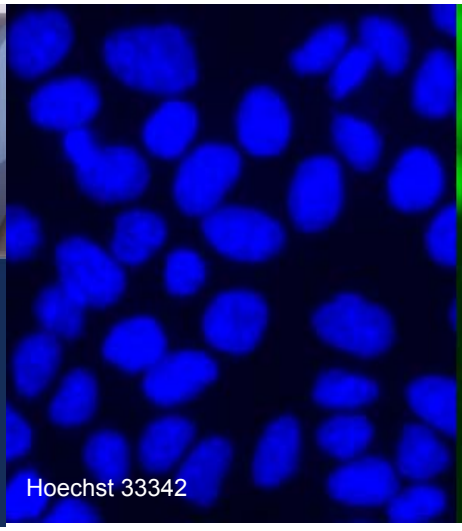
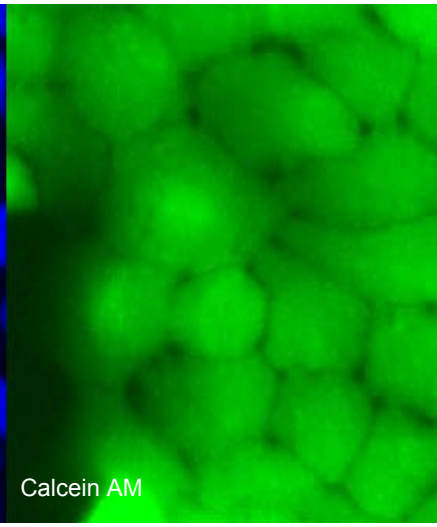
Detection of Calcein AM and Hoechst 33342

Tecan Ultra Evolution, Safire and GENios Pro





Introduction

Calcein AM (acetoxymethyl ester of Calcein) is one of the premier indicators of cell viability due to its superior cell retention and the relative insensitivity of its fluorescence to physiological pH values. Live cells may be distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant Calcein AM to the intensely fluorescent Calcein. Calcein, which is the hydrolysis product of Calcein AM, is a polyanionic fluorescein derivative. Calcein is well retained within live cells, producing intense uniform green fluorescence in live cells (Molecular Probes: C-1430).
Hoechst 33342, a bisbenzimide dye, is a cell membrane permeant, minor groove-binding DNA stain that fluoresce bright blue upon binding to DNA. The dye is quite soluble in water and relatively non-toxic (Molecular Probes: H-1399, H-3570). Hoechst 33342 stains both, live and dead cells.

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’ (Dulbecco’s Modified Eagle’s Medium DMEM, Sigma, D-5671) supplemented with 10 mM Hepes, 4 mM L-Glutamine, 1 mM Na-Pyruvat, 100 U/ml Penicillin, 0.1 mg/ml Streptomycin and 5 % FCS (PAA laboratories, Austria)) in an atmosphere of 5 % CO_2 at 37 °C. For the measurements, dilution series of cells were suspended into 96-well COSTAR plates (black, transparent bottom; Szabo-Scandic, Austria, Cat. No.: 3603): highest cell number was 30000/well, diluted 1/3 down to 780 cells/well. The cells were incubated overnight as stated above.

Staining

A 4 mM Calcein stock solution (in DMSO; Calcein: Cat. No.: C1430, Molecular Probes) is diluted with PBS to a final concentration of 0.054 mM. Hoechst 33342 (Molecular Probes) is diluted with PBS to a final concentration of 55 µg/ml. Standard medium was removed from cells and 100 µl 'basic medium' (DME low glucose, Sigma, Cat. No. 5030; supplemented with 10 mM Hepes, 4 mM L-Glutamin, 1 mM Na-Pyruvat) and 10 µl of the staining solutions (Calcein and/or Hoechst) were added. Cells were incubated for 1 and 2 hrs in an atmosphere of 5 % CO_2 at 37° C.

Instruments

Tecan Ultra Evolution; Tecan GENios Pro; Tecan Safire.

Measurements

Calcein uptake was measured after 2 hrs incubation time. Hoechst 33342 was measured after 1 and 2 hrs incubation time with different bandwidths (Safire) and different emission and excitation wavelengths.

Results

Calcein AM (acetoxymethyl ester of Calcein) is one of the premier indicators of cell viability due to its superior cell retention and the relative insensitivity of its fluorescence to physiological pH values. Live cells may be distinguished by the presence of ubiquitous intracellular esterase activity.

Spectra

Excitation and Emission Spectra of Calcein and Hoechst 33342 have been recorded (Figure 1) with Tecan Safire. The excitation maximum of Calcein was found at **~ 492 nm**, Hoechst at **~ 354 nm**, the emission maximum of Calcein at **~ 513 nm**, Hoechst at **~ 442 nm**. Based on these findings the excitation filter 485 nm and the emission filter 590 nm were selected for further measurements of Calcein. For Hoechst different emission filter settings have been tested: Excitation at 360 nm, emission at 460, 480 and 485 nm (results of 480 nm not shown). Also different bandwidths (12 nm and 2.5 nm) have been tested with a Tecan Safire.

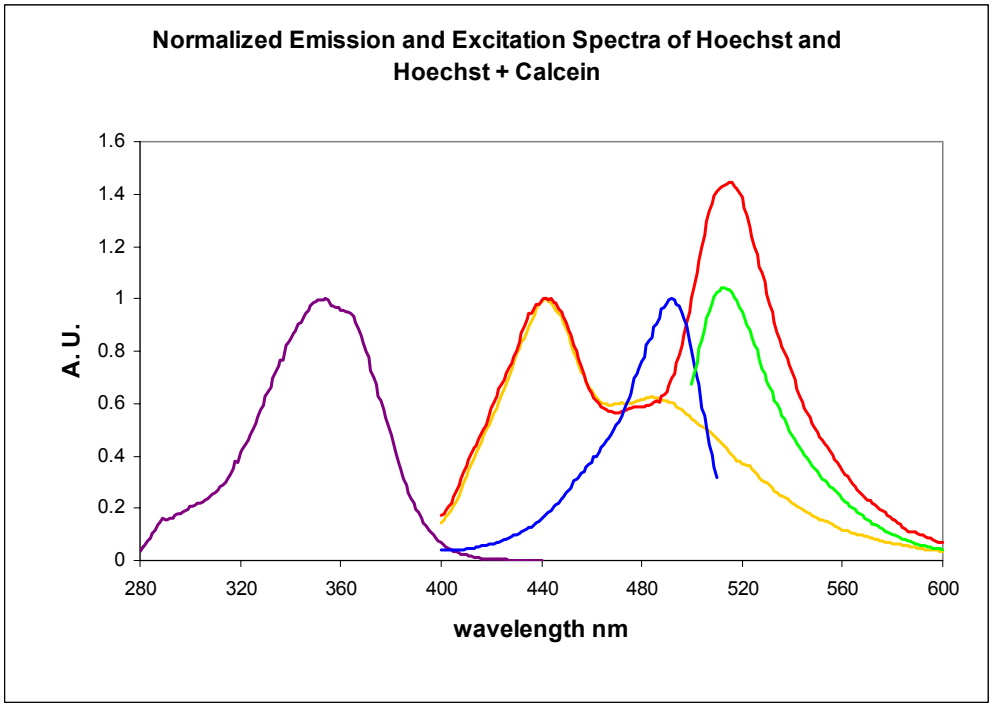


Figure 1: Normalized excitation and emission spectra of Calcein and Hoechst 33342 treated cells (■ Excitation spectrum of Hoechst 33342; ■ Excitation spectrum of Calcein; ■ Emission spectrum of Calcein and Hoechst 33342, excitation at 340 nm; ■ Emission spectrum of Hoechst 33342; ■ Emission spectrum of Calcein)

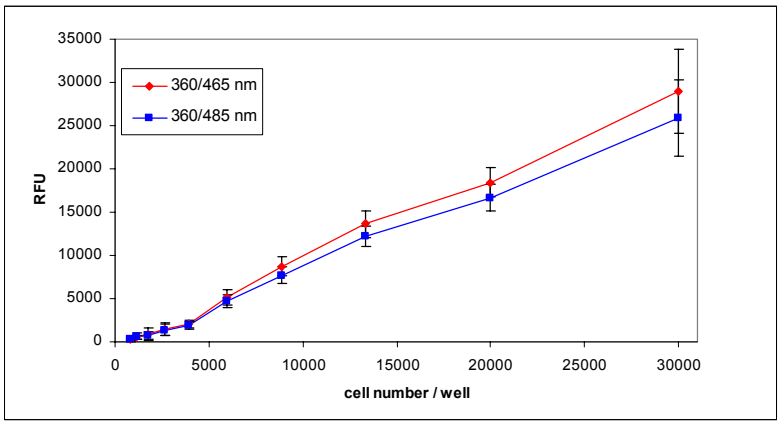


Figure 2: Measurement of Hoechst 33342 at different emission wavelengths. Measurement was performed with Tecan Ultra Evolution after 2 hrs incubation time.

Measurement of Hoechst 33342 at different emission wavelengths: Cells were incubated for 2 hrs with Hoechst 33342 and measured with a Tecan Ultra Evolution with the following measurement parameters: excitation at 360 nm (35 nm bandwidth); emission at 465 (35 nm bandwidth) or 485 nm (20 nm bandwidth), 40 µs integration time, 10 flashes (Figure 2). The (S-B)/N ratio was determined for different WL pairs and cell numbers(tab_1).

	cell number/well		
WL pairs	**30000**	**6000**	**800**
360/465 nm	5.9	5.9	1.2
360/485 nm	5.9	6.1	1.9

tab_1: (S-B)/N for different wavelengths pairs (signal – background/noise)

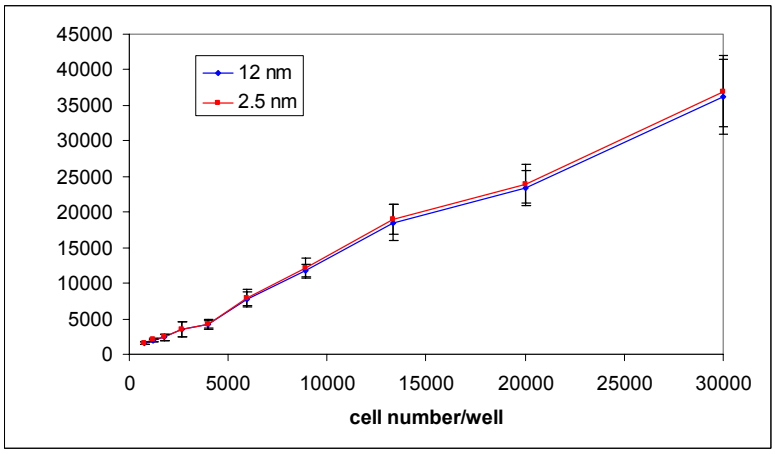


Figure 3: Measurement of Hoechst 33342 with Tecan Safire with different bandwidths, excitation at 360 nm (12 nm bandwidth), emission at 485 nm (12 or 2.5 nm bandwidth) after 2 hrs incubation time.

Measurement of Hoechst 33342 with different bandwidths: Cells were incubated for 2 hrs with Hoechst 33342 and measured with a Tecan Safire with the following measurement parameters: excitation at 360 nm (12 nm bandwidth); emission at 485 nm (12 nm and 2.5 nm bandwidth), 40 µs integration time, 10 flashes (Figure 3). The (S-B)/N ratio was calculated for different bandwidths and cell numbers (tab_2).

	cell number/well		
bandwidth	**30000**	**6000**	**800**
12 nm	6.7	6.4	2.8
2.5 nm	7.1	6.1	2.7

tab_2 : (S-B)/N for different bandwidths

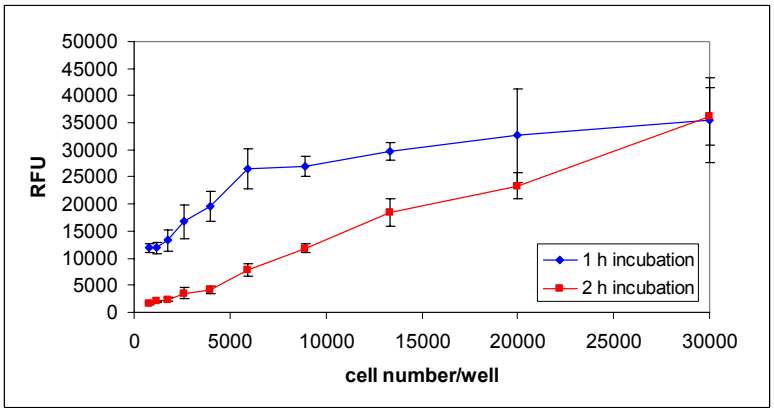


Figure 4: Measurement of Hoechst 33342 with Tecan Safire after 1 and 2 hrs incubation time (360/485 nm, 12 nm bandwidth).

Measurement of Hoechst 33342 after 1 and 2 hrs incubation time: Cells were incubated for 1 and 2 hrs with Hoechst 33342 and measured with Tecan Safire with the following measurement parameters: excitation at 360 nm (12 nm bandwidth); emission at 485 nm (12 nm) bandwidth), 40 µs integration time, 10 flashes (Figure 4).

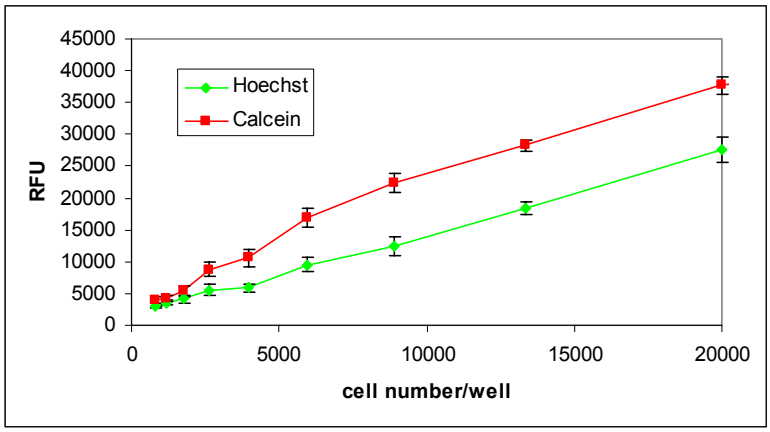


Figure 5: Measurements of cells treated with Hoechst 33342 and Calcein AM performed with Tecan GENios Pro after 2 hrs incubation time.

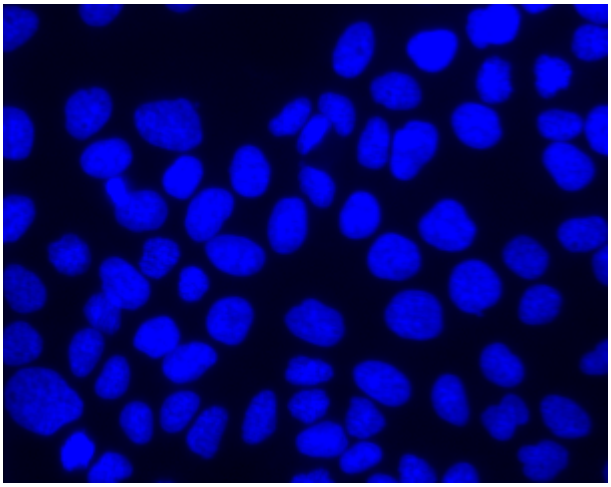


Figure 6: Cells labeled with Hoechst 33342. Cell nuclei are stained.

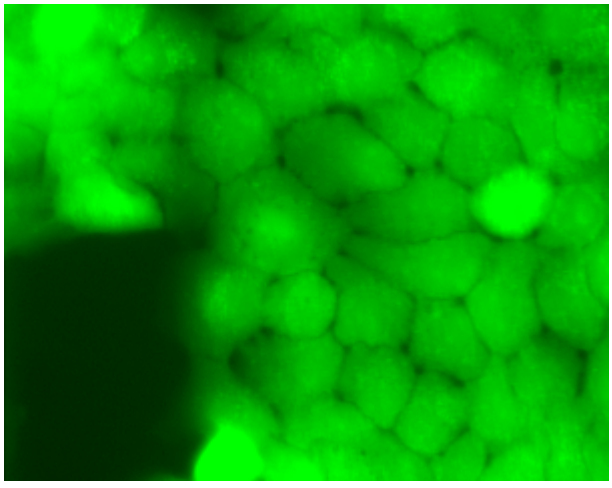


Figure 7: Cells labeled with Calcein AM. Cytoplasm is stained.

Measurement of cells treated with Hoechst 33342 and Calcein AM: The measurement was performed with all three Tecan readers. Figure 5 shows the results of Tecan GENios Pro which is representative for all three instruments. Cells were incubated for 2 hrs with Hoechst 33342 and Calcein AM and measured with GENios Pro with the following measurement parameters:
Ex Hoechst at 360 nm (35 nm bandwidth);
Em Hoechst at 485 nm (20 nm bandwidth),
Ex Calcein AM at 485 nm (20 nm bandwidth),
Em Calcein AM at 535 nm (25 nm bandwidth),
40 µs integration time, 10 flashes (Figure 5).

Cells treated with Hoechst 33342 and Calcein AM: Figure 6 and 7 were taken with an Olympus IX-70 fluorescence microscope equipped with a Spot2 digital camera (Diagnostic Instruments). Figure 6 shows the cell nuclei labeled with Hoechst 33342. Cells labeled with Calcein are displayed in Figure 7.

Discussion

The data above clearly show the ability of Tecan Ultra Evolution, Safire and GENios Pro to detect the two membrane permeant dyes, Calcein and Hoechst 33342, with the bottom reading option of the instruments.

Figure 1 shows the emission and excitation spectra of both dyes, and the emission spectrum of cells incubated with both dyes simultaneously. Even in a mixture of both dyes, the excitation and emission maxima are clearly distinguishable.

Neither different emission wavelengths (465 or 485 nm) nor different bandwidths (12 nm or 2.5 nm) seemed to have an influence on the Hoechst 33342 measurement results (Figure 2 and 3, tab_1 and tab_2). For the Calcein AM measurement we recommend for Ultra Evolution and GENios Pro to use the standard fluorescein filters.

The cells were incubated either 1 or 2 hrs with Hoechst 33342. Figure 4 shows that after 1 hr wells with higher cell numbers (> 8000 cells per well) are not completely stained. After 2 hrs the increase of signal over cell number is nearly

linear indicating that more or less all cells are stained.

Acknowledgements

We would like to thank Prof. Barbara Krammer (University of Salzburg, Institute of Biophysics) for her good collaborative work. We would also like to thank Dr. Kristjan Plaetzer, Dr. Thomas Verwanger, Tobias Kiesslich, Christian Benno Oberdanner and Monika Huber (University of Salzburg, Institute of Physics and Biophysics) for performing the cell culture and staining procedure.

Literature

For detailed information about the dyes used and their applications please see **www.probes.com**.

Glossary

A.U.	Arbitrary units
AM	Acetoxymethyl
B	Background
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
EM	Emission
EX	Excitation
N	Noise
nm	nanometre
PBS	Phosphate buffered saline
RFU	Relative fluorescence units
S	Signal
WL	Wavelength

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