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Improvement of a Fluorescence Immunoassay with a Compact Diode-Pumped Solid State Laser at 315 nm[1]

Matthias Niederkrüger[2a], Christian Salb[a], Michael Beck[b], Niko Hildebrandt[b], Hans-Gerd Löhmannsröben[b], Gerd Marowsky[a]
[a]Laser-Laboratorium Göttingen e.V., Hans-Adolf-Krebs-Weg 1, 37077 Göttingen, Germany;
[b]Institut für Chemie & Interdisziplinäres Zentrum für Photonik, Universität Potsdam, Karl-Liebknecht-Strasse 24-25, 14476 Potsdam-Golm, Germany

ABSTRACT

We demonstrate the improvement of fluorescence immunoassay (FIA) diagnostics in deploying a newly developed compact diode-pumped solid state (DPSS) laser with emission at 315 nm. The laser is based on the quasi-three-level transition in Nd:YAG at 946 nm. The pulsed operation is either realized by an active Q-switch using an electro-optical device or by introduction of a Cr^{4+}:YAG saturable absorber as passive Q-switch element. By extra-cavity second harmonic generation in different nonlinear crystal media we obtained blue light at 473 nm. Subsequent mixing of the fundamental and the second harmonic in a β-barium-borate crystal provided pulsed emission at 315 nm with up to 20 µJ maximum pulse energy and 17 ns pulse duration. Substitution of a nitrogen laser in a FIA diagnostics system by the DPSS laser succeeded in considerable improvement of the detection limit. Despite significantly lower pulse energies (7 µJ DPSS laser versus 150 µJ nitrogen laser), in preliminary investigations the limit of detection was reduced by a factor of three for a typical FIA.

Keywords: Fluorescence immunoassay, FIA, FRET, Nd:YAG, 946 nm, 473 nm, 315 nm, pulsed DPSS laser, saturable absorber

1. INTRODUCTION

The demand for fast reliable medical diagnostic techniques increased dramatically over the past years. For most of the 30,000 identified diseases there is no possibility for an early and proper diagnosis. Fluorescence immunoassays (FIA) are of growing interest for medical applications as they can detect the cause of diseases on the molecular level. There are several assays for blood diagnostics ranging from prenatal diagnostics to cancer precaution in clinical application.[1]

The homogeneous FIA scheme under consideration here (Figure 1) is based on the coupling of an antigen to two labelled antibodies, which act as donor and acceptor in a fluorescence (or Förster) resonance energy transfer (FRET). The donor reagent is excited by UV light and transfers its energy radiationless to the acceptor if they are near to each other. This is the case when an antigen is present and the donor- and acceptor-antibody both are bound to it. The acceptor returns to the ground state by emitting visible light. Typical donor-reagents for FIA applications are lanthanide chelates, such as europium cryptate. The advantage of the donor is the long excited state lifetime (in the sub-ms-regime) allowing a better discrimination of the acceptor signal against autofluorescence in time-resolved measurements [2].

Common light sources for excitation in FIA reader systems are xenon lamps combined with filters or a monochromator for wavelength selection. Compact lasers are found only in few systems and to date only nitrogen lasers with emission at 337 nm seem to be utilized (e.g. KRYPTOR® Cezanne SA, France). Beside the advantages in improvement of detection limits and reduction of the expensive reagents, there is a big drawback of applying this laser: for typical FIA donor reagents, the 337 nm emission is located on the red-spectral wing of the absorption band ranging from ca. 300 to 320 nm (Figure 2). Furthermore, the nitrogen laser possesses the typical disadvantages of pulsed gas lasers, like usually significant pulse-to-pulse energy fluctuations, limited gas reservoir lifetime and repetition rate, as well as relatively poor beam characteristics, resulting in an ineffective light delivery.

[2] Correspondence: e-mail: mniederk@llg.gwdg.de; phone: +49 551 5035-52, fax: + 49 551 5035-99, www.llg.gwdg.de

Compact pulsed DPSS lasers, as they are currently used in many analytical applications, seem to be perfect light sources for FIA systems. They are small, reliable, almost maintenance-free and provide nearly perfect beam characteristics. But up to now, compact, commercial pulsed DPSS lasers are only available with Nd^{3+} as active material and emission wavelengths (e.g., 355 and 266 nm for Nd:YAG) not suitable for current FIA readers. Here we demonstrate two approaches towards pulsed DPSS laser with emission at 315 nm and first results in combination with a FIA reader system.

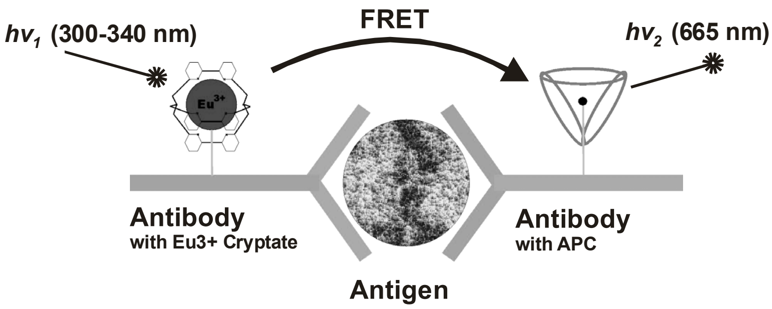

Fig. 1. Principle of the homogeneous lifetime immunoassay TRACE®

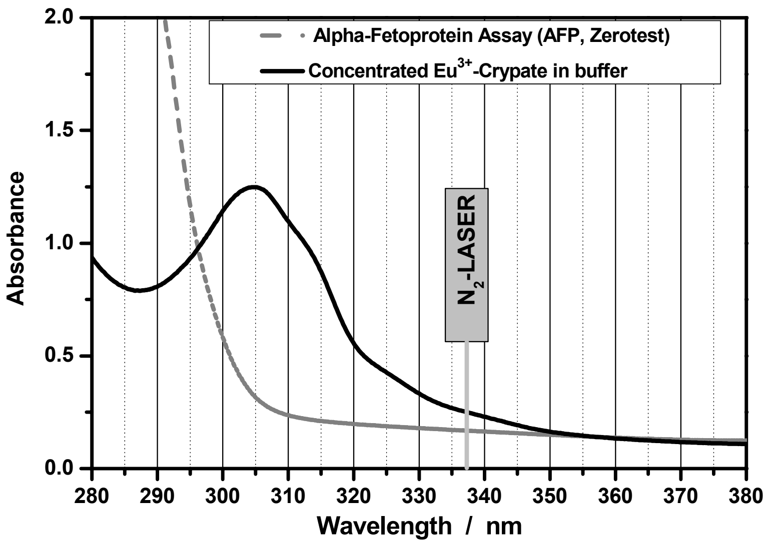

Fig. 2. Absorption spectra of FIA-donor europium cryptate and test FIA (Alpha-Fetoprotein Assay, AFP) with emission wavelength of used nitrogen laser.

2. MATERIAL AND METHODS

2.1 Fluorescence immunoassay

2.1.1 FIA principle with TRACE®

Our FIA reader system is designed for immunoassays based on the TRACE® (time-resolved amplified cryptate emission) method, a further development of investigations by the Nobel prize-winner Jean-Marie Lehn. Among his studies of supramolecular chemistry, he investigated molecular devices that perform *Absorption - Energy-Transfer - Emission processes* (A-ET-E) in which light absorption by a receptor molecule is followed by intramolecular energy transfer to a bound substrate which then emits light again. This process can be found in lanthanide-cryptates of europium(III)- and terbium(III)-cations with a macrobicyclic ligand containing three bipyridine groups.[2-4] In our experiments, the energy of the UV light absorbed by the bipyridine is transferred to the Eu^{3+}-ion bound in the molecular cavity and is emitted by the lanthanide ion with its typical long-time luminescence.

In the TRACE® technology, an antibody is labelled with an Eu^{3+}-trisbipyridinediamine (Eu^{3+}-TBP)-cryptate acting as the FIA-donor. For the FIA-acceptor, another fraction of the antibodies is labelled with cross-linked allophycocyanin (APC). The FIA-donor is excited with UV light at 337 nm. An energy transfer to the FIA-acceptor can happen if donor and acceptor both are linked by the antigen. The acceptor APC then emits around 665 nm. Otherwise, the unlinked donor emits light around 620 nm. The ratio of the observed light at 620 nm and 665 nm is a parameter for the amount of antigen in the serum.

2.1.2 FIA reader KRYPTOR® and data evaluation

We used a modified FIA reader system KRYPTOR® (Cezanne SA, France) with the schematic setup displayed in Figure 3. The laser emission is guided by a quartz fiber (diameter 1000 µm) into the reader. There it is directed by an UV high reflecting dichroic mirror and focused by a lens into a cylindrical well of a movable reader plate. The non-reflected light strikes a photodiode and causes the start signal for the detection electronics. The emission from the 150 µl assay is collected by a lens, passes the first mirror and is separated by a second dichroic mirror and two line filters (10 nm bandwidth) into the channels for the APC emission at 665 nm and that of Eu^{3+}-TBP at 620 nm. The detection light is focused on photomultipliers operating in single-photon counting mode. The FIA reader works at a repetition rate of 20 Hz, the optimal repetition rate of the implemented nitrogen laser. For other light sources, the maximum repetitions rate would be limited to ca. 200 Hz as the lifetime of the excited Eu^{3+}-TBP lies in the lower millisecond regime. The conventional laser used with the FIA reader is a nitrogen laser (150 µJ pulse energy and 3.5 ns pulse duration (FWHM)).

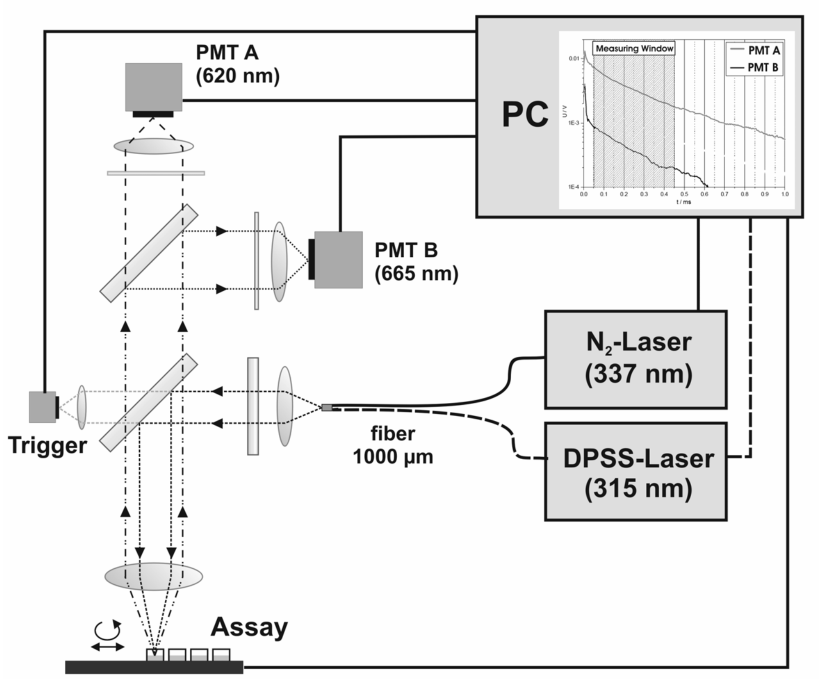

Fig. 3. Scheme of the FIA-Reader KRYPTOR ®

For evaluation of the FIA system with different lasers, two commercial immunoassays were used: one for prenatal diagnostic (Alpha-1-Fetoprotein; AFP) and one for prostate cancer diagnostic (total Prostate Specific Antigen; tPSA). Each set of samples consisted of three samples with a certain concentration of antigen and ten reference samples without antigen. The ratio of energy transfer APC emission at 665 nm is normalized by the Eu^{3+}-TBP emission at 620 nm to eliminate fluctuations. This normalized signal is proportional to the antigen concentration. The limit of detection (LOD) was obtained from the threefold standard deviation from the reference samples divided by the normalized signal of the antigen containing samples. The LOD is given in relative values for comparison of different laser combinations with the FIA-reader.

2.2 Pulsed DPSS Nd:YAG-lasers at 315 nm

2.2.1 Introduction and objectives of laser development

As explained above, a pulsed solid state laser with emission in the spectral range of 300 to 320 nm seems to be perfect for the FIA-application. But up to now there are no commercial lasers with the necessary parameters. A promising approach seems to be the frequency up-conversion of the fundamental laser transition in Nd:YAG between the energy levels $^4F_{3/2}$—$^4I_{9/2}$ with emission in the near infrared at 946 nm, subsequent second harmonic generation (SHG) to blue light at 473 nm and third harmonic generation (THG) to UV light at 315 nm by mixing of the fundamental with the second harmonic.

In contrast to the well known and widespread application of the $^4F_{3/2}$ - $^4I_{11/2}$ transition in Nd:YAG at 1064 nm, the $^4F_{3/2}$—$^4I_{9/2}$ transition at 946 nm is rarely used due to several fundamental problems based on the behavior as quasi-three-level laser transition. Firstly, the lower laser level is located in the ground state manifold that is 0.7% occupied at 293 K. Consequently, significant reabsorption losses are the result of the thermal population in the $^4I_{9/2}$ laser level. Secondly, the stimulated emission cross section is about an order of magnitude lower in comparison to the $^4F_{3/2}$ - $^4I_{11/2}$ transition. This may lead to parasitic lasing or amplified spontaneous emission at 1064 nm. Detailed theoretical investigations on the $^4F_{3/2}$—$^4I_{9/2}$ transition are reported by Fan et al.[5] and Risk et al.[6]

In spite of these problems, DPSS lasers at 946 nm and 473 nm emission are known and have been applied in the last decade for several interesting applications like data storage, optical display technology, underwater optical applications, remote-sensing of atmospheric water vapor and various kinds of biological and medical applications.[7-9] Most of these applications need continuous wave (cw) or quasi-continuous wave (q-cw) emission at the fundamental or at the second harmonic frequency. For efficient intra-cavity SHG, a more or less complex resonator design [10, 11] and/or a saturable absorber as Q-switch, generating multi-kilohertz repetition rates,[12, 13] were applied. THG to 315 nm by frequency mixing was of minor interest, as it is indicated by only few publications in this field, and only reported for flashlamp-pumped lasers with subsequent oscillator amplifier.[14, 15]

Our approach to produce 315 nm radiation was the generation of a pulsed emission at 946 nm from a compact diode pumped Nd:YAG laser with subsequent extra-cavity second and third harmonic generation. The desired parameters for the 315 nm radiation usable in the FIA-reader were more than 10 µJ pulse energy, less than 10 ns pulse duration (FWHM) and 200 Hz maximum repetition rate. The laser emission should be easily coupled into a quartz fiber of 1000 µm in diameter with negligible losses. Another important aspect was a compact cost efficient laser setup with the economical potential to compete with the nitrogen laser.

To apply extra-cavity SHG and THG processes the generation of short pulses with high energy is essential as the overall conversion efficiency depends quadratic on the power density of the initial beam. We followed two different approaches for Q-switching: utilizing an active, electro-optical Q-switch (β-barium-borate (BBO) Pockels-cell) or a passive one by utilizing a Cr^{4+}:YAG saturable absorber.

2.2.2 Pulsed DPSS-Nd:YAG-laser at 315 nm applying active Q-switching

The setup of the active Q-switched DPSS laser is depicted in Figure 4. The hemispherical laser resonator is 85 mm long and consists of a Nd:YAG rod (1.0 % Nd^{3+}, Ø 2 mm x 4 mm) with anti-reflection coating at the pump wavelength, a concave pump mirror (PM) with high transmission coatings at 808 nm (HT@808 > 95%) and high reflectivity at 946 nm (HR@946 >99,9 %), and a plan output coupler (OC) with partial reflectivity of 95 % at 946 nm. To avoid parasitic lasing at the $^4F_{3/2}$ - $^4I_{11/2}$ transition, the coating of the PM was designed for high transmission at 1064 nm (PR@1064 nm > 95%). The gain media is mounted with indium foil in a copper block and kept at 19.5°C by use of a thermoelectric cooler. A high-power, high-brightness pulsed laser diode (LD; CryLaS GmbH, Germany), operating at a central wavelength of 807.8 nm with a maximum repetition rate of 100 Hz, pump-pulse duration of 600 µs and a maximum optical output of 40 W, is deployed as pump source. The light of the LD bars was coupled into a fiber with core diameter of 600 µm to generate a homogenous pump intensity distribution and focused into the laser gain material (spot size of 400 µm diameter) by use of an optical telescope (f_1=30 mm; f_2=20 mm). Pumping the plain resonator with 11.6 mJ optical pulse energy and 600 µs pulse duration (pulse power 19.8 W at pump fiber exit) at 10 Hz repetition rate, we achieved emission at 946 nm with pulse energies up to 1.6 mJ (2.7 W). The pump threshold for this setup was 2.5 mJ (4.2 W pulse power). It was not possible to generate favorable smaller pump spot diameters in the active media as they were presented elsewhere [10, 11] due to the high losses and damages in coupling the pump light in fibers with diameter below 600 µm.

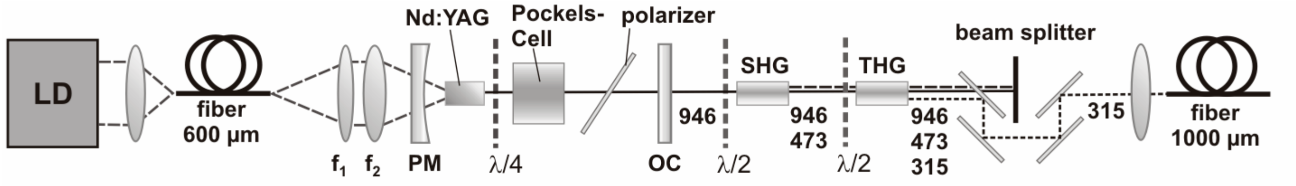

Fig. 4. Scheme of the actively Q-switched DPSS laser at 315 nm.

For short pulse generation we introduced a combination of BBO-Pockels-cell, a quarter-wave plate and a thin film polarizer at Brewster angle into the resonator. Since YAG is an isotropic material, the polarizer is necessary to fix the laser polarization, as it is necessary for phase matching in the subsequent nonlinear conversion steps, too. All components are anti-reflection coated at 946 nm. Driving the Pockels-cell with a short high-voltage pulse (5 ns; 3.3 kV) and pumping the resonator at maximum optical output of the LD, we obtained pulses at 946 nm with 450 µJ pulse energy and 22 ns pulse duration (FWHM).

Furthermore we carried out investigations with a diffusion bonded composite Nd:YAG rod (ONYX Optics Inc., USA) as gain medium (length 8.5 mm; diameter 2 mm) consisting of a 3 mm inner section with a 1.0 % Nd^{3+}- doping and two YAG caps (each 2.75 mm long). This kind of rod more easily removes the heat, minimizing thermal lensing effects like birefringence and bending of the crystal faces,[16] as was demonstrated with good results for cw-laser setups.[10, 11, 17] In our experiments, the use of a composite rod did not improve the output parameters significantly. Obviously, this is due to a small thermal load of the rod from the pulsed operation at relative low repetition rates.

The frequency conversion was realized in an extra-cavity design. There are several media which allow phase matching for SHG of 946 nm to 473 nm light at room temperature. Important parameters are the effective nonlinear optical coefficient (d_{eff}), the damage threshold and, keeping the subsequent THG process in mind, the walk-off angle between the fundamental and second harmonic beam. Suitable common crystals for SHG are potassium niobate ($KNbO_3$, FEE GmbH, Germany) and lithium borate (LBO, Castech Crystals Inc, China), as well as relatively new media like periodically-poled potassium titanyl phosphate (pp-KTP; Raicol Crystal Ltd., Israel) or bismuth borate (BiBO, Castech Crystals Inc, China). As each crystal has assets and drawbacks, we decided to investigate all these nonlinear media in our setup. With the software SNLO v36 [18] (except pp-KTP), the cut of the crystals for optimal phase matching was estimated and the crystals with anti-reflection coating were ordered from different suppliers. For polarization plane adjustment to the crystal orientation, a half-wave plate at 946 nm was inserted between the resonator and the SHG crystal, as depicted in Figure 4.

THG is only possible in β-barium-borate (BBO, Castech Crystals Inc, China) by mixing of 946 nm with 473 nm light, as this media allows phase matching and is transparent at the objective wavelength of 315 nm. As all investigated SHG processes base on type I phase matching as well as the THG in BBO, an additional phase retardation plate is required at 473 nm in front of the THG crystal. In Table 1 the parameters and results for the combination of this nonlinear media are listed for maximum LD pump power at 10 Hz repetition rate.

As expected,[17] SHG with pp-KTP yields the best conversion efficiency of 38 % and delivers outstanding pulse energies of 170 µJ at 473 nm. Surprisingly the following THG process results in poor conversion efficiency of 6.8 % and relatively low pulse energy of 10.7 µJ. The reason for this is not clearly understood to date. SHG with $KNbO_3$ and BiBO works relatively well and delivers moderate pulse energies. The small conversion efficiencies for the THG reflect the relative big walk-off angle in the SHG and the reduced interaction area of the frequency mixing beams in BBO. Nevertheless, the combination of $KNbO_3$ and BBO yields the highest laser pulse energy of 20 µJ at 315 nm for the active Q-switched Nd:YAG and was used for further investigations.

The frequency up-conversion process is followed by a four mirror configuration (HR@315 nm, 45° >99.9 %) in the setup that works as a beam splitter for efficient separation of the existing laser wavelengths. The remaining 315 nm emission is coupled into a fiber of 1000 µm diameter that is connected to the FIA-reader.

Table 1. Laser results for different combinations of SHG and THG media

Wavelength	**NLO Media** (effective length [mm])	**Pulse Energy** [µJ]	**Pulse Duration** [ns]	**Conversion Efficiency** SHG /THG [%]
946 nm	--	445	22	--
473/ 315 nm	LBO/ BBO (12/ 7)	6/ --	20/ --	1.3/ --
473/ 315 nm	$KNbO_3$/ BBO (5/ 7)	**140/ 20.0**	17/ 17	**31.5/ 14.3**
473/ 315 nm	BiBO/ BBO (6/ 7)	107/ 11.4	15/ 14	24.0/ 10.7
473/ 315 nm	pp-KTP/ BBO (10/ 7)	170/ 10.3	<20/ <20	38.0/ 6.1

2.2.3 Miniaturized passively Q-switched DPSS-Nd:YAG-laser at 315 nm

One objective of our investigations was to set up a compact and economical laser capable of competing with the nitrogen laser in the FIA-application. Therefore we built a second laser variation applying Cr^{4+}:YAG as saturable absorber for passive Q-switching and further miniaturization. In preliminary investigations with the plain resonator described above, we combined saturable absorbers of different initial transmissions (88 %, 90 %, 92 %, 94 %, 96 %, 98 %), output mirrors of different reflectivity at 946 nm (HR@946 nm= 70 %, 80 %, 90 %, 95 %, 98 %) and different focal lengths with a longitudinal pumped Nd:YAG rod. We defined the optimal configuration as it is shown in Figure 5 and described as follows: we built up a 30 mm long resonator with a composite Nd:YAG/ Cr:YAG crystal. Its Nd:YAG section (doping 1.0 % Nd^{3+}) is 4 mm long and 2 mm in diameter. The Cr^{4+}:YAG crystal with a initial transmission of 90 % is diffusion bonded to the Nd:YAG. The plan pump-side of the gain media is coated for high reflection at 946 nm (HR@946 nm >99.9 %) and high transmission at the pump wavelength. Again, to avoid parasitic lasing at 1064 nm, the transmission of the coating for this wavelength is above 85 %. The concave (f= -5000 mm) OC is partial reflective at 946 nm (90 %). The gain medium is pumped via a so called refocusing unit (RU; LIMO, Germany) which efficiently focuses the pump light from the fiber into the crystal. To obtain a stable and a highly polarized output from the resonator, a polarizer as described above is inserted. To maintain single pulse output per LD pump cycle, the current and pump-pulse duration of the LD had to be adapted. With this setup, the resonator delivers 946 nm p-polarized pulses (ratio 130:1) with 295 µJ pulse energy and 10 ns pulse duration. The pulse-to-pulse fluctuation was estimated to be below 0.6 %. The frequency up-conversion was carried out like in the active laser setup. At 315 nm, laser emission with 12 µJ pulse energy and 6.6 ns pulse duration was observed. The pulse-to-pulse fluctuation was below 1 %.

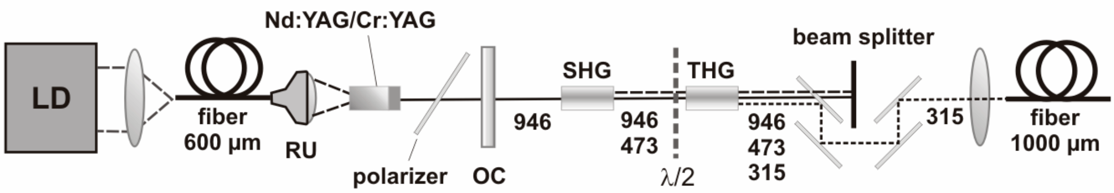

Fig. 5. Scheme of the passively Q-switched DPSS laser at 315 nm.

3. RESULTS AND DISCUSSION

3.1 FIA with actively Q-switched DPSS laser

The combination of the FIA reader KRYPTOR® with the actively Q-switched DPSS laser replacing the nitrogen laser was evaluated on the basis of the LOD for tPSA-antigen and yield remarkable results: despite significant lower pulse energy (8 µJ) for the DPSS laser versus the nitrogen laser (150 µJ), we obtained similar LOD in the standard measuring mode as demonstrated in Figure 6.

Furthermore, we found that in deploying the DPSS laser the LOD can be decreased by enhancing the number of accumulated pulses per measurement. Increasing the number of pulses from 20 to 90, the LOD decreases by a factor of two at average pulse energy of 8 µJ; increasing the number further to 180, the LOD achieved with the DPSS laser at 1 µJ was similar as with the nitrogen laser at 150 µJ. This resulted obviously from averaging over the strong pulse-to-pulse intensity fluctuations of about 15 %. These fluctuations stem from the fact that under the current soft- and hardware environment the laser is switched off between measurements of different samples. This interval operation caused instabilities due to a coarse temperature regulation of the sensitive $KNbO_3$ crystal. Among others the energy fluctuation is regarded as the main cause for the observed high variations in LOD. A demand for further laser development is therefore to minimize this fluctuation. But even in this status the DPSS laser can work at higher repetition rates as the nitrogen laser and therefore the enhancement of the collected pulses would not increase the overall FIA acquisition time.

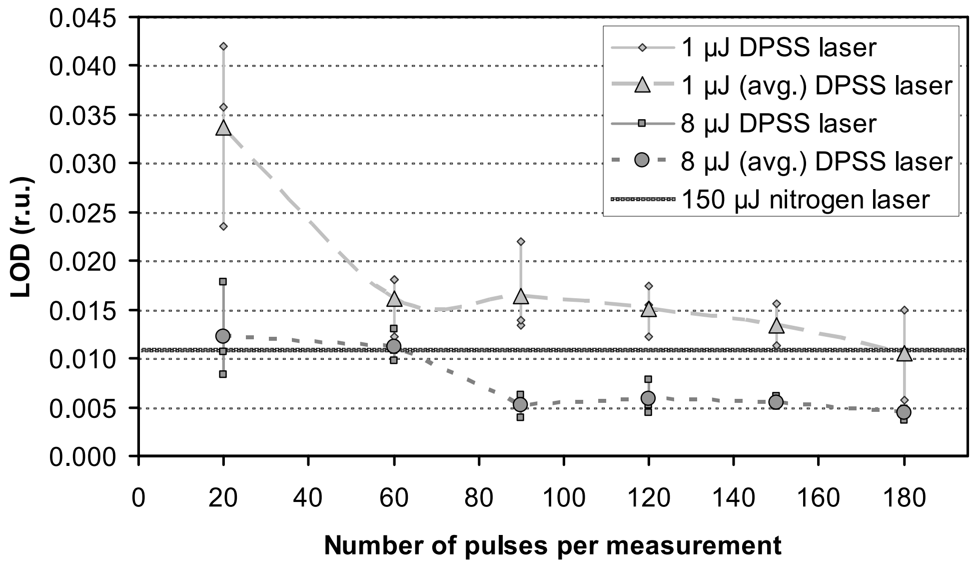

Fig. 6. Relative LOD for tPSA-FIA with actively Q-switched DPSS laser at 8 and 1 µJ for different number of pulses per measurement in comparison to nitrogen laser at fixed pulse energy (150 µJ).

3.2 FIA with passively Q-switched DPSS laser

The evaluation of the passively Q-switched DPSS laser in combination with the FIA-reader KRYPTOR® was performed in the same way as described above (Figure 7). At 7 µJ pulse energy the DPSS laser yields the same LOD as with the nitrogen laser at 150 µJ under standard measurement conditions. As demonstrated with the actively Q-switched DPSS laser, the LOD can be further decreased by increasing the number of pulses per measurement. Collecting 200 pulses per measurement increased the LOD for tPSA by a factor of 2.7 for the DPSS laser at 7 µJ compared to the setup with the nitrogen laser. At the same number of pulses the DPSS laser at 1 µJ pulse energy reaches the LOD as the nitrogen laser. With better temperature control the pulse fluctuation of the passive DPSS laser is below 1 %, and is below that of the actively Q-switched DPSS laser, there are still remarkable variations in the determination of the LOD. As these high variations were not observable in the measurements with the nitrogen laser, it is possible that there might be still some problems of adjustment or synchronization between DPSS laser and FIA reader. This will be the focus of further investigations and improvements.

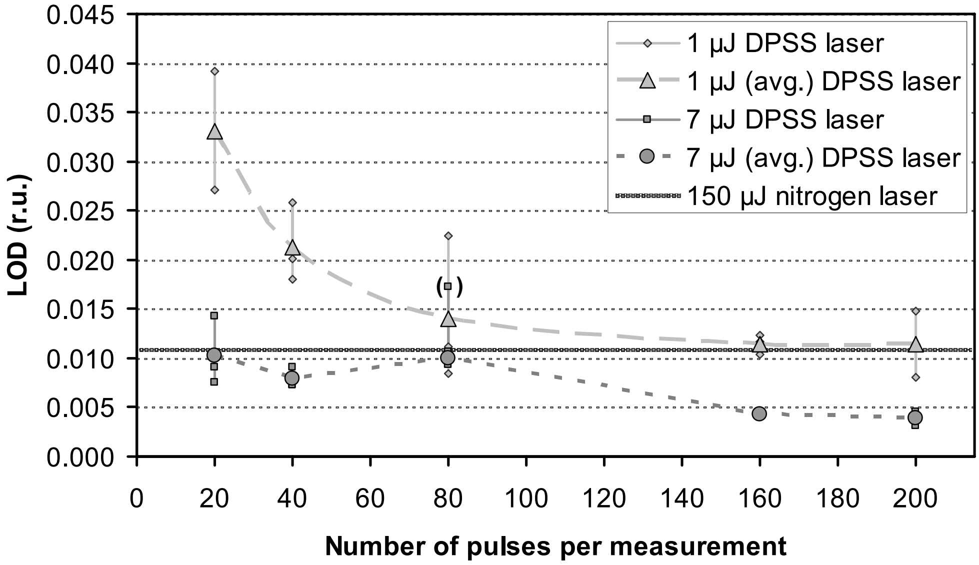

Fig. 7. Relative LOD for tPSA-FIA with passively Q-switched DPSS laser at pulse energies of 1 and 7 µJ in comparison to nitrogen laser at fixed energy and repetition rate.

4. CONCLUSION/ OUTLOOK

We demonstrate a considerable improvement of a FIA system by substituting a nitrogen laser by a DPSS laser with 315 nm emission. Despite significantly lower pulse energies (e. g. 7 µJ DPSS laser versus 150 µJ nitrogen laser), in preliminary investigations the relative limit of detection was reduced by a factor of two to three for a typical FIA. The application of DPSS laser in FIA systems is an innovative step on the way to smaller and faster medical diagnostic instruments with the future objective directed to *point-of-care* systems.

Furthermore, the developed pulsed DPSS laser with 315 nm emission represents a versatile compact UV-light source for biochemical and chemical analysis, like laser induced fluorescence spectroscopy. With an emission wavelength of 315 nm the laser fits well in the gap between the standard 355 nm and 266 nm laser lines of Nd:YAG and may be an interesting alternative to an excimer laser. Further investigations are in progress to increase the power of the DPSS laser.

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