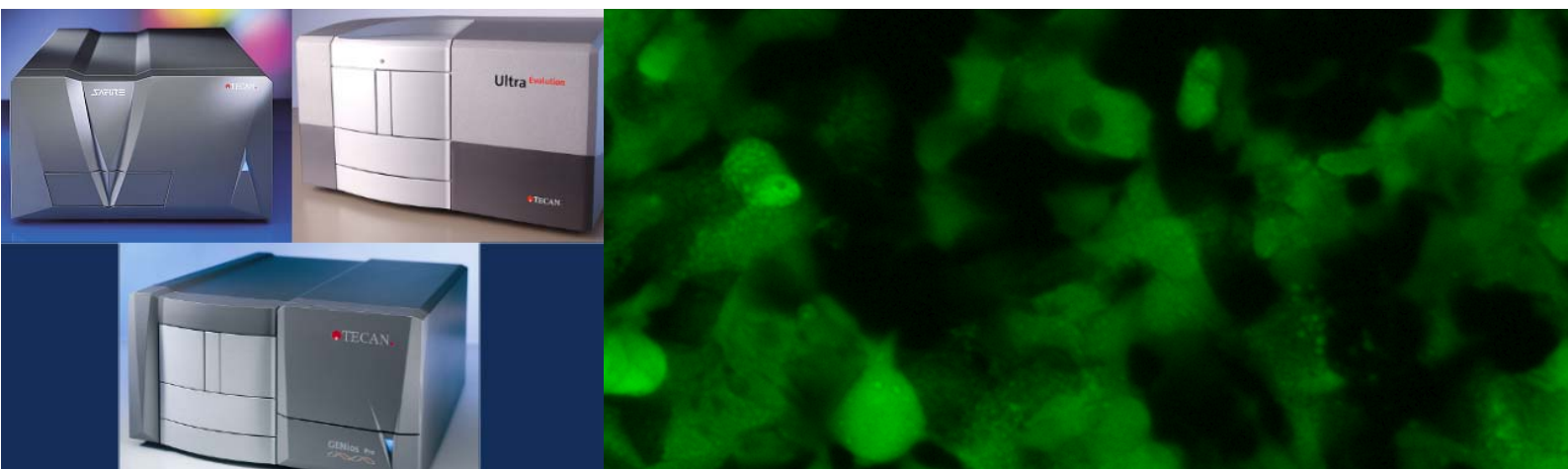


Detection of Green Fluorescent Protein (eGFP)

Tecan Ultra Evolution, Safire and GENios Pro



Introduction

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 has opened exciting new avenues of investigation in cell, developmental and molecular biology. GFP can function as a protein tag, as it tolerates N- and C-terminal fusion to a broad variety of proteins many of which have been shown to retain native function. Highly specific intracellular localization including the nucleus, mitochondria, secretory pathway, plasma membrane and cytoskeleton can be achieved via fusion both to whole proteins and individual targeting sequences.

The enormous flexibility as a non-invasive marker in living cells allows for numerous other applications such as a cell lineage tracer, reporter of gene expression and as a potential measure of protein-protein interactions.

Green fluorescent protein is comprised of 238 amino acids. Its wild-type absorbance/excitation peak is at 395 nm with a minor peak at 475 nm with extinction coefficients of roughly 30,000 and 7000 M⁻¹ cm⁻¹, respectively. The fluorescent quantum yield is about 70 – 80 %. The emission peak is at 508 nm. Analysis of a hexapeptide derived by proteolysis of purified GFP led to the prediction that the fluorophore originates from an internal Ser-Tyr-Gly sequence, which is post-translationally modified to a 4-(*p*-hydroxybenzylidene) - imidazolidin-5-one structure.

Physical and chemical studies of purified GFP have identified several important characteristics. It is very resistant to denaturation requiring treatment with 6 M guanidine hydrochloride at 90 °C or pH of < 4.0 or > 12.0.

The availability of *E. coli* clones expressing GFP has led to extensive mutational analysis of GFP function. Screens of random and directed point mutations for changes in fluorescent behavior have uncovered a number of informative amino acid substitutions (1, 2) with effects on fluorescence intensity and expression level. The combination of improved fluorescence intensity and higher expression levels yield enhanced GFP variants, which provide greater sensitivity, and are suitable for multicolor detection (3).

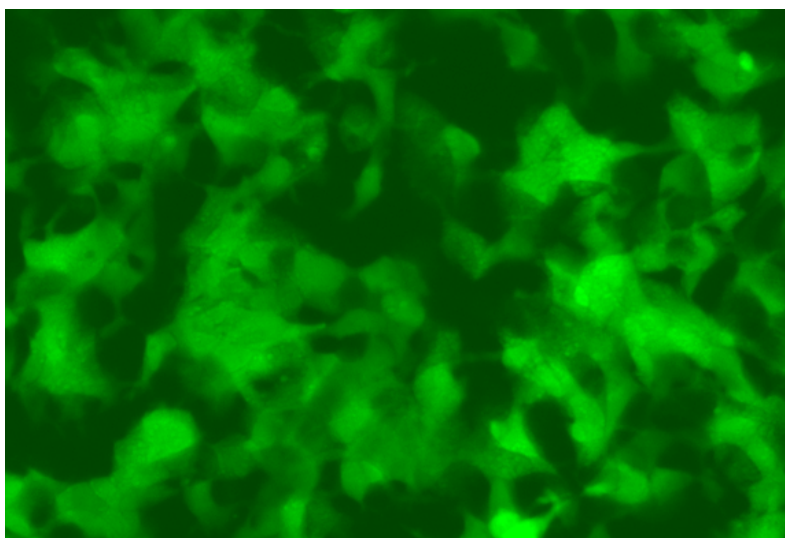


Figure 1: GFP transfected A431 cells (25000 cells/well)

The purpose of this technical note is to determine the optimal measurement conditions for eGFP, including optimal filter/wavelengths settings, reading mode (top or bottom) and plate type (Greiner or Costar).

Material and Methods

Cell culture: A431 cells (human epidermoid carcinoma cells, ATCC No. CRL-1555) were kindly provided by Prof. Barbara Krammer, University of Salzburg, and maintained in culture in 'standard medium' (DMEM high glucose, Sigma, D-5671) supplemented with L-Glutamine, Na-Pyruvat, Penicillin/Streptomycin, HEPES and 5 % heat inactivated fetal calf serum (FCS, PAA laboratories, Austria) in an atmosphere of 7.5 % CO₂ and 37 °C.

Transfection: Cells were stably transfected with the pEGFP-N1 vector (Cat. No.: #6085-1 Clontech) using Lipofectin® Reagent (18292-011, Invitrogen). GFP positive clones were selected and subcultured.

The GFP transfected cells were grown in 96-well plates (COSTAR, black, transparent bottom; Szabo-Scandic, Austria, Cat. No.: 3603 and GREINER, Cat. No.: 655090, 96 well plate, black, µClear, TC).

Plate Layout: For the measurements, 25000 transfected and non-transfected cells/well, respectively, or dilution series of transfected cells (see tab_1) were suspended into 96-well COSTAR and GREINER plates: highest cell number: 50000/well, diluted 1/2 down to 97 cells/well. However, the total number of cells/well was always 50000 for the plate containing the dilution series (see tab_1 for plate layout):

Column	transfected cells/well	non-transfected cells/well
1	0	50000
2	50000	0
3	25000	25000
4	12500	37500
5	6250	43750
6	3125	46875
7	1563	48437
8	768	49232
9	384	49616
10	192	49808
11	97	49903
12	0	0

tab_1: Dilution-series of GFP-transfected cells. Total number of cells/well is 50000.

This mixture of transfected and non-transfected cells shall simulate an environment found in transfection studies where, depending on the transfection efficiency, only a few cells out of a high cell number might be transfected. The cells were incubated overnight as stated above.

Instruments: Tecan Ultra Evolution; Tecan GENios Pro; Tecan Safire.

Measurements: The GFP signal was measured after overnight cultivation at Ex 485 (20)/Em 535 (25), Ex 465 (35)/Em 515 (20), Ex 485 (20)/Em 515 (20) and Ex 485 (20)/Em 520 (20) for determination of the optimal wavelengths pair. All further measurements (top/bottom comparison, plate comparison and detection limit) were performed with the optimal wavelength pair and the standard fluorescein

dichroic (320 – 500 nm reflection, 520 – 800 nm transmission, 505 nm break point; Ultra Evolution and GENios Pro only). Measurements were also taken before and after replacing the standard medium with low glucose medium (w/o phenol red). Single measurements (one measurement point per well) or multiple reads per well were performed (see Figure 3 for geometrical view) with the bottom reading mode or top reading mode (using optimal z-position) of the instruments. In addition, the excitation and emission spectra of GFP expressing cells in standard medium and low glucose medium (DME low glucose, Sigma, Cat. No. 5030), respectively, have been recorded with a Tecan Safire to reveal the influence of the medium on the shape of the eGFP excitation and emission spectrum.

Staining: Cells for microscopy were also stained with Hoechst 33342. Hoechst 33342, a bisbenzimidazole dye, is a cell membrane permeant, minor groove-binding DNA stain that fluoresces bright blue upon binding to DNA. The dye is quite soluble in water and relatively non-toxic (Molecular Probes: H-1399, H-3570). For staining of cells Hoechst 33342 was diluted with PBS to a final concentration of 55 µg/ml. Standard medium was removed from cells and 100 µl 'standard medium' (DME low glucose, Sigma, Cat. No. 5030; supplemented with 10 mM HEPES, 4 mM L-Glutamine, 1 mM Na-Pyruvate) and 10 µl of the staining solutions were added. Cells were incubated for 1 hr in an atmosphere of 7.5 % CO₂ at 37° C.

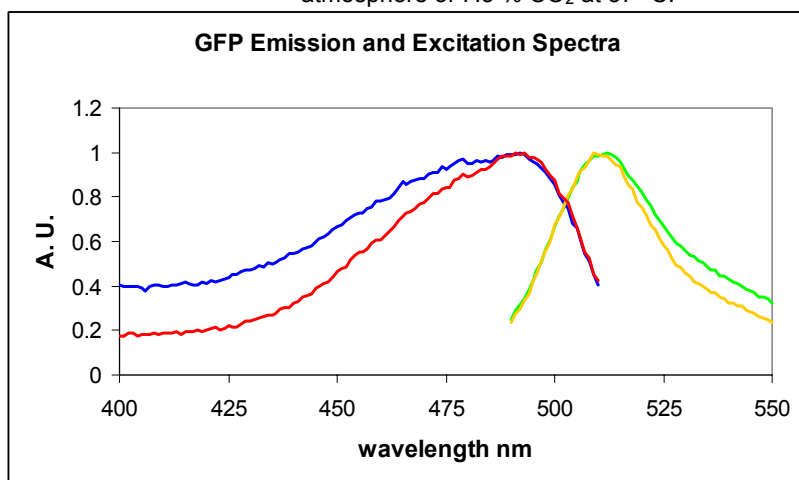


Figure 2: Normalized excitation and emission spectra of GFP expressing cells in different media (■ Excitation spectrum of GFP in standard medium; ■ Excitation spectrum of GFP in low glucose medium; ■ Emission spectrum of GFP in standard medium; ■ Emission spectrum of GFP in low glucose medium)

Microscopy: eGFP-transfected cells (Figure 1) were visualized using 600x magnification on an inverted fluorescence microscope (Olympus IX70) equipped with a Spot II CCD camera (Diagnostic Instruments). Pictures were taken with the appropriate filter block (Zeiss Filter set 17: BP485 (20), FT 510, BP 515-565).

Results

Spectra: Excitation and emission spectra of GFP were recorded (Figure 2). The excitation peak was found at ~ 490 nm, the emission maximum at ~ 510 nm. Based on these findings different filter pairs were tested to find the optimal wavelengths pair: 1) Ex 485 (20)/Em 535 (25), 2) Ex 465 (35)/Em 515 (20), 3) Ex 485 (20)/Em 515 (20), 4) Ex 485 (20)/Em 520 (20). The bandwidths of the filters are written in brackets and are valid for Tecan Ultra Evolution and GENios Pro. All Safire measurements were performed with a bandwidth of 12 nm.

Multiple Reads per Well versus Single Measurement

A pre-run was performed to check if multiple reads per well (mrpw, see Figure 3 for geometric view) is necessary to compensate an unequal distribution of cells in the wells. It turned out that single measurements are sufficient and, therefore, for all further measurements, only single measurements as described in material and methods were performed.

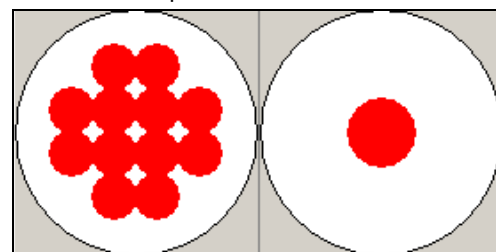


Figure 3: The picture on the left hand side shows a schematic view of the distribution of measurement points when using the multiple reads per well function (circle, 4x4). On the right side a single measurement is shown.

Filter Optimization: For filter optimization both, Greiner and Costar plates filled with 25000 transfected cells/well and 25000 non-transfected cells (both kept in medium), respectively, were measured with the following filter combinations: Ex 485 (20)/Em 535 (25), Ex 465 (35)/Em 515 (20), Ex 485 (20)/Em 515 (20), and Ex 485 (20)/Em 520 (20). The measurements were performed with the bottom

and top reading mode of Tecan Ultra Evolution, Safire and GENios Pro using 10 flashes/well, optimal z-position (Tecan Ultra Evolution for top and bottom, Safire for top measurements only), and 40 μ s integration time. Table 2 shows the results (signal to background ratio) of the bottom measurements with Tecan Ultra Evolution. Measurements with Tecan Safire and GENios Pro resulted in the same optimal wavelength pair. Measurements performed with the top measurement option (results not shown) are comparable to the bottom results.

Plate type	WL pair	S-B/B
GRE	485/535	5.7
COS	485/535	4.6
GRE	465/515	4.8
COS	465/515	3.9
GRE	485/515	9.9
COS	485/515	8.5
GRE	485/520	8.0
COS	485/520	6.5

tab_2: Signal to background ratio for different wavelength pairs obtained from measurements of Greiner (GRE) and Costar (COS) plates filled with 25000 transfected and non-transfected cells, respectively. Results of measurements with a Tecan Ultra Evolution are shown.

The standard medium was replaced with a low glucose medium without phenol red and measured (data not shown). The results were comparable to the measurements of cells kept in standard medium, Ex 485 (20)/Em 515 (20) nm turned out to be superior.

Top versus Bottom Reading: For comparison of top and bottom reading mode, both, Greiner and Costar plates filled with 25000 transfected cells/well and 25000 non-transfected cells, respectively, kept in standard medium (tab_3) or low glucose medium were measured with the filter combination which turned out to be best: Ex 485 (20)/Em 515 (20). The measurements were performed with the bottom and top reading function of Tecan Ultra Evolution, Safire and GENios Pro using 10 flashes/well, optimal z-position (Tecan Ultra Evolution for top and bottom, Safire for bottom measurement only), and 40 μ s integration time. Table 3 shows the results (signal to background ratio) of the top and bottom measurements in standard medium. Table 4 shows the effect of different media on the measurement. The ratio (S-B)/B of the standard medium to (S-B)/B of the low glucose medium (see equation 1 for calculation) is shown.

Equation 1:

$$\text{Ratio} = \frac{[(S - B)/B]_{\text{SM}}}{[(S - B)/B]_{\text{LGM}}}$$

S RFU value of GFP transfected cells
 B RFU value of non-transfected cells
 SM Standard medium
 LGM Low Glucose Medium

Instrument	Top	Bottom
Ultra Evolution	8.5	12.9
Safire	10.9	16.3
GENios Pro	4.7	8.9

tab_3: (S-B)/B ratio of top and bottom readings of cells kept in standard medium in a Greiner 96 well plate.

Instrument	Ratio Top	Ratio Bottom
Ultra Evolution	1.1	1.0
Safire	0.8	1.0
GENios Pro	0.7	1.2

tab_4: Effect of different media (standard or low glucose) on (S-B)/B measurements with the top and bottom option. The ratio standard medium to low-glucose medium is shown.

The results of the measurements of Costar plates (not shown) were comparable to those of the Greiner plates.

Costar versus Greiner Microplates: GFP transfected cells (25000 cells/well) kept in standard medium were measured in COSTAR (black, transparent bottom; Szabo-Scandic, Austria, Cat. No.: 3603) and GREINER (Cat. No.: 655090, 96 well plate, black, μ Clear, TC) plates. Non-transfected cells (25000 cells/well) were measured as control sample. The measurements were performed with bottom and top reading mode, Ex 485 (20)/Em 515 (20) nm, 10 flashes/well, and 40 μ s integration time. The signal to background ratio was calculated. The results are shown in Figure 4.

Detection Limit: Dilution series of transfected and non-transfected cells were prepared according to material and methods to determine the LOD. The cells were cultivated in Greiner and Costar plates and measured kept in standard medium or low glucose medium (w/o phenol red). The measurements were performed with the bottom reading option of Tecan Ultra Evolution, Safire and GENios Pro using 10 flashes/well, 40 μ s integration time, optimal z-position (Ultra Evolution only), Ex 485 (20)/Em 515 (20) nm and Ex 485 (20)/Em 535 (25) nm. Figure 5 shows the results of the Tecan Ultra Evolution measurements of the

dilution series of eGFP-transfected cells (left-hand side: cells kept in standard medium, right-hand side: cells kept in low glucose medium w/o phenol red). The GFP detection limits for cells kept in standard medium are shown in tab_5. The detection limit is defined as detectable transfected cells in a mixture of transfected and non-transfected cells. It was calculated according to equation 2.

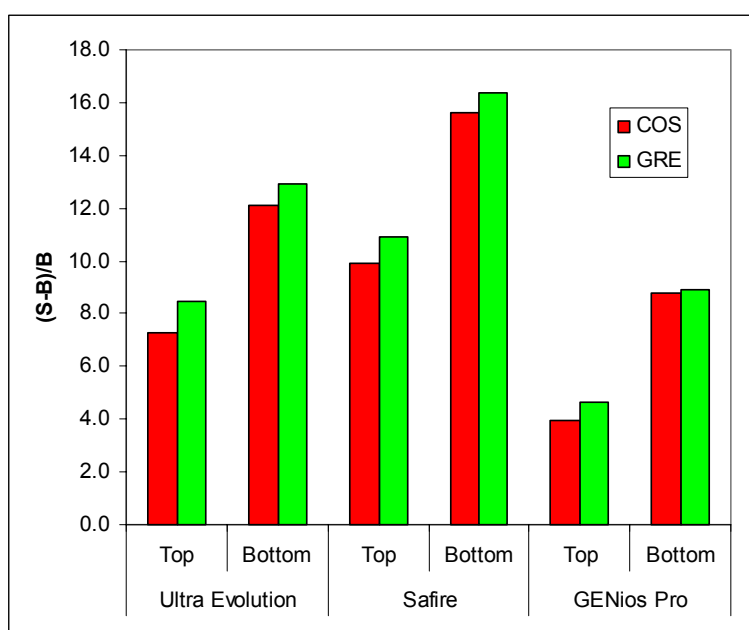


Figure 4: Comparison of Costar and Greiner plates: The (S-B)/B ratio is shown for Tecan Ultra Evolution, Safire and GENios Pro when measuring with the bottom and top reading mode.

Equation 2:

$$\text{LOD} = \frac{25000}{(F_{(25000)} - B)} * 3 * \text{Stdev}_B$$

LOD Limit of Detection
 $F_{(25000)}$ measured RFU value of 25000 transfected cells
 B measured RFU value of Blank (non-transfected cells)
 Stdev_B Standard deviation of blank

Instrument	WL pair	
	485/515 nm	485/535 nm
Ultra Evolution	185	303
Safire	175	216
GENios Pro	245	439

tab_5: Detection limit of GFP transfected cells kept in standard medium measured with Tecan Ultra Evolution, Safire and GENios Pro using two different filter sets and the bottom reading mode.

The measurements in Costar plates resulted in similar LOD values.

Figure 6 shows a sector of a well filled with 193 transfected and GFP expressing cells (left picture) out of 50000 total cell number. The picture in the middle shows a light microscopy taking of the same sector; the right-hand picture shows a Hoechst staining of the nuclei. Figure 6 displays a microscopic visualization of the LOD of eGFP.

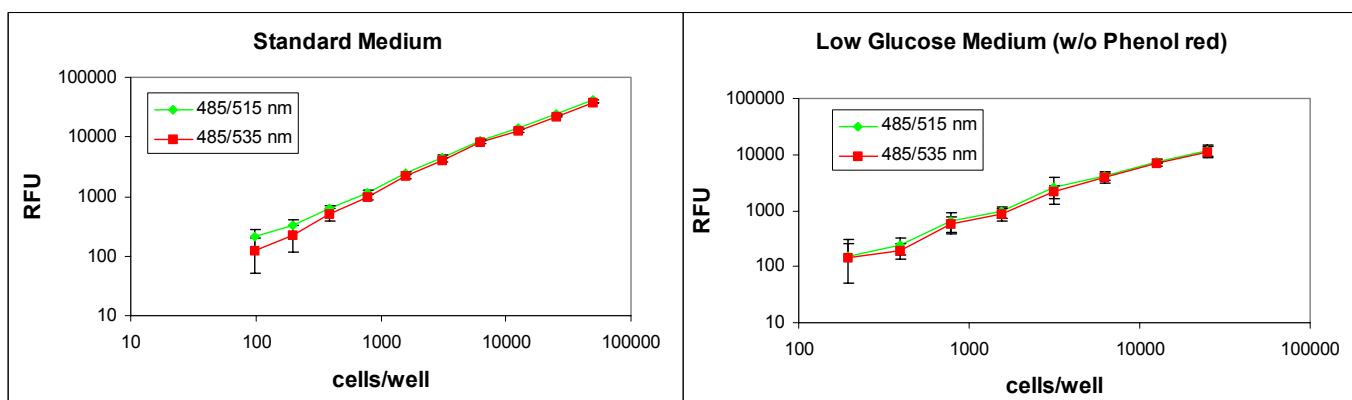


Figure 5: Measurement of dilution series of GFP-transfected cells (plate layout see material and methods) with Tecan Ultra Evolution. The dilution series were prepared in Greiner plates, and cells kept in standard medium (left picture) or low glucose medium (w/o phenol red; right picture) were measured with the bottom reading mode using two different filter/wavelength pairs.

Discussion

The purpose of this technical note was:

- a) to determine the optimal filters for eGFP
- b) to compare the efficiency of a top and bottom measurement
- c) to compare Costar and Greiner plates
- d) to determine the 'detection limit' for transfection studies

All measurements were performed with Tecan Ultra Evolution, Safire and GENios Pro.

The excitation and emission spectrum of eGFP-transfected cells (Figure 2) kept in standard medium or low glucose medium (w/o phenol red) were recorded with a Tecan Safire. The maxima were found at Ex 492 nm and Em 510 nm for both media. However, the shape of the spectra turned out to be different. Cells kept in standard medium showed an increased background (10 – 15 %) due to autofluorescence of the medium. The medium does not cause a wavelength shift of the maxima but the peaks seems to be broader than those seen with low glucose medium w/o phenol red.

Before starting the experiments it was tested if the read mode 'multiple reads per well' (mrpw, Figure 3) is superior to single measurements per well. It turned out that mrpw results are not significantly different from single measurements (data not shown). All further measurements were therefore performed with the single measurement mode.

and 4) Ex 485 (20)/Em 520 (20). The optimization was done for top (results not shown) and bottom reading (tab_2) as well as for Greiner (tab_2) and Costar plates (results not shown). Filter pair 3 (Ex 485 nm/Em 515) turned out to be superior giving the best signal to background ((S-B)/B) ratio for all, top and bottom as well as Greiner and Costar plates. Tecan Safire showed the highest signal to background ratios ((S-B)/B). In contrast to Tecan Ultra Evolution and GENios Pro, Safire measurements were performed with only 12 nm bandwidth, which is the broadest possible for this type of instrument. Tecan Ultra Evolution and GENios Pro measurements were done with filter bandwidths of 20 and 25 nm respectively. The smaller bandwidth of the Safire monochromator system might be the reason for the slightly better signal to background ratios.

The signal to background ratio was also calculated for the two measurement modes, top and bottom reading. GFP-transfected cells kept in standard medium or low glucose medium (w/o phenol red) were measured with the optimal wavelength pair (485 nm excitation and 515 nm emission). No significant differences could be observed in the (S-B)/B ratio between standard and low-glucose medium when measuring from bottom with Tecan Ultra Evolution and Safire (tab_4). GENios Pro showed a decrease of the signal to background ratio of about 20 %. The GFP-transfected cells are adherent cells, which are attached to the bottom of the microplate. The bottom optics of Tecan GENios Pro and Safire are in principal



Figure 6: GFP transfected cells (192 transfected cells/total cell number 50000): The same well sector is shown in three different ways: left: fluorescence microscopy GFP; middle: light microscopy; right picture: staining of cell nuclei with Hoechst.

Four filter combinations were tested to find out the optimal wavelengths for the measurement of eGFP: 1) Ex 485 (20)/Em 535 (25), 2) Ex 465 (35)/Em 515 (20), 3) Ex 485 (20)/Em 515 (20),

focused on the bottom of the plate, Ultra Evolution has the possibility to adjust the bottom optics to the maximum signal (z-optimization). The advantage of the bottom reading mode compared to top reading is the ability to measure the fluorescence of the cells without

the need to focus through a layer of liquid. When measuring from top the Ultra Evolution was less influenced by the different composition of media. Both, Safire and GENios Pro, showed a significant increase of the signal to background ratio when measuring cells kept in low glucose medium without phenol red. These results underline the superiority of the Ultra optics to compensate negative effects (e. g. autofluorescence) by optimizing the focus point of the optics (z-optimization).

The comparison of COSTAR (black, transparent bottom; Szabo-Scandic, Austria, Cat. No.: 3603) and GREINER (Cat. No.: 655090, 96 well plate, black, µClear, TC) plates showed no significant differences between the two plates (Figure 4).

The detection limit for eGFP was determined by measuring dilution series of eGFP-transfected cells (Figure 5) kept in standard medium, mixed with non transfected-cells to achieve a total cell number per well of 50000. The LOD measurements (tab_5) were performed with the optimal instrument settings (Ex 485/Em 515 nm, bottom reading; optimal z-position of Ultra Evolution, 10 flashes, integration time of 40 µs) using Greiner 96 well plates. Ultra Evolution and Safire were able to detect < 190 transfected cells out of 50000 (see equation 2 for calculation), Tecan GENios Pro was slightly worse (LOD < 250 cells). The reason for the superiority of Tecan Ultra and Tecan Safire might be on the one hand the ability of Ultra Evolution to optimize the Z-position and on the other hand the smaller bandwidth of Safire which reduces the background. The same measurement was performed with a second filter set (Ex 485/Em 535 nm). The detection limit was two times worse compared to the optimal filter set. Figure 5 shows the linearity of the dilution series of cells either kept in standard medium or low glucose medium (w/o phenol red). The linearity of cells kept in standard medium becomes worse with low cell numbers due to the autofluorescence of phenol red.

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Glossary

(S-B)/B	Signal to background ratio
B	Background
cDNA	Complimentary DNA (a DNA sequence that was produced from mRNA (messenger RNA) by reverse transcription. The cDNA sequence is the complement of the original mRNA sequence)
COS	Costar
DME	see DMEM
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
E. coli	Escherichia coli
Em	Emission
Ex	Excitation
FCS	Fetal calf serum
GFP	Green fluorescent protein
Gly	Amino acid Glycine
GRE	Greiner
LOD	Limit of Detection
Mrpw	Multiple reads per well
nm	Nanometer
RFU	Relative fluorescence unit
RNA	Ribonucleic acid
S	Signal
Ser	Amino acid Serine
Tyr	Amino acid Tyrosine
w/o	without
WL	Wavelength

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