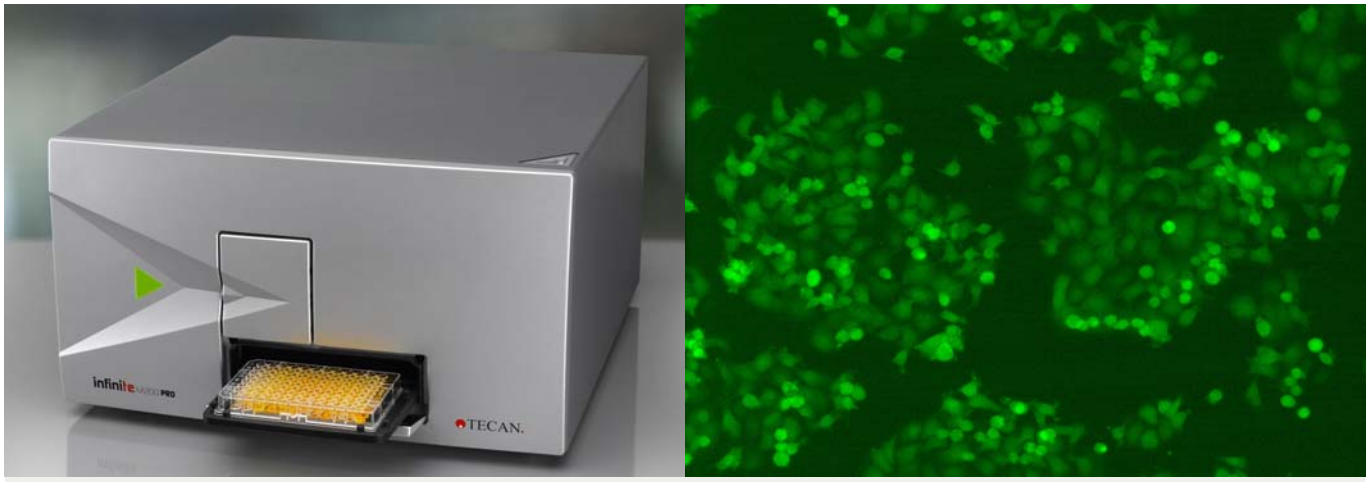


Fluorescence Bottom Reading for Cell-Based Assays

Improved Detection of Green Fluorescent Protein (GFP) in the Infinite® 200 PRO

Introduction

Cell-based applications are central to life science research. They may range from cytotoxicity, proliferation, apoptosis and GPCR signaling assays to high-throughput screening (HTS) drug discovery applications.
Adherent cell types are typically analyzed through the well bottom in order to bring the cell monolayer as close as possible to the detector and avoid unspecific fluorescence noise by the overlying growth medium. For this reason it is essential to guarantee illumination and reading of the entire well bottom, since cells are not always homogeneously distributed over the growth surface.

Green fluorescent protein (GFP)
Fluorescent proteins are well-established tools for non-invasive and real-time studies in living cells. Green fluorescent protein (GFP) is a spontaneously fluorescent protein isolated from coelenterates, such as the Pacific jellyfish (*Aequorea Victoria*) or from the sea pansy (*Renilla reniformis*).
Its role is to transduce the blue chemiluminescence of aequorin, into green fluorescent light by energy transfer.

The molecular cloning of GFP cDNA and the expression of GFP as a functional transgene have opened profitable avenues of investigation in cell, developmental and molecular biology, and have ultimately earned its discoverers Osamu Shimomura, Martin Chalfie and Roger Y. Tsien the Nobel Prize for chemistry in 2008.
Bottom reading of GFP-expressing cells, which are widely used in cellular and molecular biology, ensures maximum sensitivity and allows that plates with cover are measured to prevent compromising sterility.

The aim of the present application note is to assess the performance of the improved, fluorescence bottom fiber of the new Infinite 200 PRO in the detection of GFP-transfected cells. Measurement characteristics like well-to-well uniformity and reading reproducibility were investigated.
In addition, the new “Optimal Read” (OR) function was tested in combination with cell-based GFP measurements.

Material & Methods

Cells and cell culture

Human squamous epithelial carcinoma cells (A431, ATCC No. 1555) stably transfected with enhanced green fluorescent protein (EGFP) were grown to confluence in DMEM high-glucose (*Sigma*) supplemented with L-glutamine, sodium pyruvat, penicillin/streptomycin, HEPES and 5% heat-inactivated fetal calf serum (FCS, *PAA Laboratories*) at 37°C and 5% CO_2 in a humidified atmosphere.
The cells were harvested out of their growth flasks using trypsin/EDTA, supplied with fresh growth medium and seeded into black 96-well tissue culture plates with transparent bottom (Greiner) according to the cell density plate layout below (figure 1).

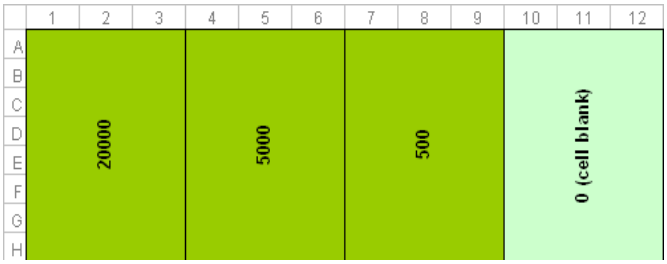


Figure 1: Plate layout used for reproducibility measurements.

The difference to the cell number displayed in the plate layout was normalized with non-transfected A431 cells to obtain a total number of 20000 cells in each well. Correspondingly, columns 1-3 contained only EGFP-transfected cells while columns 10-12 consisted of non-transfected cells only.
This mixture of cells was prepared to create realistic conditions that are similar to cell environment found in transfection experiments where, depending on the transfection efficiency, only a certain fraction of cells contain the fluorophore. The cells were allowed to adhere over night at 37°C and 5% CO_2.

Measurements

A first series of experiments was performed with cell samples in their own growth medium (DMEM high-glucose). However, the phenol red in the medium that serves as a pH indicator during culture exhibited a strong autofluorescence and massively impaired the fluorescence signal detection. Therefore all further measurements were performed after the medium had been replaced with PBS (200 µl/well).

The readers were pre-heated to 30°C to maintain optimal conditions for the cells during the measurements.
All measurements were performed in Fluorescence Bottom Reading mode with variable flash numbers and the settings summarized in table 1.

Plate definition file	GRE96fb
Measurement mode	Fluorescence Intensity Bottom
Excitation wavelength	483 nm
Excitation bandwidth	9 nm
Emission wavelength	535 nm
Emission bandwidth	20 nm
Gain	Optimal
Integration time	20 µs

Table 1: Measurement settings on Infinite 200 and Infinite 200 PRO

Optimal Read

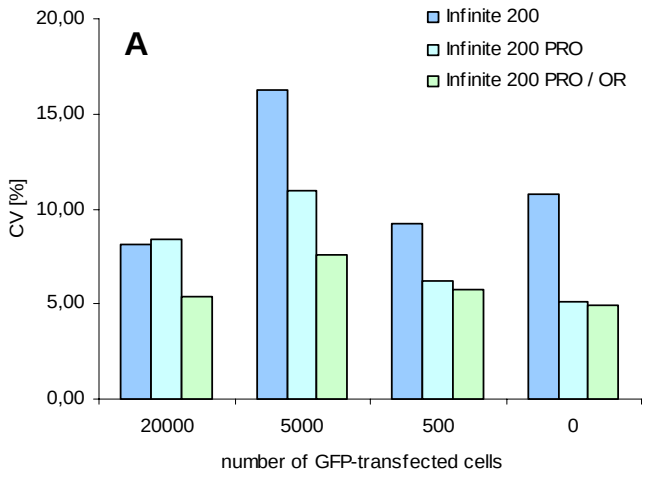
Some measurements were performed in combination with the Optimal Read function which is designed to cover the whole well bottom area of each different plate format from 6 to 384 well plate. This enhanced FI bottom reading option with its special OR (Optimal Read) capability was designed to optimize and improve cell based assays. The goal was to achieve low CVs, high intra- and inter-well reproducibility by measuring adherent cells in microplates.
The measurements were performed with 20000, 5000 and 500 GFP-transfected cells per well and a “cell blank” consisting of non-transfected cells only, using flash numbers of 4, 12, 25 or 40 flashes per well (Single Read). The same total flash numbers were used in combination with the Optimal Read function.

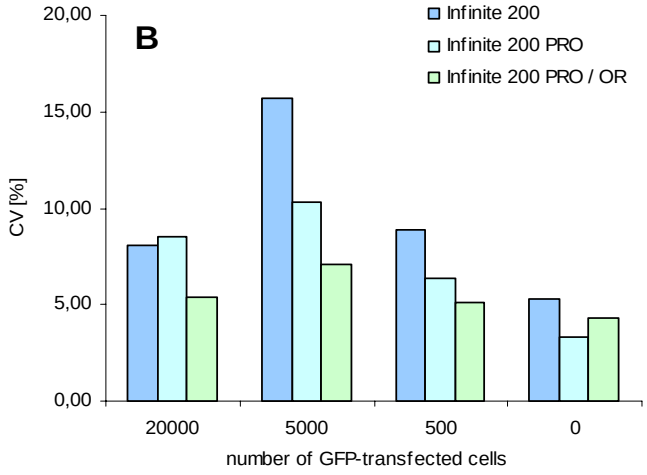
Results

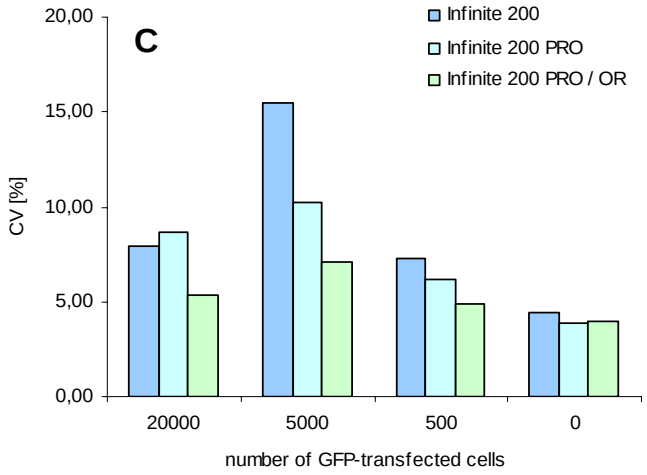
Well-to-well Uniformity

In direct comparison with the Infinite 200, the larger FI bottom fiber of the new Infinite 200 PRO results in a markedly improved well-to-well uniformity, as shown in figure 3. This effect appeared to be especially relevant for smaller numbers of GFP-transfected cells, where the bigger illumination area of the Infinite 200 PRO’s larger FI bottom fiber produces more representative signals. In contrast, the differences between the CV values were not as profound with 20000 GFP-transfected cells per well, which can be attributed to the fact that the well bottom is comprehensively and homogeniously

covered with signal-producing cells and can be effectively analyzed with both fiber sizes.







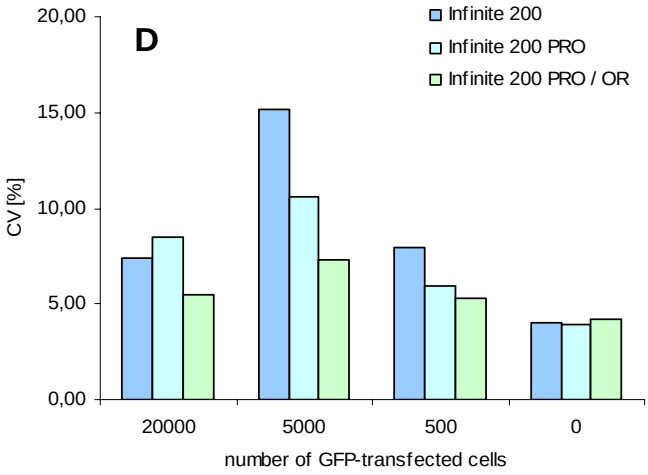


Figure 2: *Well-to-well Uniformity. (A) 4 flashes per well, (B) 12 flashes per well, (C) 25 flashes per well (default), (D) 40 flashes per well. The measurements were performed on an Infinite 200 with a standard FI bottom option, an Infinite 200 PRO with the enhanced FI bottom modul and the Infinite 200 PRO / OR were done with enhanced FI bottom with the Optimal Read (OR) function. Values represent average signals of 24 replicate wells.*

The CV values plotted in figure 2(A-D) are summarized in table 2.

	flashes	20000	5000	500	blank
I200 Single Read	4	8,12	16,21	9,24	10,77
	12	8,09	15,68	8,87	5,33
	25	7,96	15,51	7,28	4,43
	40	7,35	15,15	7,91	3,99
I200 PRO Single Read	4	8,38	10,92	6,22	5,11
	12	8,55	10,35	6,35	3,29
	25	8,63	10,27	6,16	3,84
	40	8,54	10,55	5,95	3,92
I200 PRO Optimal Read	4x1	5,37	7,56	5,73	4,95
	4x3	5,40	7,12	5,08	4,30
	4x7	5,31	7,06	4,86	3,92
	4x10	5,46	7,31	5,26	4,20

Table 2: Variation of FI Bottom measurements on Infinite 200 with single read (SR) and Infinite 200 PRO (SR and OR). The displayed values are coefficients of variation (CV) given in %.

Notably, the CV values obtained with the Infinite 200 PRO remained quite constant, regardless of the used flash number.

This shows that even at flash numbers as low as 4 the well-to-well uniformity is largely unaffected, giving the user the possibility to increase the measurement speed by reducing the flash number at uncompromised uniformity levels.
In contrast, a significant improvement of the measurement variation could be seen with increasing flash numbers on the

Infinite 200 in Single Read mode. The smallest measurement variations were observed with the Infinite 200 PRO in combination with Optimal Read, resulting in CV values only half as high as with the Infinite 200 in Single Read mode.

Reproducibility

In addition to the well-to-well uniformity, also the measurement reproducibility was tested using all three instrument configurations.

The most obvious improvement could be seen in the wells containing 500 GFP-transfected cells and in the blanks, showing that the Infinite 200 PRO in combination with Optimal Read yielded a measurement reproducibility that was approximately three times better than with the Infinite 200 in combination with Single Read.

By contrast, the reproducibility rate in the samples with 20000 cells/well remained largely unaffected, which again can be ascribed to the more homogeneous distribution of GFP-transfected cells on the well bottom (figure 3 and table 3).

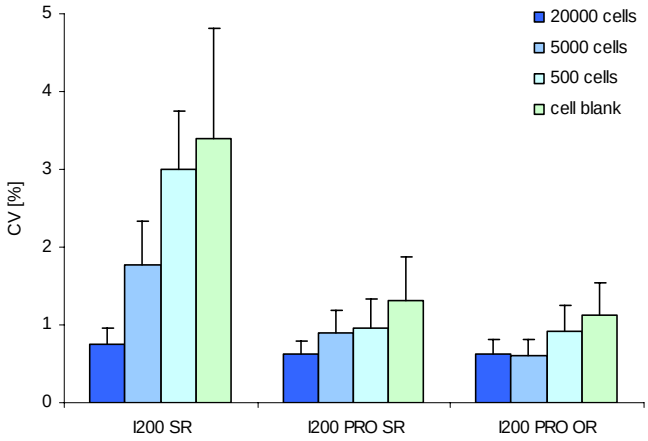


Figure 3: Measurement reproducibility. The measurements were performed on an Infinite 200 with Single Read (SR) and on an Infinite 200 PRO with Single or Optimal Read (OR). Values represent average signals of 24 replicate wells that were measured 5 consecutive times plus StDev. The CV values refer to the variation between all five measurements.

	I200 SR	I200 PRO SR	I200 PRO OR
20000 cells/well	0,76	0,63	0,63
5000 cells/well	1,77	0,90	0,61
500 cells/well	3,00	0,95	0,91
blank	3,39	1,32	1,12

Table 3: Reproducibility of FI Bottom measurements on Infinite 200 (SR) and Infinite 200 PRO (SR and OR). The displayed values are coefficients of variation (CV) given in %.

Discussion and Conclusion

The enhanced Fluorescence Bottom reading function of the Infinite 200 PRO is of great advantage especially for cell-based applications. The larger diameter of the improved bottom fiber results in a better and more homogeneous illumination of the well bottom, thus allowing for analysis of a larger number of cells.

This leads to a markedly improved well-to-well reading uniformity and better measurement reproducibility, even at lower flash numbers. In addition the special Optimal Read (OR) function could further increase homogeneity and lower the CV values, which offers a better sensitivity for adherent cell measurements.

From a scientific standpoint it should be considered that phenol red-containing media are not suitable for fluorescence measurements as their strong autofluorescence largely masks the sample-specific signals.

For optimal detection of cell-based fluorescence it is recommended to use phenol red-free media or carry out the measurements in a suitable buffer, e.g. PBS.

Acknowledgements

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Glossary

CV	coefficient of variation
DMEM	Dulbecco's modified Eagle's medium
EGFP	enhanced green fluorescent protein
FCS	fetal calf serum
FI	fluorescence intensity
HEPES	4-(2-hydroxyethyl)-1-piperazin ethanesulfonic acid
HTS	high throughput screening
OR	optimal read
PBS	phosphate-buffered saline
SR	single read
StDev	standard deviation

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