Low cost lasers challenge ultrafast systems in two-photon excitation applications

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Although excellent research tools, the ultrafast laser systems are poorly suited for routine laboratory instrumentation, simply due to their high price. Our research group has shown that application of two-photon excitation need not to be limited to those laboratories that can afford the price and maintenance of ultrafast laser systems. With certain compromises and well-designed experiments, low-cost lasers can be used in utilizing the positive properties of two-photon excitation. The purpose of this publication is to show examples of bioanalytical applications that are possible using low-cost lasers. A bioaffinity assay using microparticles as bioactive carriers and an assay for characterization of cell surface antigens are presented. By direct comparison of the example assays in two instrumental set-ups, a quantity is given to the 'compromises' that are made by replacing an ultrafast system with low cost equipment.

Keywords: two-photon excitation, bioanalytics, bioaffinity assays, in-vitro diagnostics.

1. Introduction

The use of two-photon excitation in biomedical research has been steadily growing since Denk, Strickler and Webb from Cornell first introduced the two-photon excitation microscope [1]. Originally a PhD student in Göttingen postulated the phenomenon of two-photon excitation in 1931 [2]. In her publication "Über Elementarakte mit zwei Quantensprüngen" Maria Göppert-Meyer predicted the possibility of two-photon excitation – more than 30 years before the first successful experiments. The advent of lasers in the early 60's was the triggering point for two-photon excitation experiments within spectroscopy. Other application areas were 'found' only after the discovery of two-photon excitation microscope in Cornell in 1989. The original work from Cornell was based on the use of a mode-locked sub-picosecond laser source – in fact the original thought around the lasers was that microscopy requires the use of these fast lasers. The assumption was that the NIR radiation from the laser source should be kept as low as possible to avoid damages from the laser light absorption to the sensitive biological material [3]. Since the efficiency of two-photon excitation F is dependent on the product of the peak P_{peak} and average power P_{ave} of the light source, the highest two-photon excitation efficiencies with low average power can be produced with these sub-picosecond laser sources.

$$F \propto P_{peak} P_{ave} = \frac{P_{ave}^2}{f_{rep} \tau} = FOM \qquad (1)$$

The terms f_{rep} and τ of Eq. 1 are the repeat rate and the pulse length of the laser, respectively, whereas the FOM defines the figure-of-merit for a two-photon excitation laser.

The mode-locked lasers such as used by the Cornell group are operating typically at high repetition rate around 75 to 100 MHz with pulse length in the range of 70 to 150 femtoseconds. With the average power of 50 mW, focused to the sample, the figure of merit (FOM) will then range from 200 W^2 to 500 W^2. In the mid 90's, it was discovered that the microscopical applications were possible with practically any type of laser equipment operating at a correct wavelength [4,5]. This has lead into a range of new applications including direct two-photon excitation of optically trapped objects by continuous wave (CW) lasers [6,7] and a whole range of new applications in bioanalytics [8,9]. The earlier fear of destroying the sensitive biological sample by direct absorption of the NIR light has also been shown to be exaggerated [10].

The fact remains that an ultrafast high-repetition rate laser is the ultimate choice for applications where the signal yield is of utmost importance. The purpose of this paper, however, is to show that the choice of laser is application specific – in some cases there is only little advantage in using ultrafast sources as compared to low-cost devices. In the following, two-different instrumental set-ups are compared in biomedical application examples. The first example is a bioaffinity assay using microparticles as bioactive carriers and the second example an assay for red blood cell typing.

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2. Bioanalytical applications of two-photon excitation

Since the publication of Denk *et al.*, two-photon excitation microscope has become one of the important workhorses of biomedical research. A bibliography search in ISI Web of SCIENCE® with terms "Two-photon excitation microscopy" and "Multi-photon microscopy" produces more than hundred original publications from various research groups since 1990 up to date – and these are publications whose topic is two- or multi-photon excitation microscopy. Majority of publications represent research results that benefited from the use of two-photon excitation microscopes. In this paper, however, the example assays belong to an application area with potentially a much broader user spectrum than microscopy: bioaffinity assays in fields of in-vitro diagnostics, drug-discovery studies and biomedical research.

One of the main advantages of two-photon excitation is the virtually background free observation of fluorescence from the diffraction limited focal volume. This property stems from the fact that by definition the background of two-photon excitation fluorescence at or near the fluorescence wavelength band can be only originated within the focal volume. In applications pursuing for the ultimate sensitivity of detecting individual fluorescent molecules, two-photon excitation comes in handy: removal of the background caused by immersion liquid, scattering, dense sample matrix etc., enable the use of single molecule detection techniques in applications that would otherwise not be accessible. These basic principles have been used in various publications seeking for this ultimate sensitivity of detection [11,12].

Another useful property of two-photon excitation is the ability of simultaneous excitation of multitude of fluorophores with a single laser-line. In combination with the previously mentioned background free detection, this allows construction of a simple, multiparametric detection system using a single laser. The property has been exploited in detection of biomolecule binding reactions from single molecule levels by use of cross-correlation [13,14] to multiparametric detection of binding reactions from carrier surfaces without separation of the bound and unbound fractions of the labelled "probe" molecule [15].

As compared to detection of molecules or molecule-clusters, the use of carrier particles circumvents two of the main problems of high-sensitivity detection from small volumes. First, overlap of the background and the specific signal can be largely avoided, since the presence of carrier particles in the observation volume can be reliably and independently detected from the specific signal. Secondly, the particles act as local concentrators of the label molecules boosting the signal levels and the operational assay concentration range by orders of magnitude. This principle has been applied in our earlier publications [8,9,15] using low-cost microchip laser and polystyrene microparticles as the bioactive carriers.

The polystyrene carriers may also be replaced by biological objects such as cells or cell-fragments carrying the binding site – or function to be observed. From the instrumentation point of view, the use of bioactive carriers differs drastically from direct observation of binding reactions at single molecule level. Whereas, the single molecule detection requires maximal signal yield and a quasi-continuous illumination source, the approach based on carriers can tolerate a low-repetition-rate light source and a lower signal yield.

3. Instrument set-up

The two lasers to be compared were a Nd:YAG microchip laser (Nanolase NP-17010, Meylan, France) with nominal pulse length of 1 ns, pulse repetition rate of 17 kHz and average power of 70 mW and a mode-locked femtosecond diode pumped Nd:Glass laser (Time-Bandwidth Products GLX-200, Zurich, Switzerland) with pulse length of 140 fs (sech^2), repetition rate of 110 MHz and an average power of 150 mW. The corresponding calculated FOM for the lasers are ~300 and ~1500, respectively. The optical system remained the same for comparisons with both lasers. The fluorescence detection was tuned to around 560 nm emission band of the used label. Analogue lock-in detection electronics of the standard TPX-instrument [8,15] was used for the 17 kHz laser set-up, whereas, direct photon counting electronics was used for the high repetition rate laser set-up. Channel photomultiplier tubes (C952P, Perkin-Elmer Optoelectronics, Wiesbaden, Germany) were used for fluorescence signal detection.

4. Solution measurement

As the first comparison, a solution measurement from 100 nM rhodamine-B (Rh-B) in EtOH was performed. Due to the clear single photon response of the used channel photomultiplier tubes it was straightforward to compare the two systems in absolute photon counts. The Nd:YAG microchip laser system produced ~15 000 cps fluorescence signal from this solution, whereas, the signal produced by the femtosecond system was ~80 000 cps. The ratio of 5.3 between the signals confirms the earlier FOM calculation.

5. Two-photon excitation bioaffinity assays using microparticles as bioactive carriers

In TPX-technology [8,9,15], microparticles of a size around 3 μm are used. These particles are coated with a capture biomolecule to collect specific analyte molecules from the sample – reagent mixture. As a fluorescently labelled secondary capture molecule attaches to another binding site of the analyte molecule forming a "sandwich" – or competes with the analyte molecule for the binding sites of microparticles, the amount of fluorescence from each microparticle becomes representative of the amount of

analyte molecules in the sample. In case of sandwich-type assay, i.e., immunometric assay format, the signal from microparticles is directly proportional to the concentration of the analyte, whereas in the competitive assay format this relationship is the inverse.

The TPX-instrument (Fig. 1) analyses the samples by observation of individual microparticles within the reaction suspension. As an individual microparticle enters the focal volume of two-photon excitation, the signal from the confocal scattering detector (SD) will interrupt the XY-search-scan of particles. At this point, optical forces from the focused laser light will laterally trap and guide the particle axially through the focus (see insert of Fig. 1). Once the particle signal disappears from the scattering detector, the search-scan is re-engaged. The time that a particle spends in the focus is directly proportional to the average power of the laser – in our case the respective maximum focus times were about 50 ms for the modelocked Nd:Glass laser and 100 ms for the Q-switched Nd:YAG. The scanning system enables us detection of up to about 10 particles/second. The signals of individual particles are filtered to remove large variations and then integrated into a single result that is representative of the concentration of the analyte molecules in the sample. The total measurement

time for each sample is determined by the requirement of result precision. For example, a typical case would call for measurement coefficient of variation (CV) better than 5%. This would mean that with particle-to-particle variation of 50%, minimum of 100 particles needs to be measured for 5% CV (improvement in CV is proportional to the square root of the number of measured events).

To compare the different lasers in an actual application we chose to use human thyroid stimulating hormone (hTSH) immunoassay as the model. A full TSH-dose response curve was recorded using both lasers (Fig. 2). Each point of the response curve was measured 60 seconds and repeated three times. For clearer presentation and comparison the 0-control values were subtracted from signals. The 0-control was measured 10 times to be able to predict the lowest limit of detection. In Fig. 2, the 3σ ($3 *$ standard deviation) of the 0-control is drawn for both curves – 3σ or 2σ values of the background are commonly used as "confidence values" in determination of the detection limit. The actual assay format is shown as a sketch in insert within Fig. 2.

In a second step at 50 mIU/l concentration level a set of individual particles were measured for a prolonged period of time to acquire good statistics for individual particle CV calculation. The individual particle CVs for microchip and mode-locked lasers were 35% and 50%, respectively. This difference can be fully accounted for the different observation times of the microparticles and is on the other hand compensated by the fact that with shorter trapping time the number of microparticles becomes higher in a fixed-time measurement.

From the response curves it is apparent that the mode-locked laser exhibits better sensitivity. This, however, is not due to lower signals of the Nd:YAG microchip
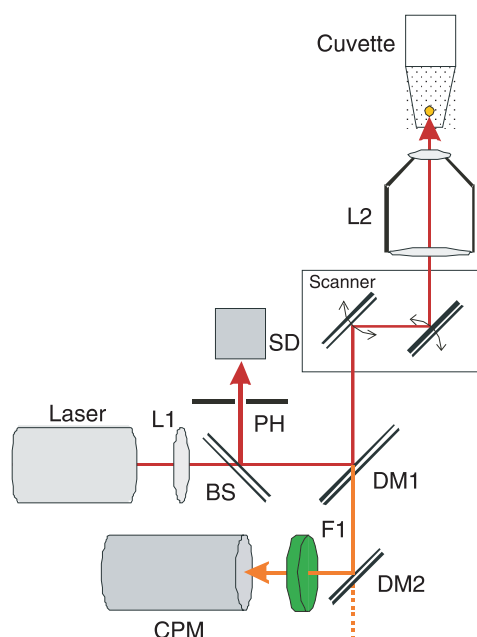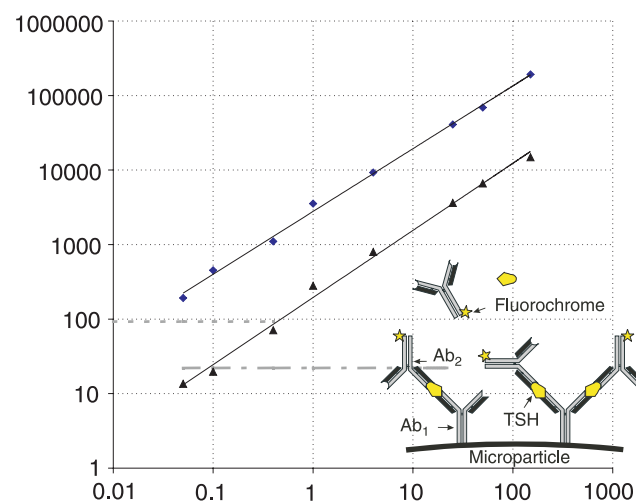

Fig. 1. The experimental setup. The laser (either a mode-locked or Q-switched) is directed via an expansion lens to the optical system. The scanner is used for deflecting the beam in the sample cuvette in order to find the targets within the sample. After the scanner a microscope objective lens (Leica C-Plan 40x 0.65, Leica Microsystems, Bensheim, Germany) is used for focusing the beam and collecting the scattered and fluorescence light. The scattered/reflected light from the target is guided via the confocally arranged pinhole (PH) to the scattering detector (SD). Fluorescence light from the sample is filtered with dichroic mirrors DM1 (transmission 530-700 nm) and DM2 (reflection 530-600 nm) (UAB-Standa, Vilnius, Lithuania) and a filter (F1, BP560-50, UAB-Standa) and directed to the detector (CPM).

Fig. 2. hTSH dose-response curves for the mode-locked laser system (diamond) and Q-swithed system (triangle). The detection limits calculated from the 3σ value of the 0-control are drawn for each curve (dotted, dash-dot). The respective detection limits are 0.02 mIU/l and 0.1 mIU/l for the mode-locked and Q-switched systems.

system. At the detection limit even the low cost laser produces in average 140 cps during microparticles presence in the focus. This signal level projected to the 60 s measuring time and the duty cycle of 15% (85% of time is spent in searching for particles) would suggest less than 3% CV between consequent measurements. However, the measured CV is about 6% indicating that the particle-particle fluctuation is the main cause for variations.

Looking at the signal ratios of microparticles at similar concentration levels, we find that the ratio is 13 in average, in favour of the mode-locked system. Since, this is considerably more than what is expected from the calculation, and what was observed by solution measurements, we can only conclude the reason for the lower detection limit to be the saturation of label molecules that becomes pronounced in microparticle measuring mode. The individual pulses of the microchip laser are extremely intense – too intense. Higher repetition rate at the cost of the peak-power would be preferable – unfortunately such lasers are not on the market yet. One could argue that by reducing the laser power the sensitivity would improve. This, however, is a difficult compromise, since, also the particle trapping forces would be weakened and the lowered signal would start limiting the sensitivity. On the other hand, the achieved sensitivity and dynamic range are already sufficient for most immunoassays as shown in our previous publications [8,9]. More sensitive assay detection may be achieved by using nanoparticle labels and dyes that do not saturate under the intense pulses.

6. Cellular two-photon excitation assays

Microparticles have many positive properties when used in bioaffinity assays. However, microparticles cannot be used in assays where the biomolecules of interest cannot be isolated from their natural environment, or when the actual observation target is a biological system such as a cell. An obvious solution for these cases is the direct observation of the biological object, i.e., the cell itself. One of the areas where two-photon excitation of fluorescence can be used is the identification of cells. In this application the cells are marked with specific labels that attach to the identifying target of the cell – in our example to an antigen at the cell surface. As compared to the microparticles, the direct use of cells adds a different "noise" component to the system: the biological noise. Whereas the microparticles can be produced with very low variation in size and surface capacity, the nature rather creates variation – to protect its existence by enabling quick adaptation to changing environment. Another important difference of cells and microparticles from fluorescence application point of view, is the amount of binding targets: whereas we can concentrate binding targets artificially on the microparticle surface – the amount of targets in a cell can be so low that binding of the label molecules is difficult to detect.

In our example the Rhesus factor determination from a blood sample was performed using both laser systems. The detection of Rhesus factor was based on the use of a labelled monoclonal antibody (Anti-D) against red blood cell surface antigen for the Rhesus factor (see protocol and inserts of Fig. 3). In case of a positive Rhesus factor, an antibody binds to the antigen on the surface of the blood cells, whereas the antigen is missing in Rhesus negative cells. In our example, the interest was not of the subtype of Rhesus factor, but only of its existence. As compared to other blood group antigens, the number of Rhesus factor antigens on cell surface is low – under visual observation using a fluorescence microscope the positive cells differentiated only weakly from the negative cells.

Instrumentally the detection of cells in the focus of two-photon excitation differs from that of detecting microparticles: Cells are considerably larger than the microparticles that were used and the cells have an index of refraction near the surrounding medium. The scattering detectors of the instrument set-ups showed only very weak and ambiguous signals as the cells entered the focal volume. Instead of the scattering coincidence, the samples were analysed by fluorescence burst analysis – detection of fluorescence bursts from the Rhesus positive cells was considered a sufficient proof of principle, since we were only interested in answers without quantity. Due to the lack of reference signal, the measurements were also performed with the xy-scanners fixed to the middle of the scanning field.

Figures 3(a) and 3(b) show typical scans of an Rhesus negative sample respectively with Q-switched and mode-locked laser. Comparison with the Rhesus positive samples of Figs. 3(c) and 3(d) reveals that there is some apparent non-specific binding to negative cells but there is no problem in identifying positive and negative samples – especially with the fast mode-locked laser system. The peak photon count values for the positive cells were about 2 500 cps and 15 000 cps with the Q-switched and mode-locked systems, respectively, yielding a signal ratio of 6. As compared to microparticle measurement we believe that the improved ratio stems from the fact that red blood cells are much larger than the 3 μm particles: the saturation is compensated by the fact that a larger portion of the cell becomes visible.

7. Conclusions

The use of ultrafast mode-locked lasers in applications of two-photon excitation can be avoided by careful planning of the experiments and by the use of low-cost Q-switched microchip lasers. The limits that are imposed by the use of these lasers are mostly related to the available wavelengths and in some cases to the recording speed that can be achieved. However, as we have shown, there are applications where there is only very little advantage in using mode-locked lasers, since signal yield is not always the limiting factor. It is our belief that in the near future two-photon excitation will find numerous new application areas once laboratory instruments utilizing low-cost micro-
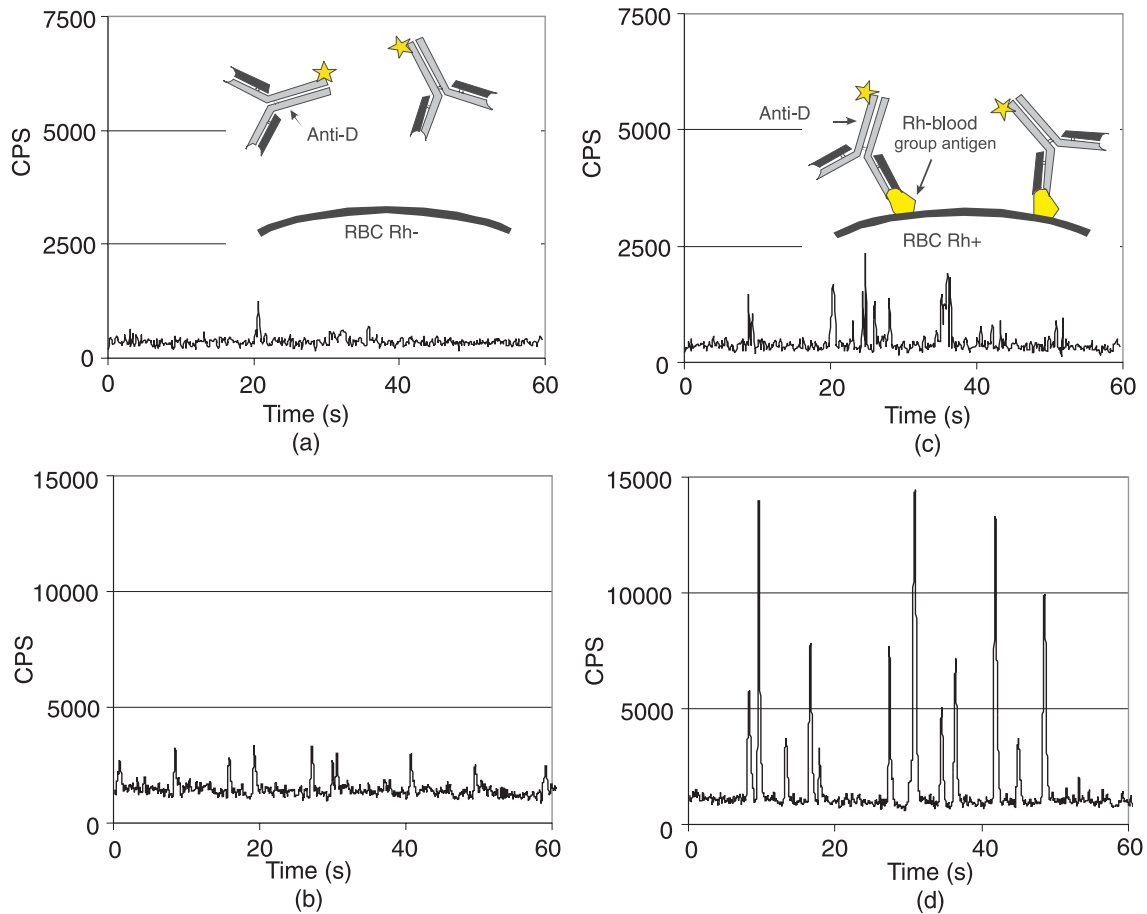
Fig. 3. Determination of the Rhesus factor. The figures a and c have been recorded with the Q-switched system, whereas b and d are the corresponding recordings with the modelocked laser system. The inserts of a and d show the actual assay formats. The Rhesus negative samples (a, b) and positive samples (c, d) reveal the function of the system.

chip lasers become commercially available. We also set high hopes in new developments of near-infrared lasers.

8. Experimental protocols

TSH – immunoassay: The microparticles (3.22 µm carboxy modified, Bangs Laboratories, Fishers, IN) were coated by first passively coating the particles with the antibody (Medix Biochemica, Espoo, Finland, clone 5404) and then generating the covalent bonds by EDC reaction. For each assay, 25 000 microparticles in total assay volume of 20 µl were used. The number of particles/volume unit was checked every time by counting the particles under a microscope in a Bűrker chamber. As a tracer we used succidinimidyl ester of the orange fluorescent dye BF 560.8 (l_{ex} 560 nm, l_{em} 580 nm) (Arctic Diagnostics Oy, Turku, Finland) linked to an antibody against another epitope of the TSH molecule (Medix Biochemica, Espoo, Finland, clone 5409). The tracer concentration was 1.2 nM in the assay. For the dose-response curves the sample, TSH standard was used (Scripps Laboratories, San Diego, CA, catalog No: T0133, 2.4 IU/mg) in final assay concentrations of 0, 0.05, 0.1, 0.4, 1, 4, 25, 50 and 150 mIU/l.

Rhesus group determination assay: 3 ml EDTA--blood from a Rhesus positive (Blood Group B+) donor and from a Rhesus negative (Blood Group O-) donor was centrifuged for 20 minutes (500 G) and RBC-fraction below serum and leucocytes was used without further preparations. B+- and O- -cells were diluted by factor of 400 in ISOTON II -buffer (Beckman Coulter Inc.), 0.01% Tween-20, 0.5% bovine serum albumin, 10 mM NaN_3 and from this dilution 5 µl was pipetted to the cuvettes. The RBC-fractions were estimated to have 4×10^6 cells/µl, so approximately 5 000 RBC/µl was used in the final concentration. As a tracer reagent 5 µl BRAD 3 human IgG3 anti-RhD antibody, (Batch 681L, International Blood Group Reference Laboratory, Bristol, UK) labelled with BF560.8 (Arctic Diagnostics; BF560.8) in 11 nM concentration was used (reaction concentration of 5.5 nM). Reactions were incubated 1.5 h (37°C, 1100 rpm, Comfort Thermomixer, Eppendorf) before measurements.

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