Fluorescence polarization assays with the EnVision.

Introduction

Fluorescence polarization is a homogenous technology, which has proved to be a powerful tool in the study of molecular interactions. The technology is based on molecular movement and rotation. Fluorescent molecules are excited with polarized light. If the molecules are large they rotate little during the excited state interval. The collected emission light is parallel with the excitation angle. However, if the excited molecule is small, it rotates out of the polarized plane during the excited state. The collected emission signal is then in a different plane from that of the initial excitation. The emission signal is called depolarized.

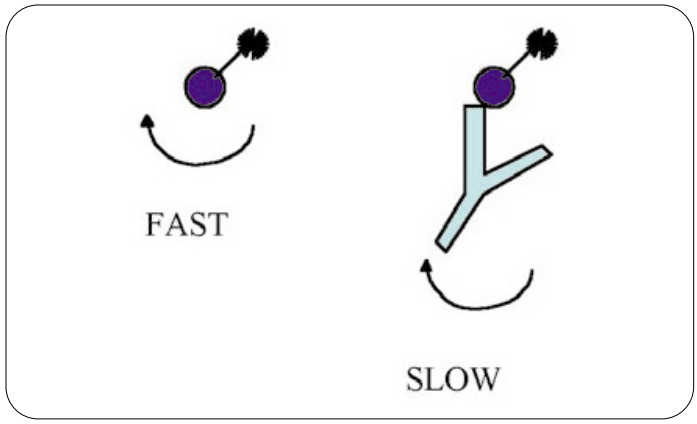
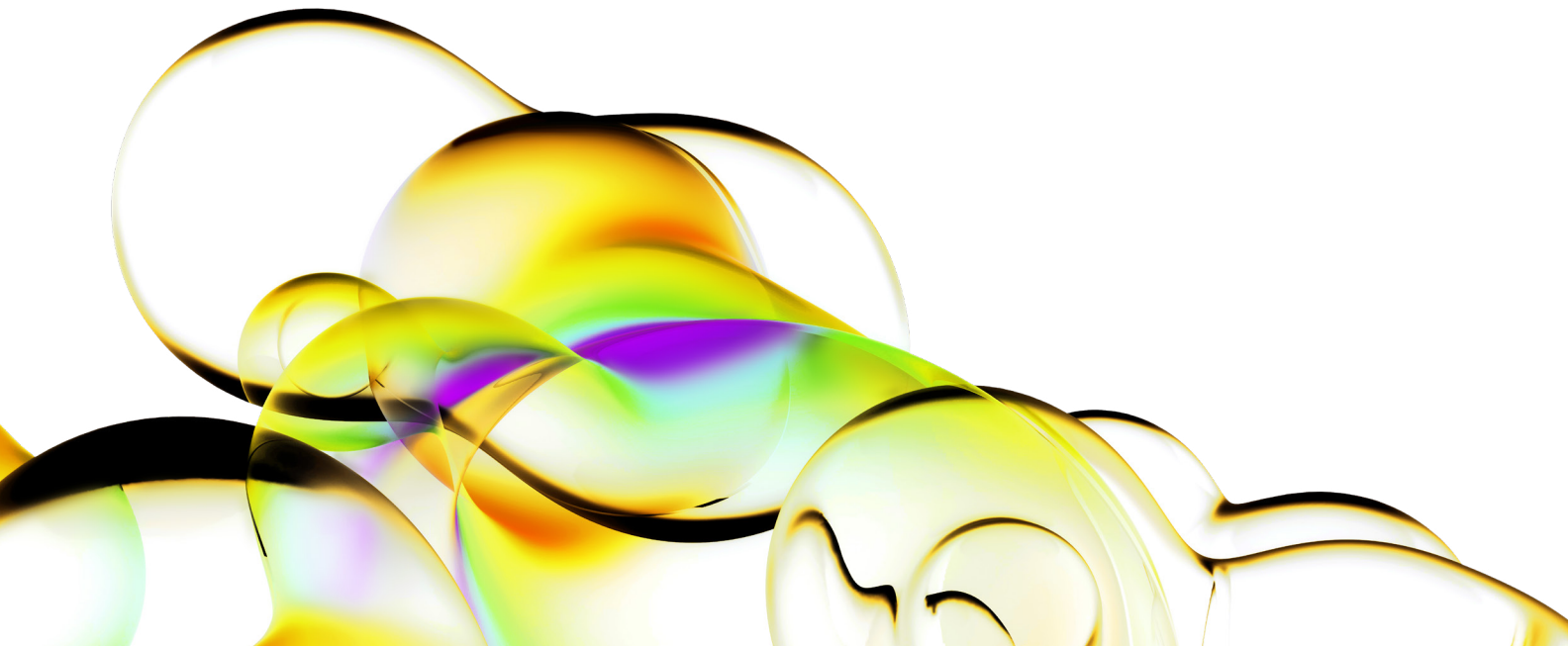


Figure 1: Small molecules rotate fast and change the plane of light during the excitation. Large molecules move slowly and the plane of light does not change during the excitation.



To detect fluorescence polarization two readings are needed: one using a polarized emission filter parallel to the excitation filter (S-plane) and the second with a polarized emission filter perpendicular to the excitation filter (P-plane). These readings can be obtained simultaneously with a suitable dual detector instrument. The fluorescence polarization response unit is normally mP (milli-polarization) level, which is obtained from the equation

Polarization (mP)=1000*(S-G*P)/(S+G*P),

where S and P are background subtracted fluorescence count rates and G (grating) is an instrument and assay dependent factor.

The emission filter transmissions may vary within and between instruments. This causes some bias in the polarization values. The bias is also assay dependent. To correct this bias, the G-factor is calculated from results obtained from pure fluorophore solution.

G=(S/P)*(1-L/1000)/(1+L/1000)

where S and P are background subtracted fluorescence count rates and L is the literature value for the fluorophore (mP). The literature value for free 1 nM fluorescein, RT, is 27 mP.

EnVision in fluorescence polarization applications

The EnVision™ multimode plate reader is an instrument with modular design, supporting all of the following five technologies: photometry, luminometry, time-resolved fluorometry, fluorescence polarization and fluorescence intensity. In fluorescence polarization applications you may choose between sequential single and simultaneous dual measurements. In the former measurement mode S and P channel signals are measured one after the other, while in the latter measurement mode the S and P channel signals are measured simultaneously to save time.

To optimize the response level, prepare a 1 nmol/L fluorescein solution that should give a mP value of 27 at RT. With a single detector system, the G-factor can be used to adjust the mP level (normally between 0.8 and 1.2). Where the instrument uses two detectors and dual optical module, the G-factor can be kept as 1 and the gain settings can be adjusted to obtain 27 mP (for example gain 925 for detector 1 and 1000 for detector 2.)

In EnVision fluorescence polarization measurements the light is guided through the excitation filter and the optical module to the sample. The optical module includes the polarizer for the excitation light. The emission signal is guided through the optical module and the emission filters to the photomultiplier tube -detector. In the emission light path the filters include the polarizers. The EnVision software supports the automatic background correction. You may select whether you want to have mP values or background corrected mP values, which requires background samples on the plate map.

Methods

To evaluate the performance of the EnVision for fluorescence polarization, a fluorescein dilution series (Cat# C557-100 from Revvity) was studied. Samples were pipetted into a 384-well plate using volumes of 50 µL/well. Standard deviation for the whole plate using 1 nM fluorophore was plotted against measurement time/plate. The fluorophore was diluted into polarization Buffer. Also the MC5 Receptor Binding FP-assay (Revvity Cat# FPA104) was measured with EnVision.

Samples were measured using ex. 480 nm

(Cat# 2100-5150) and FP FITC dual optical module (Cat# 2100-4070) for fluorescein measurements and ex. 531 nm (Cat# 2100-5050), em. 595 nm S-channel (Cat# 2100- 5160), em. 595 nm P-channel (Cat# 2100-5170) and BODIPY TMR FP dual optical module (Cat# 2100-4080) for BODIPY-TMR measurements. Sample volumes 200 µL/96-well plate, 50 µL/384-well plate and 5 µL/1536-well plate.

Plates that were used during the studies:

- 96-well plate Black Cliniplate Labsystems, Cat# 9502817
- 384-well plate Black Costar, Cat# 3710
- 1536-well plate Black Greiner, Cat# 782076

Labels — FITC FP

Fluorescence Polarization - FITC FP

Name	FITC FP
Mirror	FITC FP Dual - Slot 4
Excitation filter	FITC FP 480 - Slot 5
Emission filter	FITC FP P-pol 535 - Slot 2
2nd emission filter	FITC FP S-pol 535 - Slot 1
Detector gain	950
2nd detector gain	1000
G - factor	1
Number of flashes	100
Number of flashes per A/D conversion	1
Measurement done on-the-fly	No
Reference signal	506649
AD gain	1
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Figure 2: Label properties for fluorescein FP measurement. For BODIPY measurement BODIPY filters and optical module were used. 27 mP for 1 nmol/L solution was adjusted by changing the gain settings. Measurement height 9 mm.

Results

Fluorescein

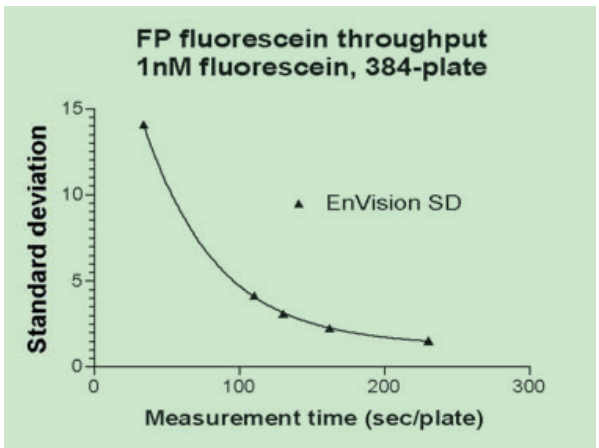

Figure 3: Standard deviation plotted against measurement time (sec/plate).

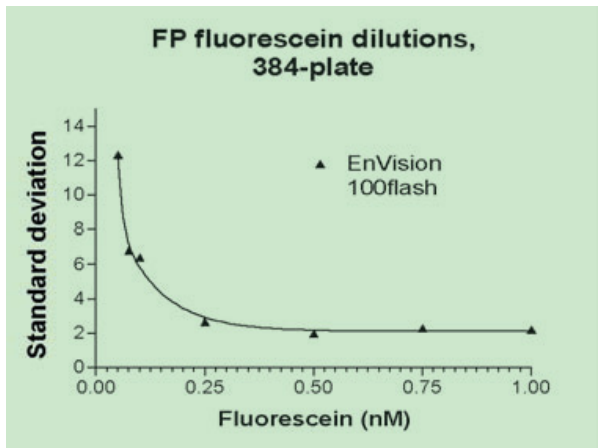

Figure 4: Standard deviation plotted against fluorescein concentration (nM), measurement time / plate 2 min 43 s, 100 flashes.

Table 1: Results for the uniformity plate. 1 nM Fluorescein solution was pipetted into wells of a 384-well plate (50 µL/well) and standard deviation across the plate was analyzed using two different flash amounts.

flash	time/plate	SD (mP)
25	1 min 37 s	4.7
100	2 min 43 s	2.8

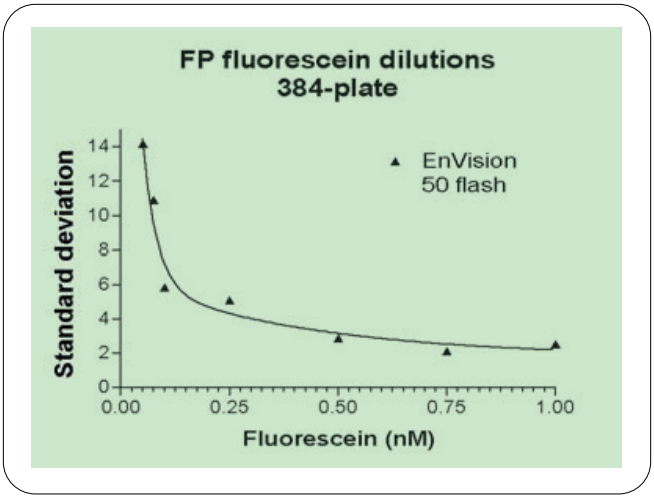

Figure 5: Standard deviation plotted against fluorescein concentration (nM), measurement time/plate 1 min 56 s, 50 flashes.

Results

BODIPY-TMR

MC5 Receptor binding assay in a 96-well plate format

Table 2: The effect of measurement time on Z′-factor in fluorescence polarization MC5 receptor binding assay in a 96-well plate format, of=on the fly.

Read time/plate	Flashes	Z′	Average SD
1 min 34 s	200	0.81	3
1 min 21 s	150	0.67	6
1 min 7 s	100	0.68	6
55 s	50	0.75	5
48 s	25	0.53	9
33 s	1, of	0.14	14

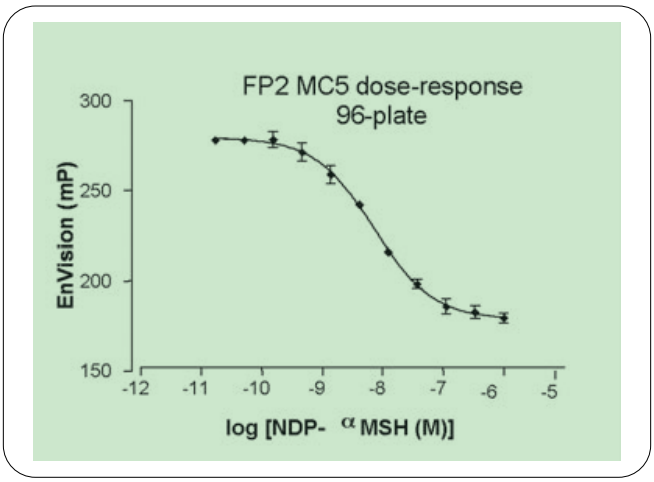

Figure 6: The displacement curve of BODIPY-TMR labelled NDP-alphaMSH from hMC5 GPCR membranes using native ligand in a 96-well plate format.

MC5 Receptor binding assay in a 384-well plate format

Table 3: The effect of measurement time on Z′-factor in fluorescence polarization MC5 receptor binding assay in a 384-well plate format, of=on the fly.

Read time/plate	Flashes	Z′	Average SD
4 min 26 s	200	0.82	5
3 min 26 s	150	0.78	5
2 min 43 s	100	0.73	6
1 min 56 s	50	0.70	8
1 min 37 s	25	0.62	10
31 s	1, of	0.48	34

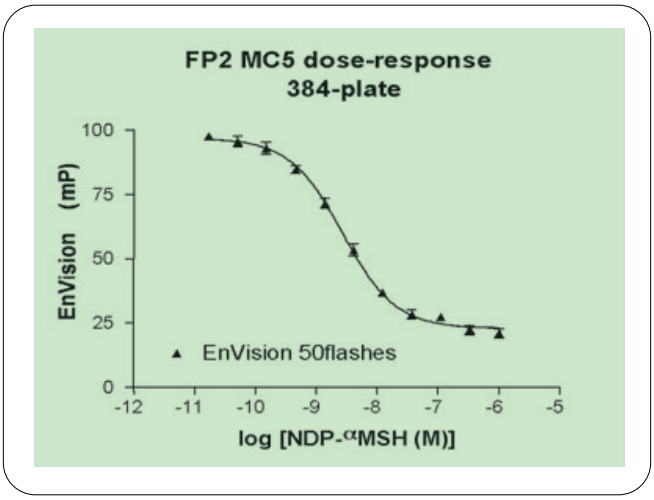

Figure 7: The displacement curve of BODIPY-TMR labelled NDP-alphaMSH from hMC5 GPCR membranes using native ligand in a 384-well plate format, measurement time / plate 1 minutes 56 seconds.

MC5 Receptor binding assay in a 1536-well plate format

Table 4: The effect of measurement time on Z'-factor in fluorescence polarization MC5 receptor binding assay in a 1536-well plate format.

Read time/plate	Flashes	Z'	Average SD
16 min 7 s	200	0.62	6
12 min 49 s	150	0.49	8
9 min 32 s	100	0.60	7
6 min 14 s	50	0.33	11
4 min 33 s	25	0.20	13

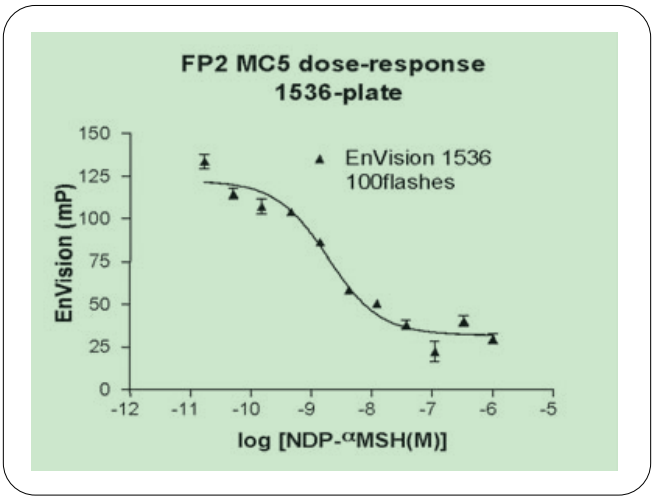

Figure 8: The displacement curve of BODIPY-TMR labelled NDP-alphaMSH from hMC5 GPCR membranes using native ligand in a 1536-well plate format, 100 flashes, measurement time / plate 9 minutes 32 seconds.