DEVELOPMENT OF TIME-RESOLVED LASER-INDUCED FLUORESCENCE SPECTROSCOPIC TECHNIQUE FOR THE ANALYSIS OF BIOMOLECULES

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Abstract. *Our developments of the time–resolved laser–induced fluorescence (TR–LIF) detection system for biomolecules are presented. This system is based on the tunable (320 nm to 475 nm) Nd:YAG laser pulses used to excite various biomolecules. The detection part is the Streak System for Fluorescence Lifetime Spectroscopy (Hamamatsu, Japan). The system consists of a C4334–01 streakscope, as a detector, DG 535 digital pulse/delay generator, C5094–S Spectrograph and HPD–TA System, as a temporal analyzer. The TR–LIF spectrometer is designed primarily to study the temperature and pressure effects on fluorescence behavior of biomolecules upon excitation with a single nanosecond pulse. The design of this system has capability to combine laser–induced breakdown (LIB) with fluorescence, as well to study optodynamic behavior of fluorescence biomolecules.*

Key words: *Fluorescence, Laser–induced spectroscopy, biomolecules, optodynamic*

INTRODUCTION

Laser–induced fluorescence (LIF) spectroscopy is one of the most widely used spectroscopic techniques in the fields of life sciences, biochemistry and molecular biophysics today [1,2]. LIF introduces advantages such as short pulse excitation, wavelength tunability, and narrow bandwidth excitation. This technique is of great importance for environmental monitoring of bioaerosols, due to capabilities for *in situ* analysis and remote sensing [3]. LIF technique has been applied for imaging and tracking a single molecule or particle in a biological cell [4,5]. Fluorescence has been used to study the structure and conformations of DNA and proteins with techniques such as

fluorescence resonance energy transfer [6]. Different LIF designs have been employed to induce and collect fluorescence with the aim of probing a wide range of fundamentally different physical, biological and chemical processes.

Time-resolved (TR) detection modalities is a promising tool for examining microenvironment, molecular dynamics of proteins in complex environment crowded by other molecules, dynamics of excited state, etc. [1,2]. There are two types of measurements: the first is the measurement of the decay of the total fluorescence intensity following the excitation (pulsed or modulated), and the second type involves measurements of the polarization anisotropy decay. In this report, we describe a temperature dependent TR experiment in different samples (solutions, powders, etc.) based on pulsed excitation on a home-made sample cell.

Laser–induced breakdown (LIB) method has been applied for analysis of liquids, solids or gases [7,8]. A limited number LIB and LIF combined techniques have been reported up to now [9-11]. A new design of LIB–LIF system will be developed in future effort in order to investigate biomolecules.

When matter is irradiated with a laser pulse a part of the incoming light energy is converted to mechanical energy. Optodynamic (OD) waves are created and they propagate outward from the source. By detecting and analyzing the outgoing waves the characteristics of the light – matter interaction can be determined [12,13]. Joint analyses by OD and LIB–LIF can afford new possibilities in investigation of biomolecules in different environments. This is the reason we are going to set a combined system that can simultaneously detect OD and LIB–LIF effects.

EXPERIMENTAL METHODS

Time-resolved Laser Induced Fluorescence

Time-resolved laser-induced fluorescence (TR-LIF) is a standard technique for measuring radiative lifetimes of excited atomic states. TR-LIF is carried out by two methods: pulse method (time domain) and harmonic or phase-modulation method (frequency domain). The proposed experimental system is based on the pulse method TR-LIF and only this method will be briefly described here.

The excitation of the sample is done with short laser pulses, and the intensity of the sample emission versus time is recorded using detectors with high temporal resolution such as e.g. fast detection electronics or streak camera. In ideal case, the excitation laser pulses are much shorter than the radiative lifetime under investigation. For a simple system having a single fluorophore, the fluorescence intensity decay, $F(t)$ is described by an exponential decay law:

$$F(t) = F_0 \exp(-t/\tau) . \tag{1}$$

The F_0 is the fluorescence intensity at the time excitation, and τ is the excited-state fluorescence lifetime. τ can be expressed in terms of the radiative, τ_r, and the non-radiative de-excitation time τ_{nr} ($\tau = \tau_r + \tau_{nr}$). The most often proposed mechanism in proteins and peptides, as the principal quenching mechanism is the electron transfer and can be regarded as an extra deactivation part ($\tau = \tau_r + \tau_{nr} + \tau_{et}$) [21].

In a complex system (an ensemble of many molecules), the $F(t)$ represents the sum of fluorescent intensities of all species

$$F(t) = \sum_{i=1}^{N} F_i \exp(-t/\tau_i),$$ (2)

where F_i is the fluorescence intensity of species i at $t = 0$, and τ_i the corresponding fluorescence lifetime.

Experimentally measured $F(t)$ is not the true fluorescence response function from the sample under investigation. The recorded profile, $F_{OBS}(t)$ (Fig. 1) is a convolution of the instrument response function (excitation pulse) $I(t)$ and the $F(t)$

$$F_{OBS}(t) = \int_{o}^{t} I(\tau) F(t - \tau) d\tau .$$ (3)
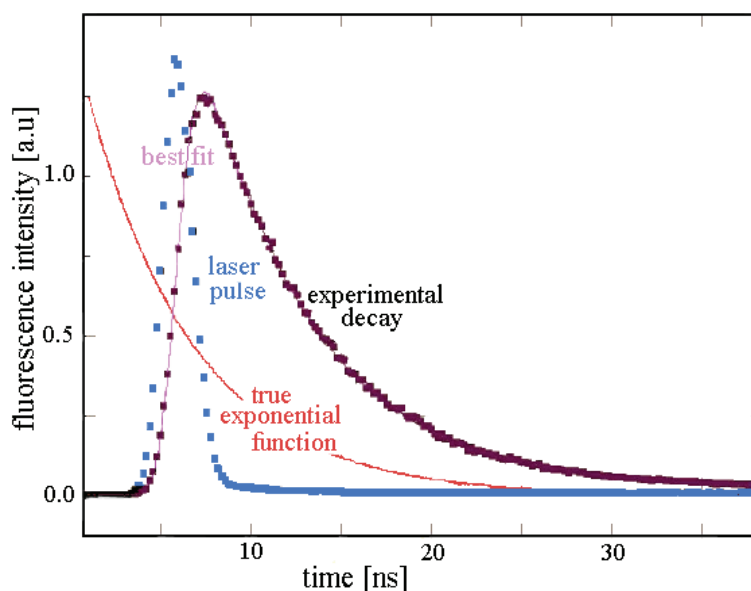

Fig. 1. Laser pulse (blue squares), experimental decay (black squares), the best numerical fit (true experimental function convoluted with laser pulse, violet line) and true exponential function (hypothetical decay if the laser pulse was infinitely narrow, red line) [1].

Different methods of analysis are used to extract $F(t)$ from $I(t)$ and $F_{OBS}(t)$ [1]. Most commonly used is the nonlinear least-squares iterative deconvolution method. This method is based on comparing the value of measured signal $F_{OBS}(t)$ at time t with the calculated response, $C(t)$ at the same time t. Deconvolution of data is achieved using a numerical technique applying an estimated function and iteration to minimize chi-squared [14,15].

$$\chi^2 = \sum_{i=1}^{np} w_i [F_{\text{OBS}}(t) - C(t)]^2 , \qquad (4)$$

where w_i is weighting factor, $C(t)$ is calculated response, and np is number of data points in decay file. To fit decay curves to the reconvoluted data, a numerical fitting routine for multidimensional fluorescence data is employed [1]. Beside this methods, the method of moments, maximum entropy method (MEM) [16], Stretched-exponential analysis for systems having continuous distribution of lifetimes [17], time resolved area normalized emission spectroscopy (TRANES) [18] are developed to analyze the fluorescence intensity decays of complex systems.
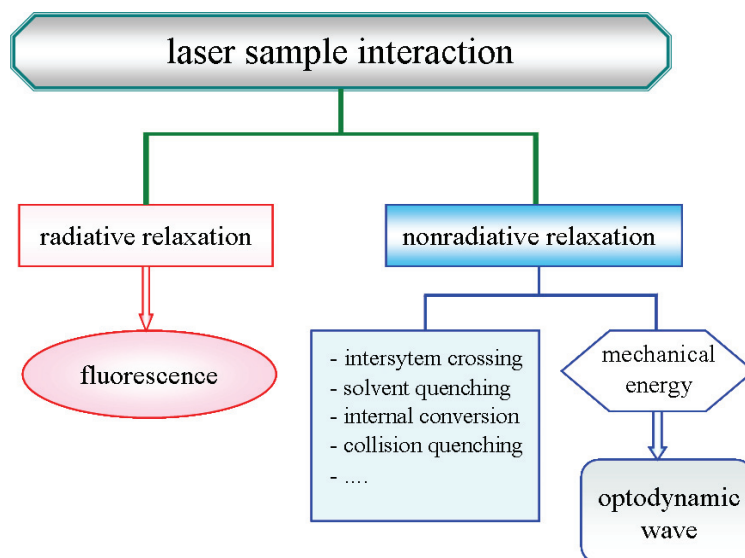

Fig. 2. Schematic representation of fluorescence and some possible competing non-radiative routes.

The measured average fluorescence lifetime, τ_{av}, is proportional to the total area under the fluorescence decay curve defined by

$$\tau_{\text{av}} = \sum_{i=1}^{N} F_i \tau_i \bigg/ \sum_{i=1}^{N} F_i . \qquad (5)$$

It will be analyzed by fitting it to a function with the sum of exponentials. τ_{av} is a function of many rate (probability per unit time) parameters. The use of the fluorescence lifetime of a molecule as a probe of the microenvironment can be illustrated by considering the dependence of τ_{av} on the rates of competing decay pathways [2,19,20] (Fig. 2).

According to the theory and experimental results [21], k_r is independent on temperature. Therefore, the fluctuation of fluorescence lifetime with the temperature variation of sample originates from nonradiative rate. k_{nr} splits the nonradiative rate into

temperature independent k_{nr}^0 and temperature dependent contribution, $k_{nr}(T)$, which can be described by the Arrhenius relationship

$$\frac{1}{\tau} = k_r + k_{nr}^0 + k_{nr}(T) = k_r + k_{nr}^0 + A\exp(-E^*/RT) \ , \tag{6}$$

where A is the is the Arrhenius pre-exponential term, E^* is the activation energy, which characterizes the principal dynamic quenching process, R is the universal gas constant, and T the absolute temperature.

Time-Resolved Laser–Induced Breakdown

Laser–induced breakdown (LIB) or also sometimes called laser induced plasma (LIP) because it is the laser light which transforms matter in solid, liquid or gaseous state to the ionized state [7]. The LIB technique has been applied successfully for qualitative and quantitative purposes [22,23]. Modern detectors, based on CCD or ICCD technology are capable of achieving good temporal resolution and are contributing to make LIBS into a more useful analytical tool. Some authors have named this technique time-resolved laser induced breakdown spectroscopy (TRELIBS) [23]. Using TR imaging, it is possible to directly and indirectly monitor the breakdown dynamics induced in water [24], to observe the shock waves and cavitation bubbles in water and corneal tissue [25], for analysis of aerosols [26], for investigation cell lysis process [27].

Optodynamic

When the sample is illuminated by a laser source (continuous or pulsed), excited molecules (atoms) decay back to their initial state by radiative (e.g. fluorescence) or nonradiative mechanisms. A part of nonradiative relaxation is converted to mechanical energy (Fig. 2). Optodynamic (OD) waves are created and propagate outward from the source. The characteristics of the OD waves are influenced by the properties of the incoming light pulse (wavelength, pulse energy, pulse duration), as well as, optical, thermal and mechanical properties of the irradiated sample. Thus, by detecting and analyzing the outgoing waves the characteristics of the light – sample interaction can be determined [12,13,28].

The experimental setup described in this presentation is designed for simultaneous detection of both radiative (fluorescence) and nonradiative mechanisms by LIB–LIF and OD technique. By using the time-resolved spectroscopy one can test the transient energy states of the molecules under study.

INSTRUMENTATION

The instrumental of LIF spectroscopy consists of a laser excitation source with specified narrow band-width, focusing and collection optics, a spectrometer, and a sensitive spectroscopic CCD detector. To suppress the excitation light, a cut off filter in front of the spectrograph is required. These instruments can be put together in different ways, depending on desired application. We have designed the experimental setup which facilitates TR–LIF spectroscopy and combines laser–induced breakdown with laser

induced fluorescence/optodynamic processes (Fig. 3). The main components of this system are described as follows.
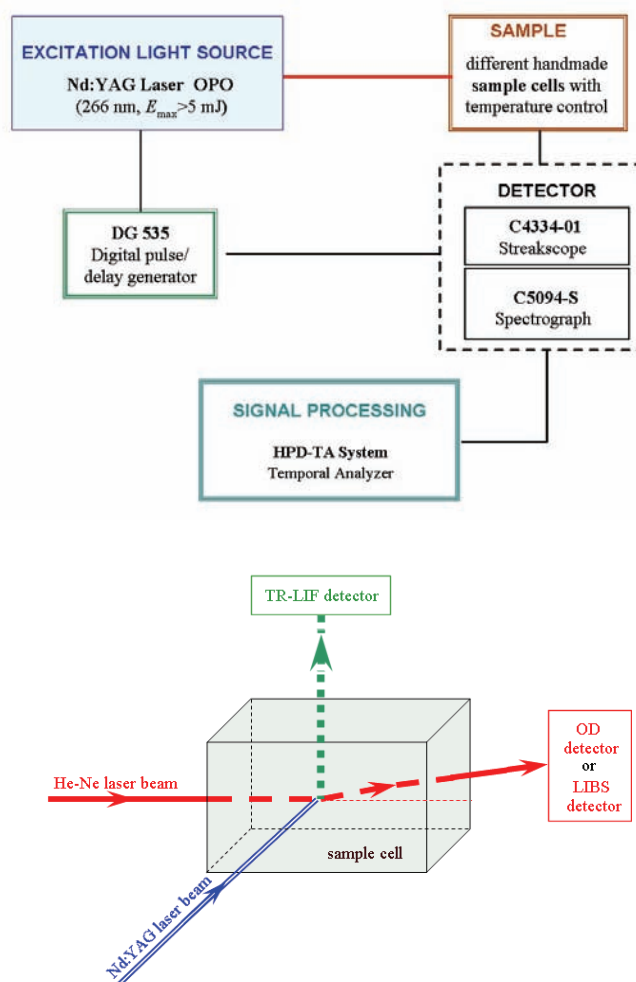

Fig. 3. Schematic illustration of experimental setup for TR–LIF spectroscopy. and schematic principle of simultaneous detection TR-LIF and OD/LIBS

Continuously Tunable Laser System

The fluorescence of biomolecules is produced by using a tunable laser system (Vibrant models 266-I made by Opotek, Inc.). This system (Fig. 4) includes the optical

parametric oscillator (OPO) that is pumped by the fourth harmonics of the Nd:YAG Brilliant laser at 266 nm, and control electronics. The sample can be excited over the wavelength range from 320 nm to 475 nm with pulse widths of 5 ns at a repetition rate of 10 Hz. The linewidth of OPO increases dramatically as the output wavelength approaches the degeneracy point.
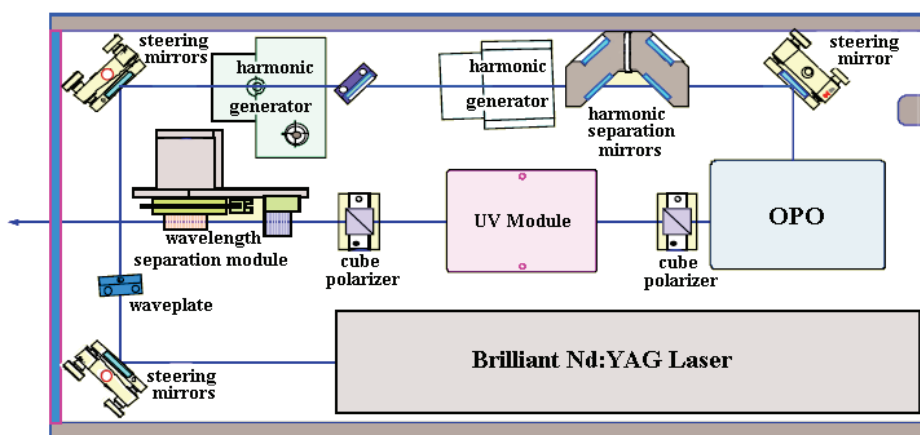

Fig. 4. Schematic representation of tunable laser System.

The main part of the laser beam is focused on the sample in different hand-made sample cells. Part of laser pulse (~1%) is transmitted to trigger the system. The trigger is controlled by software, which also initiates the CCD camera. Trigger delays are generated by DG535 delay generator (Stanford Research Systems, Sunnyvale, CA).

Detection System

Picoseconds time-resolved fluorescence spectroscopy is performed by a streakscope (Hamamatsu model C4334-01) with integrated video streak camera and enables a wide range of fluorescence lifetime measurement from ps to ms with high accuracy. The heart of the streakscope is an electron tube (S– tube). The spectroscope covers the wavelength region from 200 to 850 nm, with the temporal resolution better than 15 ps, and with repetition rate up to 2 MHz.

The video streak camera has high sensitivity and high time resolution, and is designed to take the spectral image from the spectrograph and convert it into a two-dimensional image. Such performances enable the TR-LIF measurement with short acquisition time. Operating principle of the streak camera (Fig. 5) in brief:
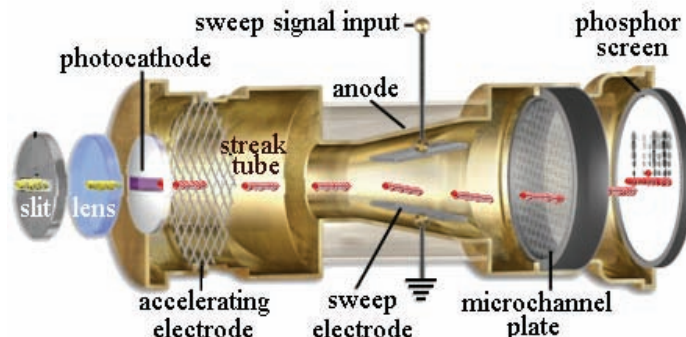
Fig. 5. Streak camera. [Hamamatsu, C4334 manual].

The pulses with different optical intensity and which vary slightly in time and space passing through the slit and optics form the image on the photocathode of the streak tube. On photocathode the photons are converted into a number of electrons proportional to the intensity of the incident light. The electrons are then accelerated and conducted towards the photocathode. Then they pass between another pair of sweep electrodes where a high voltage synchronized to the incident light is applied. The applied voltage steers the electron paths away from the horizontal direction at different angles depending on their arrival time at the electrodes. Then electrons are conducted to the microchannel plate (MCP), where they are multiplied several thousands of times, and then bombard against the phosphor screen, and are converted again into light.

The brightness is proportional to the intensity of the incident pulses, and positions in horizontal direction correspond to the horizontal location of the incident light. The vertical axis represents the time.

The combination of the streakscope (Hamamatsu, C4334-01) with the spectrograph (Hamamatsu, C5094–S, focal length 250 mm, with 3 gratings) enables simultaneous multiple–wavelength measurements to be made. In the same design of the system it is also possible to use another spectrograph C5094-S-S3 (Acton Research, Princeton Instruments) with focal length of 300 mm, with interchangeable grating.

The delay units (Stanford Research Systems DG535) allows for delays on time scales of 10 ns and higher.

Signal Processing

The HPD–TA (Temporal Analyzer) streak software controls the C4334–01 spectroscope, spectrograph C5094–S and delay generator on a PC in order to carry out data acquisition, data correction and data analysis. The HPD-TA software is designed specifically for reading out the image on phosphor screen of streak and framing cameras. It enables precise, quantitative acquisition and pre-analysis of two dimensional streak data.

All fluorescence data will be acquired in single photon-counting mode using HPD-TA software. The collected data will be fitted with advanced adaptive least-squares fitting algorithm and deconvoluted for the laser pulse profile using the same software package (HPD-TA) including "fluorescence lifetime fitting module for HPD-TA" (Hamamatsu TA-Fit) for data analysis. The quality of fit results will be tested via chi-square.

EXPERIMENT

Amino acids play central role as building blocks of proteins and as intermediates of metabolism. Nonaromatic amino acid alanine (Ala) and glycine (Gly) can be inside or outside of the protein molecule. At the beginning w*e plan* to study the temperature effect on fluorescence behavior (TR–LIF) on these molecules as well as a formic acid, in different environments upon excitation with a single nanosecond pulse.

The obtained results of the TR–LIF spectroscopy can be used to test the transient energy state of the molecules under study and thereby understand the mechanisms of the damages induced by the radiation. The more detailed data can be obtained if one combines the TR–LIF and the high resolution energy loss spectra [29-32]. At the same time we planed to adopt a model for the analysis of air fluorescence induced by electrons [33] for our experimental conditions.

Brief overview of similar experimental systems

During the last decade, different trials with instruments for measurement of fluorescence behavior of biomolecules for many application in physics, chemistry, engineering, biology and medicine have been reported. We presented in Tab 1. a brief survey of significant results of various studies with similar detection systems (or instruments), as we planned to develop.

Table 1. A brief summary of results with Hamamatsu detection systems

Detection system	Excitation	Application	Reference
C4780 Hamatsu picoseconds fluorescence lifetime system consisting of a C1334 Hamamatsu streakscope, C5094 Hamamtsu spectrometer, DG535 pulse delay generator, A2513optical delay unit.	Nd:YAG laser 1064 nm, 35 ps, 3-5 mJ, third harmonic at 355 nrn, and fourth harmonic at 266 nm.	TR ps emission spectroscopy of xanthone in aqueous solution solvents and various surfactants.	34
C4780 Hamatsu picosecond fluorescence lifetime system 4334 hamamatsu streak scope	Mode-locked Ti:Sapphire laser, 485 nm, pulse width less than 2 ps, and repetition rate 2 MHz.	TR fluorometric method for observing hybridization in living cells using fluorescence resonance energy transfer	35

4334 Hamamatsu streakscope	Third harmonic of a continuum PY-61 ps Nd:YAG laser (355 nm, 35 ps, <3 mJ pulse,	Monitoring photo-decomposition of dibenzyl ketone within NaY zeolite with a pre-fluorescent nitroxide compound	36
C4334-11 Hamamatsu spectrograph-streak camera system	fs mode-locked Ti:sapphire laser, 800 nm (400 nm frequency doubling), repetition rate 76 MHz, pulse width 200 fs FWHM, average laser power 15-25 mW.	ps TR-LIF of PRODAN dissolved in 1 [bmim][PF6] at 298 K as a function of solubilized water in the [bmim][PF6] phase.	37
C4334 Hamamatsu streakscope.	20 ps pulsed dye laser synchronously pumped by a mode-locked Ar^+ ion laser, energy 2.19 eV (565 nm)	TR photoluminescence study of exactions in thin PTCDA films at various temperatures	38
C4334 Hamamatsu streakscope	Titanium: Sapphire 860 nm, 200 fs.	Multiphoton Fluorescence Lifetime Imaging Microscopy system using a Streak Camera	39
C4334 Hamamatsu streak camera	Ti:S Spitfire laser, 520 nm and 575nm, pulses duration of 1.5 ps at the repetition rate of 1 kHz.	Fluorescence lifetimes of DDPT in methanol-in-oil reverse micelles.	40
C4334-01 Hamamatsu streakscope	775 nm, from a fs Ti:Sapphire regenerative amplifier seeded by the second harmonic of a mode-locked Er-doped fiber laser 535 nm.	ps TR-LIF of the excited state of sulforhodamine B (SRB) molecules adsorbed in the mono- and multi-layered Langmuir–Blodgett (LB) films of octadecylamine.	41
C4780 Hamatsu ps fluorescence lifetime system C4334 Streakscope C4792-01 synchronous delay generator HPD-TA Hamamatsu software	Ti:sapphire laser 550 nm.	TR-LIF to determine the deactivation pathways following photoexcitation of monomeric 6 in MCH.	42
C4334-01 Hamamatsu streakscope	ps Nd:YAG laser, 355 nm FWHM30 ps, 2-5	TR-LIF singlet excited-state	43

C5094 Hamamatsu	mJ/pulse	properties of the block copolymers of oligothiophene and oligosilylene in solution.	
C4780 Hamatsu ps fluorescence lifetime system consisting of a C4334 streak camera C5094 spectrograph	fs Ti:sapphire laser, 395 nm, repetition rate 250 kHz	Time dependent energy shifts in PL of fluorescent thin films of aluminum tris-(8-hydroxyquinoline) (AlQ3) at different temperatures.	44

CONCLUSION

We report here the development of the time–resolved laser induced fluorescence life time (TR–LIF) system using a streak camera. In future, with small modifications this system can be adapted to *s*imultaneously detect OD and TR–LIF effects in biomolecules. Combination of these two complementary spectroscopic techniques, OD and TR-LIF, can provide a useful method for the study of the dynamics of biomolecules. Beside this, we have tried to present in short, the basic theory of experimental methods which we planned to use and review similar instrumentation.

The streak camera is capable of measuring time-resolved emission spectra over a 100 nm wavelength range in real time. This makes possible direct comparison of kinetics of different spectral features.

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RAZVOJ VEMENSKI RAZLOŽENE LASERSKI INDUKOVANE FLUORESCENTNE SPEKTROSKOPSKE TEHNIKE ZA ANALIZU BIOMOLEKULA

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Prikazan je razvoj vremenski-razloženog laserski indukovanog fluorescentnog (TR–LIF) detekcionog sistema za biomolekule. Sistem se zasniva na podesivim (320 nm do 475 nm) Nd:YAG laserskim impulsima za pobudu različitih biomolekula. Detekcioni deo čini „streak" sistem za fluorescentnu spektroskopiju vremena života (Hamamatsu, Japan). On se sastoji od C4334–01 streakscope, kao detektora, DG 535 digitalog impulsog/delay generatora, C5094–S spektrografa i HPD–TA sistema za vremensku analizu. TR-LIF spektrometar je prevashodno dizajniran za proučavanje temperaturnih efekata i efekata pritiska na fluorescentno ponašanje biomolekula prilikom njihove ekscitacije pojedinačnim nanosekundnim impulsom. Sistem je projektovan tako da omogućuje i kombinaciju laserski indukovanog proboja (LIB) sa fluoroscencijom, kao i za proučavanje optodinamičkog ponašanja fluorescentnih molekula.