Comparison of Fluorescence Lifetime and Fluorescence Intensity Readouts using a Homogeneous Protease-assay

CyBio

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Introduction

The majority of fluorimetric assays uses fluorescence intensity as readout parameter which can be determined either from prompt intensity measurements (FI) or temporally delayed measurements (TRF). These methods as well as others depending on intensity information (e.g. FRET) can suffer from several side-effects, which affect the readout itself, namely stray light, scattering effects, turbidity and the auto-fluorescence of probes.

Measuring the decay time of a fluorescence signal can overcome these secondary effects, since this parameter distinctly displays a fluorophore in its direct molecular surrounding – comparable with a fingerprint. In this work we compare two measurement parameters, the fluorescence intensity (FI) and fluorescence lifetime (FLT), which could be acquired efficiently in a multi parameter readout from one and the same measurement.

The biochemical reaction being investigated consists of a fluorescent labelled enzyme-substrate (casein as a biopolymer carrying BODIPY FL), which is cleaved by the protease α-chymotrypsin. In the beginning of the assay the fluorescence signal is internally quenched as a result of energy transfer interactions among the BODIPY labels. The continuous fragmentation of the biopolymer backbone in consequence of enzymatic degradation leads to a rise of the fluorescence signal while the quenching effects decrease. In the assay this is expressed in an increase of both, intensity and lifetime.

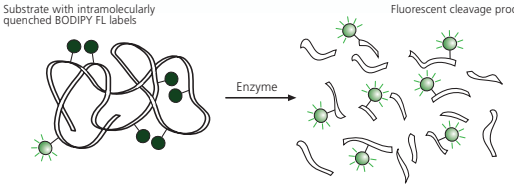


Figure 1:
Assay-scheme of the enzymatic degradation of BODIPY-FL-labelled casein (from Molecular Probes, Inc).

Experimental

Materials:
These studies were performed with a commercially available protease testing kit (Molecular Probes: EnzChek® Protease Assay Kit green, E-6638) containing BODIPY-FL labelled casein and a Tris-HCl digestion buffer. α-Chymotrypsin was purchased from Sigma (C-4129) and Pefabloc® SC from Fluka (76307). All measurements were done in Greiner 96 well microplates (standard medium binding, 781076).

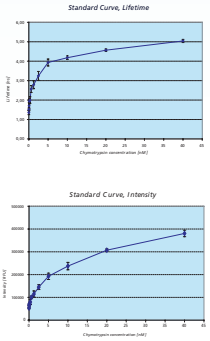
Instrumentation:
All experiments were measured on a CyBi®-NanoScan HT fluorescence plate reader, capable of analysing steady-state and time-resolved fluorescence. For excitation a dye-laser module emitting at 488nm was used. The fluorescence was detected at 535/30nm - in our experiments we used a minimum of 16 excitation flashes per well. The reader was controlled by Mikrotek's MikroWin 2000 software including plugins made by IOM GmbH (Berlin, Germany) for addressing the device and deconvolution of fluorescence curves. For kinetic measurements we used CyBio® Control Software. For nonlinear curve-fitting of the resulting IC_{50}-Curves we used Graphpad Prism® 4. Pipetting was performed on a CyBi®-Well.

Experiments:
The assay instructions suggest to work with concentrations of 10µg/mL (ca. 400nM) BODIPY-casein substrate, which in initial experiments turned out to be too high. If not stated different we used a working concentration of 1µg/mL (ca. 40nM). All other assay conditions were kept as specified in the instructions.

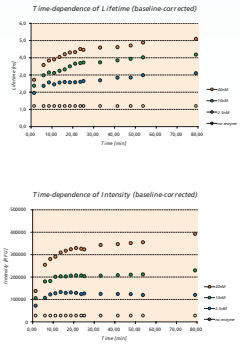
1.) Standard curves:
The standard curves for chymotrypsin were generated by incubation of variable enzyme-concentrations with the substrate for 1h. The respective fluorescence signal was plotted vs. protease activity.

2.) Enzymatic turnover:
Kinetic measurements were performed by monitoring the development of the fluorescence signal immediately after enzyme was pipetted to the labelled biopolymer solution. Several enzyme concentrations were investigated.

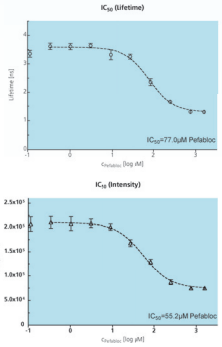
3.) The inhibitory effect of Pefabloc® SC on the enzymatic cleavage was analysed by preparing a serial dilution of Pefabloc SC in the concentration range as proposed in the instruction leaflet. The inhibitor was incubated with chymotrypsin for at least 30min prior to the start of the reaction. After an incubation time of 1h the fluorescence signal was plotted vs. inhibitor concentration.



The graphs display standard-curves for both, fluorescence lifetime and fluorescence intensity, measured after an incubation time of 1h. The curves show the progression of the respective fluorescence signal depending on various enzyme concentrations within the specified reaction time.



In order to monitor the temporal development of both, the fluorescence lifetime and intensity signal, measurements were performed in predefined intervals immediately after starting the reaction. During the first 30 min. all measurements were executed consecutively – leading to an interval of ca. 2.5 min. Later the time intervals were extended to 5 or 15 min. respectively.



The graphs display a dose-response curve of serial dilution of the protease inhibitor Pefabloc® SC. The particular inhibitor concentrations were mixed with the enzyme and incubated for 30 min. before the addition to the biopolymer-substrate solution.

Chymotrypsin Concentration [nM]	Z'-Factor Lifetime [ns]	Z'-Factor Intensity [RFU]
40	0.85	0.84
20	0.85	0.82
10	0.79	0.65

Z'-Factors characterize the actual screening window as a quality parameter for HTS-assays. These coefficients were calculated after the incubation time proposed by the supplier of this protease assay for both, fluorescence lifetime and fluorescence intensity.

Conclusions:

Fluorescence lifetime measurements were successfully applied to the investigated protease assay, which is originally intended to be analysed with fluorescence intensity measurements. Both, lifetime and intensity experiments, could be performed with only 10% of the proposed amount of labelled casein substrate, when the CyBi®-NanoScan HT was used as the reader and we yielded results suitable for HTS, as can be concluded from the Z'-Factors, which were above 0.5.
Compared to the intensity readout we found the lifetime-parameter to be more sensitive regarding to small changes of enzyme activity, which is displayed in the slope of both dose-respose-curves (standard curve for enzymatic dose-response and IC_{50} curve of the inhibitor).
If the robustness with respect to side-effect is taken into consideration, fluorescence lifetime ***per se*** is a qualified alternative to fluorescence intensity in this bioassay.
Additionally lifetime measurements always contain the intensity data, which gives easy access to a valuable multi parameter readout.

- » Fluorescence Lifetime is a robust measurement parameter.
- » Fluorescence Lifetime was successfully applied to an Intensity-Assay.
- » Good results were achieved with only 10% of proposed substrate-concentration.
- » Lifetime is more sensitive to small changes in enzyme-activity
- » Z'-Factors > 0.5
- » Easy multi parameter readout with CyBi®-NanoScan HT