep Red are 573 and 662 nm, respectively. The delay of the trigger signal to the streak camera was adjusted so that the excitation supercontinuum was tuned out of the detection time window.

From the image of the streak camera output, we obtained not only the entire unfiltered fluorescence spectra of these two dye molecules (Fig. 4a), but also their fluorescence decay curves (Fig. 4b). The two spectra are well enough separated that each decay curve can be fitted with a single exponential at their respective center wavelengths. Deep Red has a fluorescence lifetime of 0.60 ns, while 6-TAMRA has a 2.4 ns lifetime, which agrees well with other measurements [10]. Although the spectral overlap between these two dyes is minimal, the difference in decay lifetimes would allow one to distinguish different dye molecules even with overlapping spectra. These results demonstrate that the time-resolved measurements of the supercontinuum-excited fluorescence emissions from multiple dye molecules can be achieved and provide both spectral and lifetime data simultaneously.

#84077 - $15.00 USD Received 12 Jun 2007; revised 24 Jul 2007; accepted 25 Jul 2007; published 2 Aug 2007

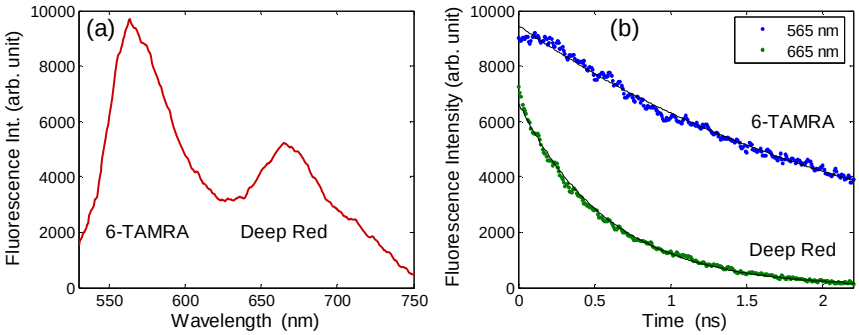

Fig. 4. (a) Fluorescence spectra of 6-TAMRA and Deep Red 0.2 ns after excitation. (b) Fluorescence decay curves of 6-TAMRA and Deep Red with a 2.4 ns and 0.6 ns fluorescence lifetime respectively.

3.*3. Potential applications*

Owing to the above mentioned advantages of this whole-spectrum fluorescence detection, this new detection mechanism opens up a wide range of potential applications. Following the demonstration of the fundamental detection mechanism, we expect that successful development of this technology will lead to significant improvements in many different kinds of fluorescence based instruments, such as microplate readers, fluorescence microscopes, endoscopes, and flow cytometers, etc. As a first demonstration, we chose flow cytometry as an example to demonstrate the applicability of this technology for multicolor fluorescence measurements.

With a long track record of being the most accurate and well–defined technology for measuring properties of single cells, flow cytometry might appear to be commonplace and mature. To simultaneously detect a large variety of cells stained with different fluorescent markers, people continue to expand the number of excitation sources and detection channels in a system. For example, one of the most sophisticated commercially available systems, the FACSAria from BD Biosciences, has three lasers with wavelengths at 488 nm, 633 nm, and 407 nm as the excitation sources, and a complicated signal collection system composed of 20-30 sets of filters and dichroic mirrors and 14 photomultiplier tube detectors. In spite of the complexity, this system is still not capable of exciting all fluorescent markers in the visible and near infrared region since there are only three isolated excitation wavelengths available. Due to the complexity of such systems, they are reaching geometrical limitations, *i.e*., in practice the optical configuration does not allow adding additional excitation sources and detection channels. In our system, only a single laser source is needed to excite all different fluorescent biomarkers over the whole spectrum range from visible to near infrared. In addition, no filters and dichroic mirrors are required, thus significantly simplifying the optical configuration.

As an initial test of applying the whole-spectrum fluorescence detection technique to flow cytometry, we have carried out measurements on two kinds of standard fluorescent beads with quite different absorption and emission spectra. We used a simple flow system that consists of a square fused silica capillary (100x100 μm inner dimension) and a syringe pump. The square capillary was held perpendicular to the supercontinuum excitation source and with its surface adjusted perpendicular to the source. The capillary was connected to a syringe pump with plastic tubing. Fluorescent bead samples were loaded into a syringe and pushed by the syringe pump through the capillary at a constant flow rate (5μL/min.). Fluorescence was collected at a right angle to the capillary and to the excitation source, spectrally dispersed in a spectrometer and detected using the streak camera and photon counting system as described above. Figure 5a shows the fluorescence signals from the beads with absorption maximum of 430 nm and emission maximum of 465 nm, while Fig. 5(b) shows the signals from the

#84077 - $15.00 USD Received 12 Jun 2007; revised 24 Jul 2007; accepted 25 Jul 2007; published 2 Aug 2007

fluorescent beads with absorption maximum of 665 nm and emission maximum of 680 nm obtained with the same excitation and detection system. The signal levels (photon counts per detected bead) are similar to those obtained using standard cw-laser excitation and photon counting detection. The use of a photomultiplier detector array would enable straightforward multichannel cytometry, limited only by the number of channels in the detector array.

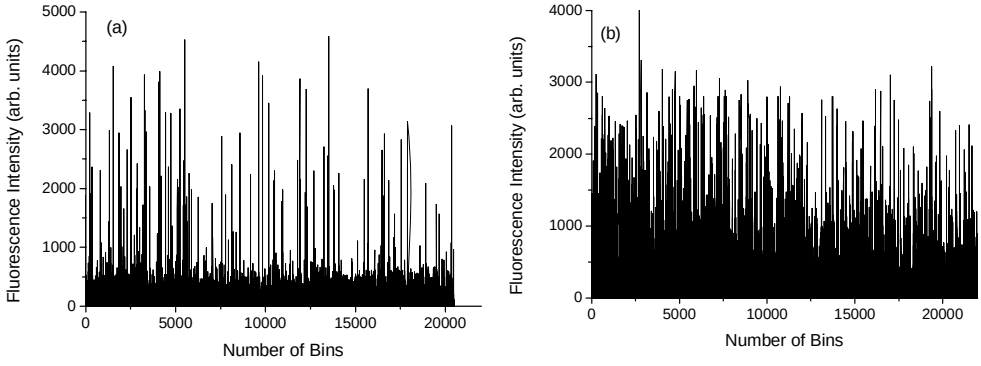

Fig. 5. Measurements of fluorescent beads having different absorption and emission spectra with a single ultrafast supercontinuum excitation. Each peak represents a fluorescence burst from an individual bead passing through the excitation volume. The bin width was set to 1.3 ms. (a) Fluorescent beads with absorption maximum of 430 nm and emission maximum of 465 nm, and (b) fluorescent beads with absorption maximum of 665 nm and emission maximum of 680 nm.

In conclusion, a new mechanism for multicolor fluorescence detection has been explored. We demonstrate the possibility for true whole-spectrum coverage from visible to near infrared for fluorescence detection using an ultrafast supercontinuum excitation source without spectral filtering in conjunction with time-domain filtering by a streak camera. We believe this new detection mechanism can significantly improve the performance of conventional multicolor fluorescence instruments and open up a wide range of applications.

#84077 - $15.00 USD Received 12 Jun 2007; revised 24 Jul 2007; accepted 25 Jul 2007; published 2 Aug 2007