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New Challenges in Biophotonics: Laser-based Fluoroimmuno Analysis and in-vivo Optical Oxygen Monitoring*

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ABSTRACT

Two examples of our biophotonic research utilizing nanoparticles are presented, namely laser-based fluoroimmuno analysis and in-vivo optical oxygen monitoring. Results of the work include significantly enhanced sensitivity of a homogeneous fluorescence immunoassay and markedly improved spatial resolution of oxygen gradients in root nodules of a legume species.

Keywords: Biophotonics, nanoparticles, FRET (Förster Resonance Energy Transfer), immunoassay, luminescence, quantum dots, lanthanides, oxygen, optode.

1. INTRODUCTION

Biophotonics is the exciting marriage of photonics and biology.¹ Biophotonic research is addressing important issues in many disciplines, such as life, medical and pharmaceutical sciences. Key fields of biophotonics include optical diagnostics and therapy, photobiology and biophotonic materials. New frontiers are reached, e. g., with novel laser technology as well as with novel nanoscopic materials, such as bio-labelled nanoparticles. In this paper we demonstrate sensitivity improvements in immunoanalysis by implementing new DPSS laser technology and by utilization of nanoparticles (quantum dots). Moreover, it is shown that self-built micro-optodes can successfully be employed for in-vivo monitoring of molecular oxygen with high spatial resolution.

2. FLUOROIMMUNOANALYSIS

Fluoroimmunoanalysis (FIA)[†] is a good example for a well-proven technology reaching new frontiers in diagnostics. A comprehensive overview of immunoassay principles and practices is given in Ref. 2. FIA overcomes a typical diagnostic challenge by detecting small amounts of, specific proteins in a 'heterogeneous sea' of other proteins, fibres, etc. The unequivocal answer of the assay is ensured by the specific reaction between antibody and antigen (see Figure 1).

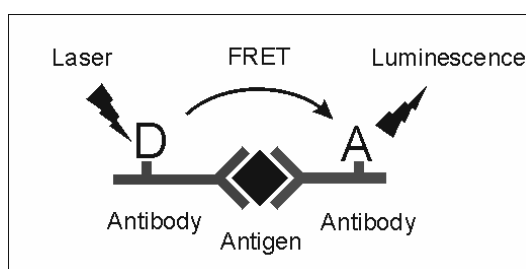


Figure 1 Scheme of Förster Resonance Energy Transfer (FRET). D and A mark the luminophores working as donor and acceptor, respectively.

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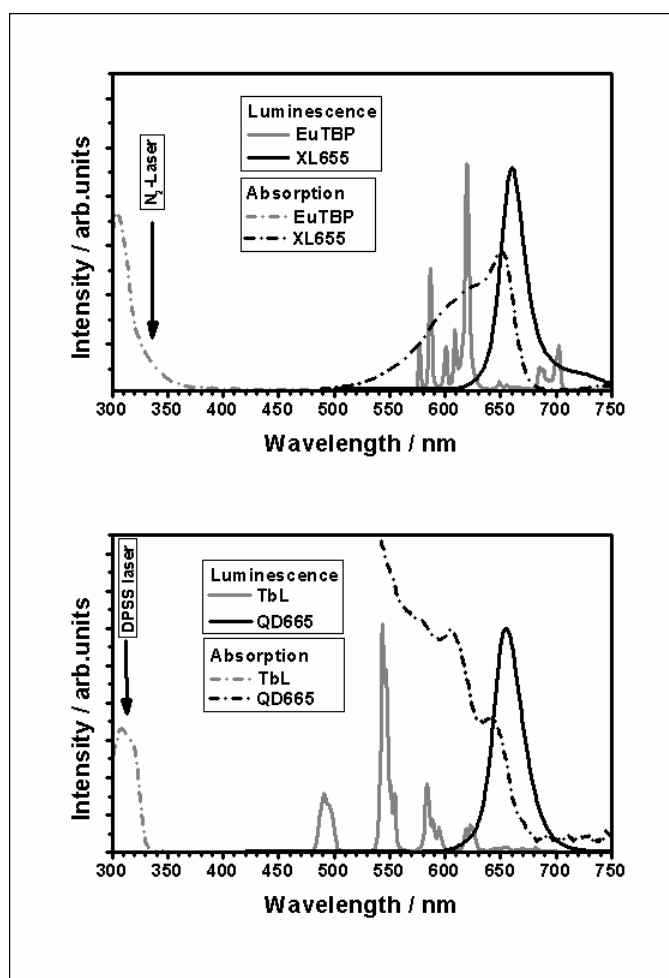
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[†] FIA, referring to *fluorescence* in immunoassays, is commonly used throughout the literature. We prefer the term *luminescence* as it is a more general notion for radiative transitions.

With homogeneous assays it is possible to detect proteins in the picomolar range in blood serum without washing and separating steps. This is achieved by the ‘sandwich’ architecture, with two antibodies bound to the same target antigen. The donor luminophore of one antibody is excited by laser light. Only when both antibodies are bound together via the antigen, energy transfer towards the acceptor luminophore of the second antibody occurs. Thus, the emission of the first luminophore is quenched while the latter is then able to luminesce. This is known as Förster Resonance Energy Transfer (FRET). That way, the acceptor luminescence is a measure for the antigen concentration. Due to spectral and temporal separation of excitation and emission, background signals from the surrounding matrix can be eliminated. This technique is already applied in commercially available systems (such as Kryptor™, Cezanne S.A.S., Nîmes, France). A product technology overview can also be found in Ref. 2.

In an on-going R&D-project (FluoroImmunoAnalysis with miniaturized solid-state LASers, FIA-LAS), the FIA system is improved by implementing new DPSS laser technology and new nanoparticle-based luminophores. Spectroscopic properties of the assays employed are shown in Figure 2. For testing, a simplified scheme as shown in Figure 3 is used.

A)



B)

Figure 2 Spectral scheme of the assays employed. Assays consist A) of EuTBP and XL655 excited by the N₂-laser and B) of TbL and QD665 excited by the frequency-tripled 946 nm Nd:YAG-DPSS-laser emission. The absorption of the donor is dominated by the organic compound, its emission by the lanthanide.

The excitation laser in the commercial Kryptor™ system so far is a N₂-laser which does not match the donor absorption band well (Figure 2). New laser technologies, such as DPSS- and μ chip-lasers, make a more efficient excitation feasible without increasing the size (table-top) of the whole device. The 946 nm emission of a Nd:YAG laser (developed by the FIA-LAS partner LaserLaboratoriumGöttingen e.V., Göttingen, Germany) is frequency-tripled to allow excitation

directly at the maximum the donor absorption band (315 nm). For testing reasons, the here presented results were obtained with the laser emission of a Nd:YAG-OPO system. The energy donor is another crucial part of the FIA system. It comprises a lanthanide ion (Eu^{3+}) as luminophore chelated by the so-called cryptate trisbipyridin (TBP). Due to internal energy transfer from the cryptate to Eu^{3+} , a spectral shift of about 300 nm is achieved between absorption and luminescence. Lanthanide luminescence is characterized by an extraordinarily long decay time (about 1 ms). Part of the R&D project is the replacement of Eu^{3+} by Tb^{3+} and optimization of the chelate. As a substitute, a ligand (abbreviated L) consisting of two 6-carboxybipyridyl units organized on a glutamat framework was chosen.^{3,4} Whereas Tb^{3+} shows better luminescence behaviour, the new ligand seems to provide better internal energy transfer properties. The acceptor XL655 is a modification of the protein allophycoprotein (APC) containing three luminophores.⁵ The overlap with the emission bands of Eu^{3+} is good. No influence of the serum is detectable in the spectrum. XL655 alone shows a luminescence lifetime of some ns. With lanthanides as FRET donors, the XL655 luminescence decay time increases by several orders of magnitude to about 1 ms. This allows the complete suppression of the short-lived assay autofluorescence by temporally gated detection.

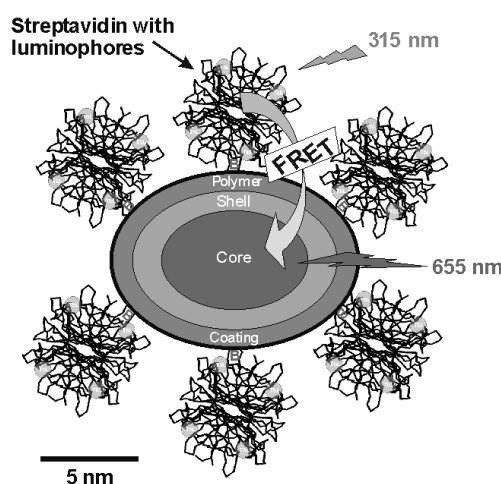


Figure 3 Geometry of the test scheme using nanoparticles (quantum dots). The donors attached to streptavidin are excited by the laser light and transfer the energy to the nanoparticle which is bound to the streptavidin by biotin.

As substitute for XL655, semiconductor nanoparticles (also called quantum dots) were chosen. Due to the confinement effect, these show broad and strong absorption and narrow intense luminescence bands. Comparison of the spectral properties of APC and a specific nanoparticle (QD665; emitting at 665nm; Quantum Dot Corporation, Hayward, California, USA) reveals that the latter shows much broader absorption spectra, resulting in a better overlap with the donor luminescence. Using Tb^{3+} as donor increases the overlap even further. This results in a more intense signal from the small amount of bound nanoparticles. The narrow emission of the nanoparticles can be tailored to desired wavelengths, thus offering the possibility of a multiplexing immunoassay. This will enable the detection of more than one antigen in one assay by using more than one type of antibody labelled with different nanoparticles.

The experiments with the different assays result in high energy transfer rates up to 65% ($\text{EuTBP} \rightarrow \text{XL655}$ and $\text{TbL} \rightarrow \text{QD665}$). The quality of the immunoassay can be defined by the limit of detection (LOD). Whereas the FRET efficiency at 315 nm excitation wavelength does not change much, the LOD of the different assays vary by about two orders of magnitude down to $3.3 \cdot 10^{-12}$ mol/l ($\text{TbL} \rightarrow \text{QD665}$). This is mainly due to the better luminescence of the nanoparticle. Optimization of the excitation wavelength (315 nm vs. 337 nm) yields an additional decrease of LOD by a factor of 3. These results indicate an achievable improvement of about two orders of magnitude for the immunoassay.

Thus, by using new laser sources and novel biophotonic materials, like bio-labelled nanoparticles, better immunoassays can be envisioned which significantly enhance the sensitivity of homogeneous fluorescence immunoassays. This means a further step towards earlier diagnosis with better chances of counteracting diseases.

3. IN-VIVO OPTICAL OXYGEN MONITORING

The concentration of molecular oxygen in living tissue can be determined optically by use of phosphorescent probes.⁶ It is possible to introduce spherical nanoparticles, which contain the oxygen sensitive dye, into cells.^{7,8} In the case of transparent tissue, it is straight-forward to detect the ratio between the signal of the oxygen probe and a reference dye.⁹ For plant cells, such a ratiometric method is not suitable since the cell content often shows absorption and fluorescence in the spectral range of the oxygen sensor phosphorescence signal. However, it has been shown previously that a two-frequency phase modulation technique allows evaluation of the actual sensor signal even in the presence of strong biogenic fluorescence.¹⁰ This technique is based on the fact that the phosphorescence lifetime of the oxygen probe is much longer than the lifetime of the biogenic fluorescence. Figure 4 shows a cell of the green algae *Chara corallina*, in which oxygen sensitive beads are injected by use of a microinjection capillary. This method works perfectly well for intracellular measurement, although injection can be very tedious, especially when cell turgor pressure is very high.

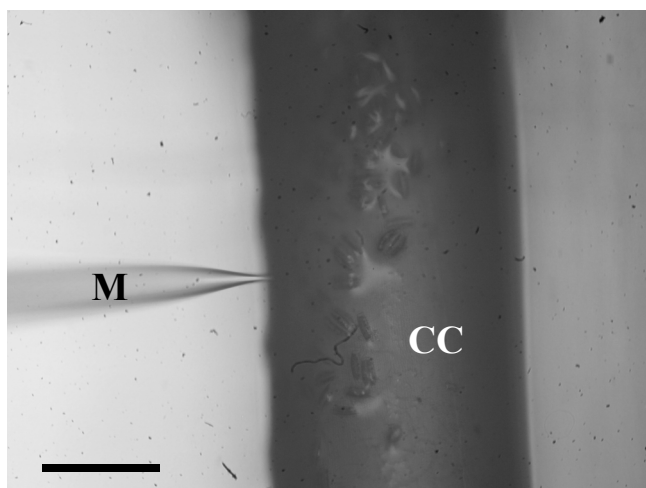


Figure 4 Microinjection of the micro-beads in a *Chara* cell (CC) using a glass micro-capillary (M). The scale bar represents 500 μm .

An alternative approach for in-vivo optical oxygen measurements with a high spatial resolution is the use of miniaturized optodes. An optode for oxygen measurements usually consists of a tapered optical fiber with the tip surface impregnated with a polymer containing the oxygen sensitive dye. To circumvent interferences caused by fluorescent samples, an opaque coating, which is permeable for oxygen, can be added. It is a drawback of this method that it increases the reaction time of the sensor and thickens the fiber tip. The above-mentioned two-frequency phase modulation technique can be easily adopted for fiber optodes and allows the utilization of very thin fibers. By tapering 100/140 μm step index fibers over a gas flame tip diameters of less than 20 μm were produced. Our self-built fiber probes can thus be regarded as micro-optodes with superior spatial resolution. The sensor solution consisted of 30 mg polystyrene, 0.5 mg Pt-tetrapentafluorophenylporphin¹¹ and 450 μl 2-butanone. The optode was obtained by dipping the fiber tip into the viscous solution.¹² After 1 day curing, the sensor was ready to use. Figure 5 shows a self-built fiber probe in comparison with a commercially available optode (Presens, Regensburg, Germany). The fiber diameter of our micro-optode remains extendedly thin, which allows penetration depths in the range of some millimetres without further damage of the tissue around the puncture after injection. Damaging of tissue has to be avoided as much as possible, since it may cause oxygen exchange with the surface. Furthermore, inserting a fiber with a small diameter requires less force which results in easier handling and a reduction of the risk of breaking the optode. To verify the performance of the self-built optodes, the following setup was used: the 400 nm line of a frequency-doubled titan sapphire laser (Tsunami, Spectra Physics) was sinusoidally modulated with 4 and 6 kHz by use of an acousto-optic modulator (60X, HBT-Laser, Schwäbisch Gmünd, Germany). The laser intensity was adjusted with a grey filter to a few milliwatts. The laser light was coupled into the fiber of the optode using a collimator. The phosphorescence signal, which is generated at the fiber tip, is guided back through the fiber and led into the photo-multiplier (Hamamatsu R955, Hamamatsu, Japan) by use of a dichroic mirror. The current of the photomultiplier is converted into voltage by a preamplifier (Femto DLPCA-200, Berlin, Germany). Since the preamplifier bandwidth at the selected gain of 10^6 V/A is limited to 200 kHz, the laser behaves as a quasi continuous-wave source, even though there are actually pulses at a repetition rate of 82 MHz. The respective phase shifts

of the two sinusoidal waves are measured simultaneously with a dual reference lock-in amplifier (EG&G Signal Recovery 7260, Workingham, United Kingdom).

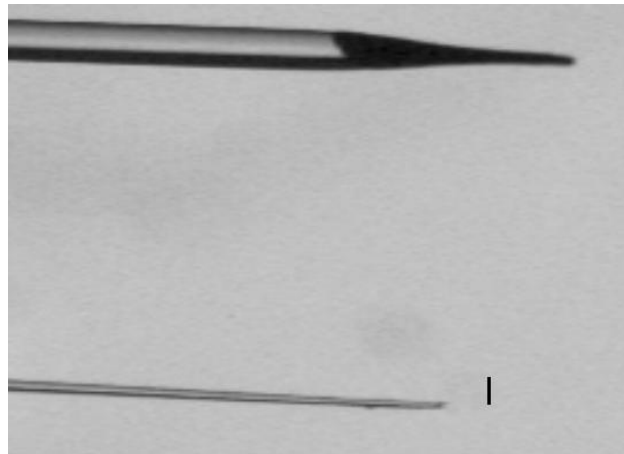


Figure 5 Top: Commercially available oxygen optode (Presens GmbH, Regensburg, Germany). Below: Self-built optode. The vertical black scale bar in front of its tip represents 100 μm .

The performance of the self-built sensors was verified by measuring oxygen in living root nodules of the legume species *Lotus japonicus*. These specialized plant organs of about 1 mm diameter contain nitrogen-fixing rhizobia that are able to fix and metabolise inorganic nitrogen into organic nitrogen compounds that can be used by the plant. This reaction is catalysed by an enzyme, nitrogenase, which is inactivated in the presence of oxygen. Therefore, the nodules contain high amounts of the protein leghemoglobin, which strongly reduces the concentration of free molecular oxygen within the tissue. This function of leghemoglobins has been validated by measuring oxygen contents within nodules of a wild-type plant as well as within nodules of a genetically modified line in which no leghemoglobin protein was detectable: wild-type nodules show a steeper oxygen gradient than the leghemoglobin-free mutant.¹⁵ Figure 6 outlines a comparison of the results obtained with the thin self-built micro-optode (diamonds) and of the previous measurements with the commercial optode (circles). The slopes obtained with the self-built optode are clearly steeper. There is an abrupt oxygen decrease around 0.1 relative penetration depth for both wild-type- and leghemoglobin-free mutant. This behaviour results

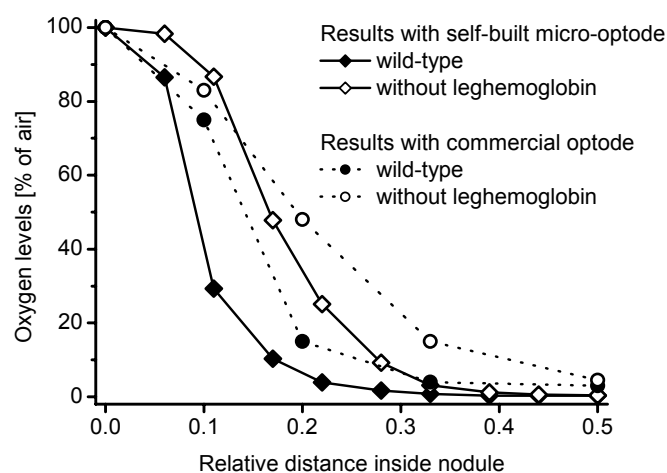


Figure 6 Oxygen gradients in root nodules of the legume species *Lotus japonicus*. 0.0 relative distance indicates the surface, 0.5 the center of the nodule.

from a permeability barrier located directly below the surface of the nodule. This layer restricts oxygen diffusion into the tissue.¹⁴ This characteristic phenomenon is barely recognizable in the measurements with the commercial optode's lower spatial resolution. Furthermore, the oxygen values measured in the centre with the self-built optode are found to be below 1 % of air content. This is lower than the 5 % of air content measured with the commercial optode. The thin optode seems to effectively prevent a permeation of oxygen from the surface through the penetration hole. We are confident that further miniaturization is possible and that more accurate measurements with an enhanced resolution, even on a cellular level, will be practicable soon.

4. ACKNOWLEDGEMENT

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