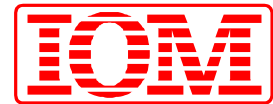


# high precision analysis of oxygen-sensitive microplates by microsecond lifetime analysis



Lutz Pfeifer, IOM Innovative Optische Messtechnik GmbH

Application Note No. 9

## 1. Introduction

Oxygen-sensitive microplates are used for the analysis of cellular assays in primary and secondary screening levels of drug development where kinetic information about proliferation or viability of cells in dependency of e.g. cytotoxic or antibiotic influences are required. The consumption of oxygen by cells influences the concentration of oxygen dissolved in the liquid media which can be measured using fluorescence techniques. Presently two suppliers of oxygen plates are known, BD Biosciences (USA) and Presens GmbH (Germany).



The basis of this oxygen biosensor is a fluorescent dye embedded in a gas permeable matrix placed at the bottom of all cavities of a microplate. The fluorescence signal of the fluorescence compound (Ruthenium-complex) is more or less quenched by oxygen molecules depending on the oxygen concentration. The physical law describing this behaviour is the well-known Stern-Volmer equation:

$$F_0/F = 1 + k_D[Q].$$

Here,  $F_0$  and  $F$  are the fluorescence intensities in the absence and presence of the quencher,  $k_D$  is the Stern-Volmer quenching constant and  $[Q]$  is the concentration of the quencher.

Usually according to the Stern-Volmer equation the fluorescence intensity of the sample with an unknown quencher concentration is measured and the concentration is recalculated according to a calibration procedure. This procedure requires in the special case of sensitive plates a highly precise production process of the plate with constant amounts of the sensitive dye in each cavity in order to make different cavities comparable.

Furthermore, it requires the absence of other influences which may disturb the measurement of the fluorescence intensity. Such influences could result from varying filling heights in the cavities, light scattering due to cells or coloured media.

**IOM** presents a different approach to a precise measurement of the fluorescence signal of the sensitive dye – the measurement of its **fluorescence lifetime**. This is possible since in the case of dynamic quenching like with oxygen the following formula holds:

$$\tau_0/\tau = F_0/F$$

Here,  $\tau_0$  and  $\tau$  are the fluorescence lifetimes of the dye in the absence and presence of the quencher. The fluorescence lifetime as a physical parameter is not dependent on influences leading to wrong intensity values. In a wide range it is not concentration dependent, insensitive to stray light or geometric-optical disturbances like refraction at meniscus on the surface of the liquid sample.

## Aim

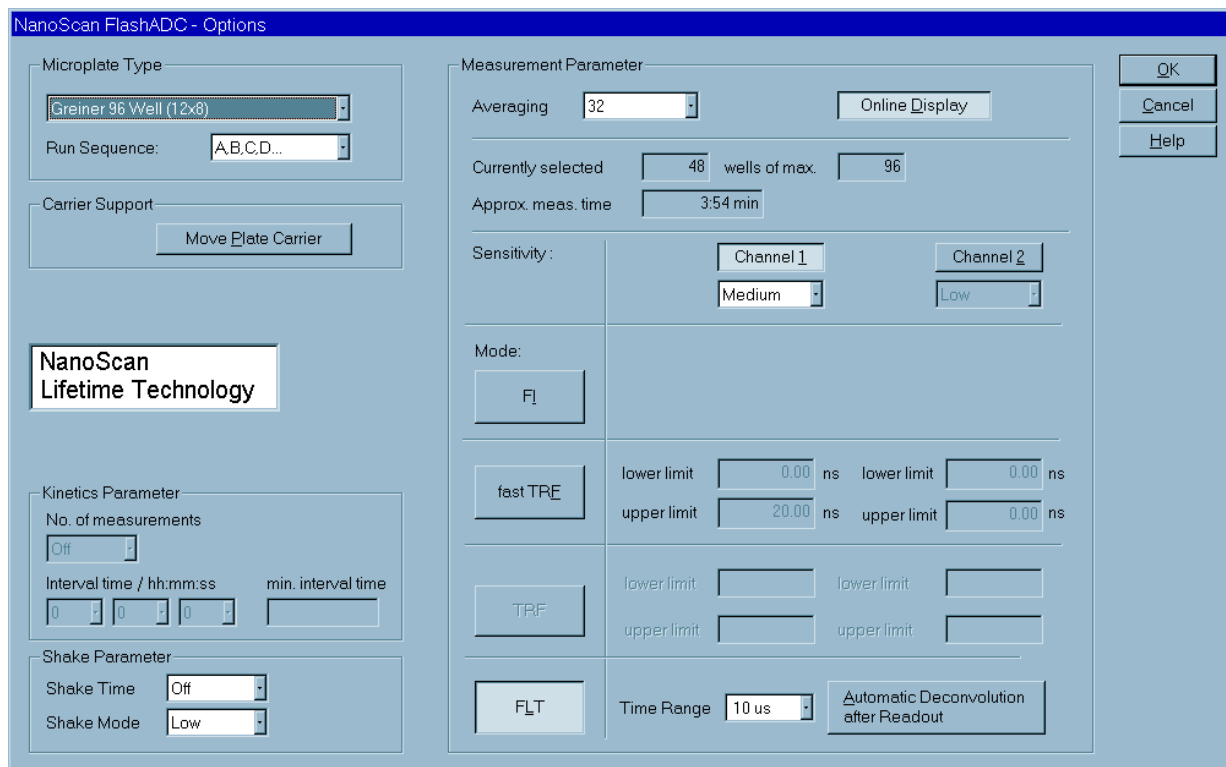
The aim of this paper is to demonstrate that lifetime-resolved measurements yield more exact oxygen concentration results than the conventional intensity measurements.

## 2. Method

Measurements have been performed with the BD Oxygen Biosensor System as comparative measurements between a conventional fluorescence intensity reader and the **LF 401 NanoScan FLT**.

Groups of 16 wells with 0.7% oxygen concentration or 96% oxygen concentration respectively were measured. The wells were filled with 250 µl sample solution each. The conventional reader was used according to instructions published in the internet (BD Biosciences).

To produce “zero oxygen solution” (0% oxygen concentration) 1 g of sodium sulfite was dissolved in 100 ml distilled water. To produce a high oxygen concentration fresh de-ionised water was shaken powerfully in a screw bottle for 2 minutes. The oxygen concentration was checked with an OXI 340 (WTW, Germany).

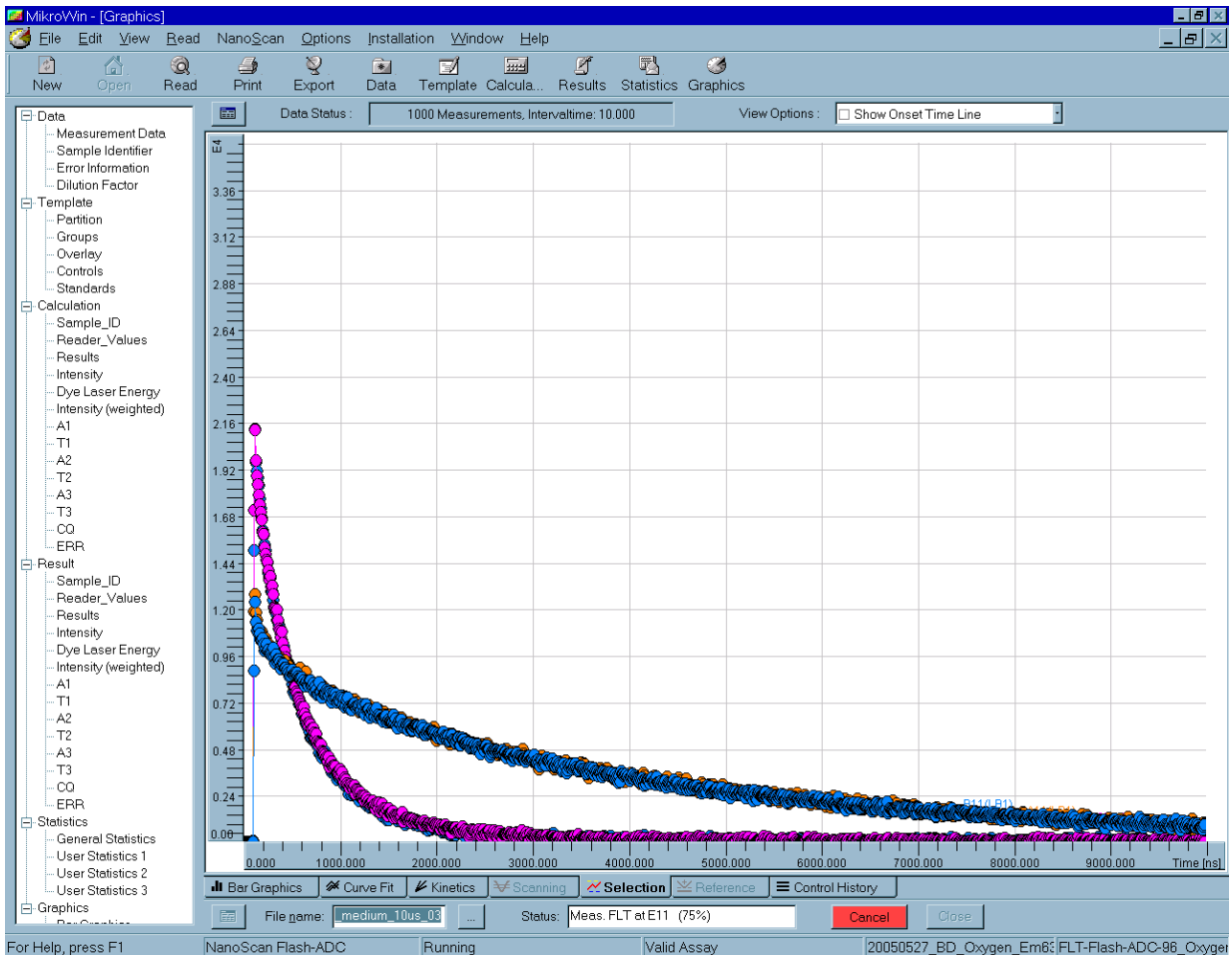


The instrument set-up occurred as indicated in the following table I:

	Intensity reader	LF 401 Nanoscan FLT
Excitation wavelength	488 nm	456 nm
Emission wavelength	620 nm	630 nm
Integration time	500 µs	10 µs (for intensity results)
time-window for lifetime measurement	-	10 µs
Number of flashes	16	16

## Results

Typical decay curves of the detected fluorescence traces are shown in the next picture as a “screen shot” of the Mikrowin 2000 reader software. In this example for both air-saturated and oxygen-free solutions two fluorescence decay curves of the biosensor membranes on the bottom of the microplate are shown.



The time axis of this plot spans 10  $\mu$ s. The fast decaying signals are resulting from wells with air-saturated water whereas the slow decaying signals result from wells with 0 oxygen concentration.

For analysis of fluorescence intensities the measured decay curves are integrated. In order to obtain the fluorescence lifetime values the decay curves are fitted with a single-exponential decay law using an automatic curve fitting module inherent to the MikroWin 2000 software package. The results are summarised in the following table II:

	signal intensity with 100 % oxygen	signal intensity with 0 % oxygen	fluorescence lifetime with 100 % oxygen	fluorescence lifetime with 0 % oxygen
<b>Intensity reader</b>	6366 $\pm$ 262 rfu cv = 4,1 %	32792 $\pm$ 1450 rfu cv = 4,4 %	-	-
<b>LF 401 NanoScan FLT</b>	11051827 $\pm$ 1230785 rfu cv = 11,1 %	33473818 $\pm$ 2098954 rfu cv = 6,3 %	548,3 $\pm$ 8,7 ns cv = 1,6 %	3943,5 $\pm$ 57,7 ns cv = 1,5 %

The red numbers indicate the normalised standard deviations of the signals. The precision of the lifetime values with the **LF 401 Nanoscan FLT** in this example is higher than that of the intensity values. It should be expected that in cases where circumstances like scattering media or variations in medium volume have an influence on the intensity measurement this higher data quality of the oxygen measurement by fluorescence lifetime should be even stronger. Since the fluorescence lifetime parameter is a photo-physical characteristics of the Ruthenium complex in the Biosensor matrix it is not dependent on the actual amount of this indicator dye immobilized in each well. Thus, no calibration is required.

## Conclusion

**The measurement of the fluorescence lifetime for the analysis of oxygen-sensitive microplates is a more robust means of analysing the fluorescence signal, provides more precise oxygen values, eliminates the need for calibration and therefore will lead to an improved assay quality.**