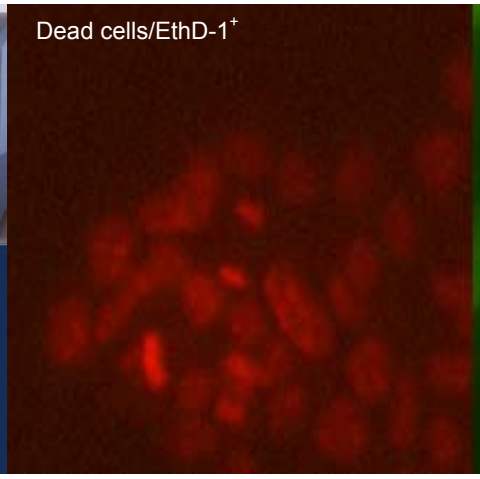
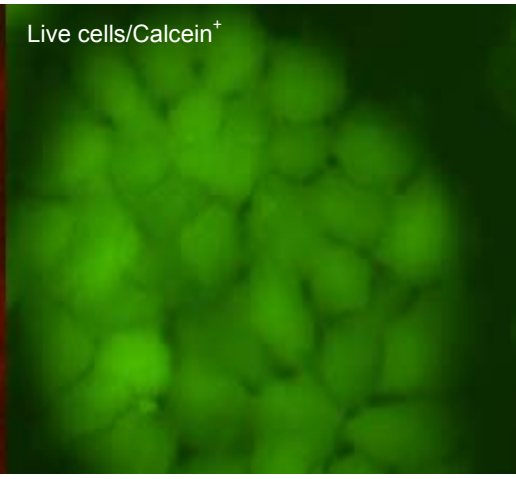
LIVE/DEAD® Viability/Cytotoxicity Assay

Tecan Ultra Evolution, Safire and GENios Pro





Introduction

Live cells are 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. The polyanionic dye Calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (EX/EM ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (EX/EM ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. The determination of cell viability depends on these physical and biochemical properties of cells. Cytotoxic events that do not affect these cell properties may not be accurately assessed using this method. Background fluorescence levels are inherently low with this assay technique, because the dyes are virtually nonfluorescent before interacting with cells (*1*).

The LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Molecular Probes, L-3224) provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cell viability — intracellular esterase activity and plasma membrane integrity. Molecular Probes has found that Calcein AM and ethidium homodimer (EthD-1) are optimal dyes for this application. The kit is suitable for use with fluorescence microscopes or fluorescence multiwell plate readers and easily adaptable for use with flow cytometers and other fluorescence detection systems. The assay principles are general and applicable to most eukaryotic cell types, including adherent cells and certain tissues, but not to bacteria or yeast. This fluorescence-based method of assessing cell viability can be used in place of trypan blue exclusion, ^{51}Cr release and similar methods for determining cell viability and cytotoxicity. It is generally faster, less expensive, safer and a more sensitive indicator of cytotoxic events than alternative methods. Several laboratories have established

validity of the LIVE/DEAD Viability/Cytotoxicity assay for animal cell applications. Published applications include measuring the cytotoxic effects of tumor necrosis factor (TNF), β-amyloid protein, adenovirus E1A proteins, tetrodotoxin (TTX) binding to Na^+ channels, methamphetamines, and mitogenic sphingolipids. The assay has also been utilized to quantify apoptotic cell death and cell-mediated cytotoxicity (*1*).

For this technical note cell death was induced by applying different concentrations of Ethanol or Saponin. Saponins are natural surfactants or detergents, found in many plants, but they are most abundant in the desert plants Yucca and Quillaja. Saponins also have commercial applications such as ore separation in industrial and mining operations, and are useful in products such as photographic emulsions, cosmetics, and shampoos. Legumes, such as peas and soybeans, also contain small quantities of Saponins, which are proving their worthiness as phytochemicals. In the diet, phytochemical Saponins have a wide spectrum of activity as antifungal and antibacterial agents, lowering of blood cholesterol, and inhibition of cancer cell growth.

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, Sigma, D-5671) supplemented with L-Glutamine, Na-Pyruvat, Pen/Strep, HEPES and 5 % heat inactivated fetal calf serum (FCS, PAA laboratories, Austria) in an atmosphere of 7.5 % CO_2 and 37 °C. The cells were grown in 96 well plates (Greiner. Cat. No.: 655090, 96 well plate, black, µClear). For the measurements 12500 cells were suspended into 96-well Greiner plates and incubated overnight as stated above.

Staining: The staining solution was prepared by adding 10.32 µl Calcein-stock solution (4 mM, C1430, Molecular Probes) and 20.6 µl Ethidium-Homodimer-1-stock solution (1mM, E1169, Molecular Probes) to 720 µl PBS. After overnight incubation cells were treated with either 100 µl of different Ethanol (Sigma, 02854) concentrations (% v/v: 100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.391 and 0; in standard medium) or 100 µl of different Saponin (Sigma, S-4521) concentrations (µg/ml: 100, 66.67, 44.44, 29.63, 19.75, 13.17, 8.78, 5.85, 3.90 and 0; in standard medium) to investigate the effect of both agents. After adding Ethanol or Saponin the cells were incubated 10 minutes as stated above. 10 µl of the staining solution were added. After a short incubation time measurements were performed before and after washing the cells and replacing the medium containing Ethanol or Saponin with standard medium. A second experiment was performed with a second set of Ethanol and Saponin gradients to show the switching point between live and dead in more detail (Ethanol: 50, 44.5, 39, 33.5, 28, 22.5, 17, 11.5, 6 % v/v, and 0; Saponin: 40, 35.5, 31, 26.5, 22, 17.5, 13, 8.5, 4 µg/ml, and 0; both dilution series were prepared with standard medium as diluent). The cells were treated in the same way as described above. Only the results after washing are shown.

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

Measurements: The Calcein signal was measured before and after washing (see staining) at EX 485 nm/ EM 535 nm, the Ethidium-Homodimer-1 (EthD-1) signal at EX 535 nm/ EM 635 nm.
Either single measurements (one measurement point per well) or multiple reads per well (circle, 4x4; see Figure 1 for geometrical view) were performed with the bottom reading mode of the instruments. In addition the excitation and emission spectra of Calcein and EthD-1 were recorded with a Tecan Safire.

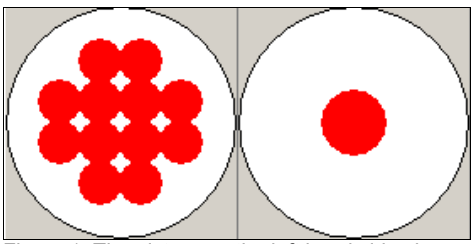


Figure 1: The picture on the left hand side shows a schematic view of the distribution of measurement points when using the multiple reads per well function (circle, 4x4). On the right side a single measurement is shown.

Microscopy: Stained cells were visualized using a 600 x magnification on an inverted fluorescence microscope (Olympus IX70) equipped with a Spot II CCD camera (Diagnostic Instruments). Pictures (Figure 6 and 7) were taken with the appropriate filter block swb (Ex 420 nm, Em 515 nm, 500 nm dichroic filter) for Calcein and swg (Ex 480 – 550 nm, Em 590 nm, dichroic filter 570 nm) for EthD-1.

Results

Spectra: Excitation and emission spectra of Calcein and EthD-1 were recorded (Figure 2). The excitation peak of Calcein was found at ~ **492 nm**, of EthD-1 at 535 nm; the emission maximum of Calcein was at ~ **514 nm**, of EthD-1 at ~ **610 nm**. Based on these findings the following filters were used for further measurements: Calcein: EX 485 nm/ EM 535 nm; EthD-1: EX 535 nm/ EM 635 nm.

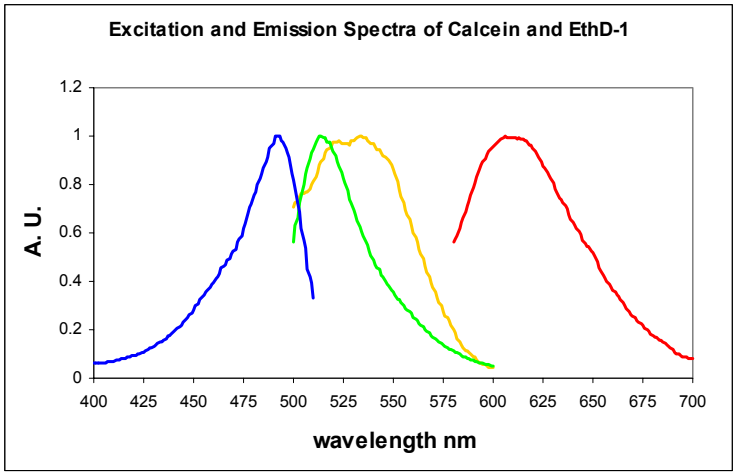


Figure 2: Normalized excitation and emission spectra of Calcein and EthD-1 (Excitation spectrum of Calcein; Excitation spectrum of EthD-1; Emission spectrum of Calcein; Emission spectrum of EthD-1)

Multiple Reads per Well versus Single Measurements: Figure 3 shows the comparison of a single measurement versus multiple reads per well (mrpw). Stained cells were treated with different concentrations of EtOH or Saponin as stated above and have been measured with either 10 flashes per well (single) or multiple reads per well (1 flash, 4x4, circle) at EX 485 nm (FWHM 20 nm, Safire: 12 nm)/EM 535 nm (FWHM: 25 nm; Safire: 12 nm) for Calcein and at EX 535 (FWHM: 25 nm; Safire: 12 nm)/EM. 635 (FWHM: 35 nm, Safire 12 nm) for EthD-1. Figure 3 shows the results of measurements of an EtOH dilution series performed with a Tecan Ultra Evolution. The results were comparable to those of Tecan GENios Pro and Safire (results not shown).

EtOH Dilution Series: Cells stained with Calcein and EthD-1 were treated with different concentrations of EtOH to determine the lethal dose. Figure 4 shows the results of the measurements performed with a Tecan Safire. Measurement wavelengths are the same as stated above, multiple reads per well. Tecan Ultra Evolution and GENios Pro showed comparable results (results not shown).

Saponin Dilution Series: Cells stained with Calcein and EthD-1 were treated with different concentrations of Saponin to determine the lethal dose. Figure 5 shows the results of the measurements performed with a Tecan GENios Pro. Tecan Ultra Evolution and Safire showed comparable results (results not shown).

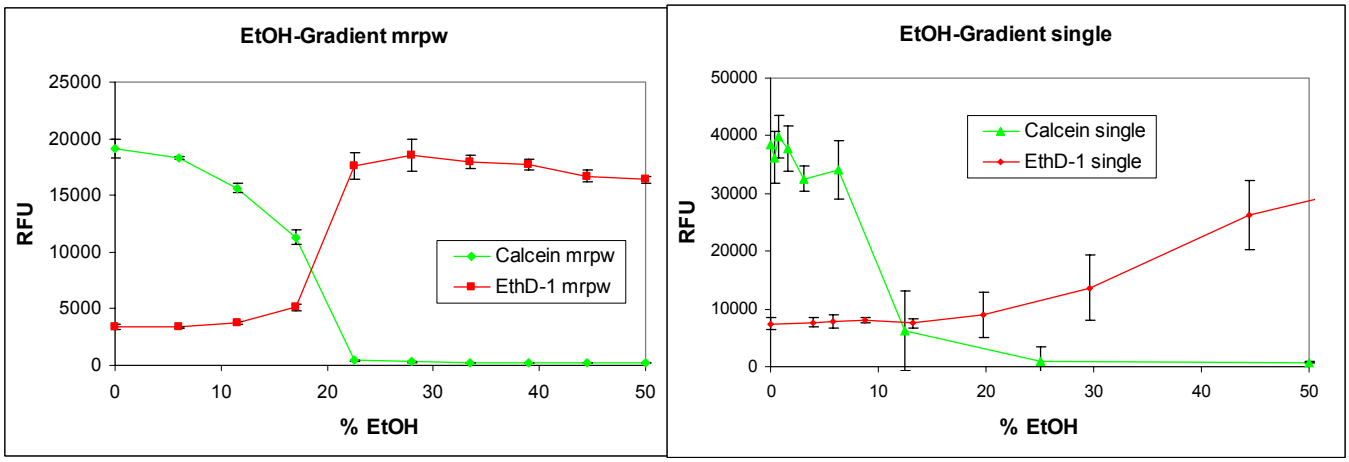


Figure 3: Mrpw (left picture) versus single (right picture) measurement of cells treated with different EtOH concentrations is shown in the pictures above. The measurement was performed with a Tecan Ultra Evolution.

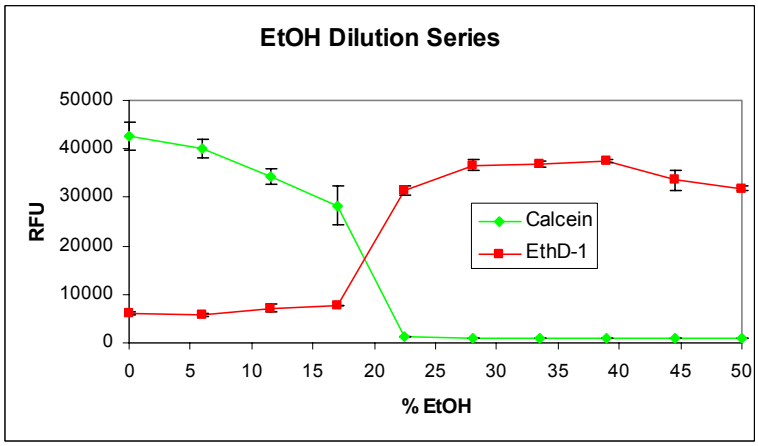


Figure 4: Measurement of Calcein and EthD-1 of EtOH dilution series with a Tecan Safire to determine the lethal EtOH concentration

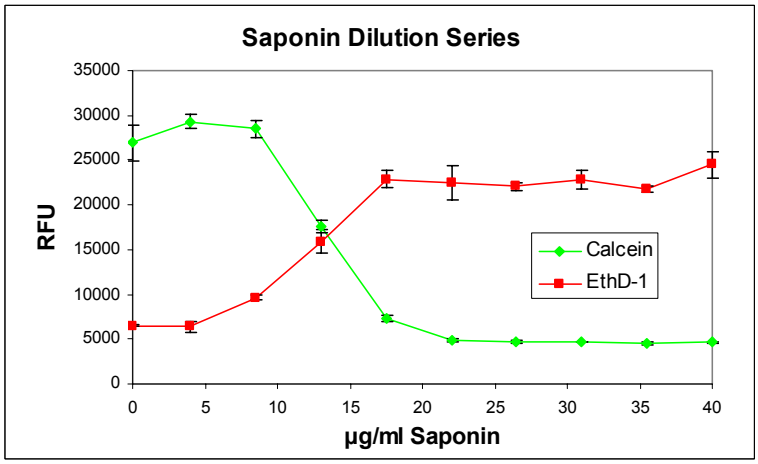


Figure 5: Measurement of Calcein and EthD-1 of a Saponin dilution series with a Tecan GENios Pro to determine the lethal Saponin concentration

Measurements with Magellan: When performing the measurements with Magellan, the software allows you to calculate automatically the percentage of live and dead cells using the following formulas (see equation 1 and 2); please refer to the LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, L-3224) description to get more details about necessary controls and plate layout:

Equation 1:

$$\%LiveCells = \frac{F(535)_S - F(535)_{min}}{F(535)_{max} - F(535)_{min}} * 100\%$$

$F(535)_S$ RFU of sample labeled with Calcein and EthD-1 at 535 nm
$F(535)_{min}$ RFU of control sample at 535 nm where all cells are alive, labeled with EthD-1 only
$F(535)_{max}$ RFU of control sample at 535 nm where all cells are alive, labeled with Calcein only

Equation 2:

$$\%DeadCells = \frac{F(635)_S - F(635)_{min}}{F(635)_{max} - F(635)_{min}} * 100\%$$

$F(635)_S$ RFU of sample labeled with Calcein and EthD-1 at 635 nm
$F(635)_{min}$ RFU of control sample at 635 nm where all cells are dead, labeled with Calcein only
$F(635)_{max}$ RFU of control sample at 635 nm where all cells are dead, labeled with EthD-1 only

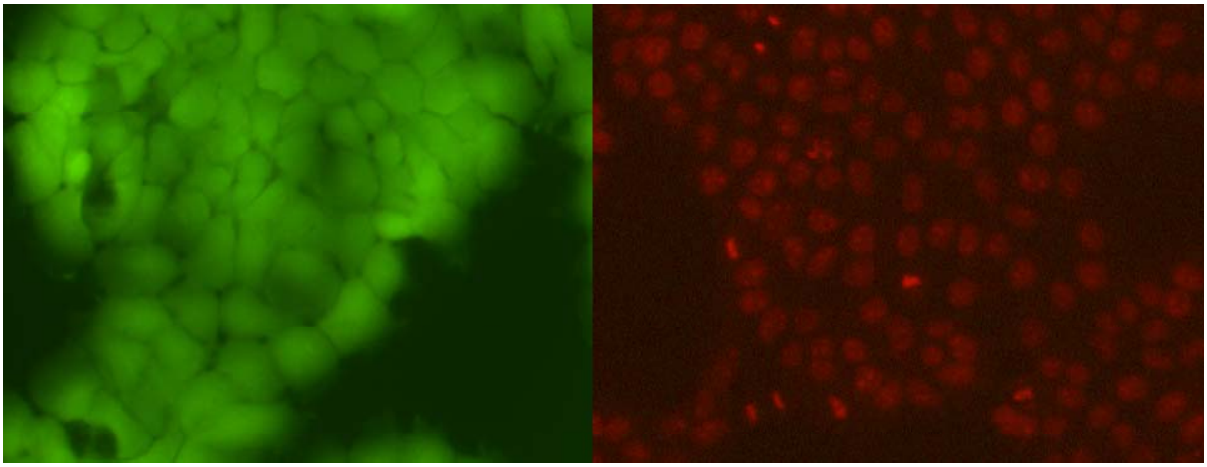


Figure 6: Live control cells (left picture: Calcein staining of the cytoplasm) and (dead) cells after treatment with 100 % Ethanol (right picture: EthD-1 staining of the nuclei)

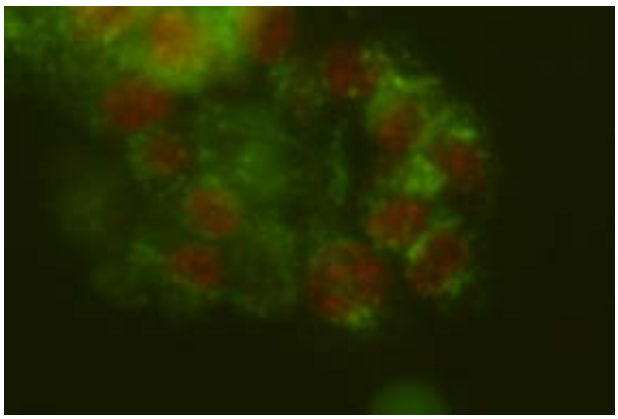


Figure 7: Cells treated with 21 µg/ml Saponin. Picture shows a mixture of live (green, Calcein staining of cytoplasm) and dead cells (red nuclei, EthD-1 staining)

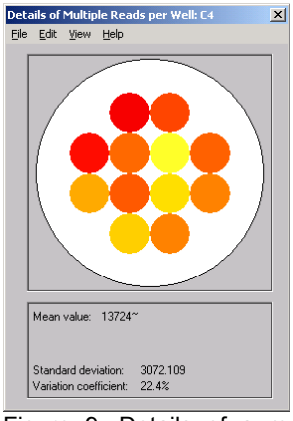


Figure 9: Details of a multiple read per well measurement of one well. Single measurement data are shown as colors. The darker the color the higher is the measured RFU value.

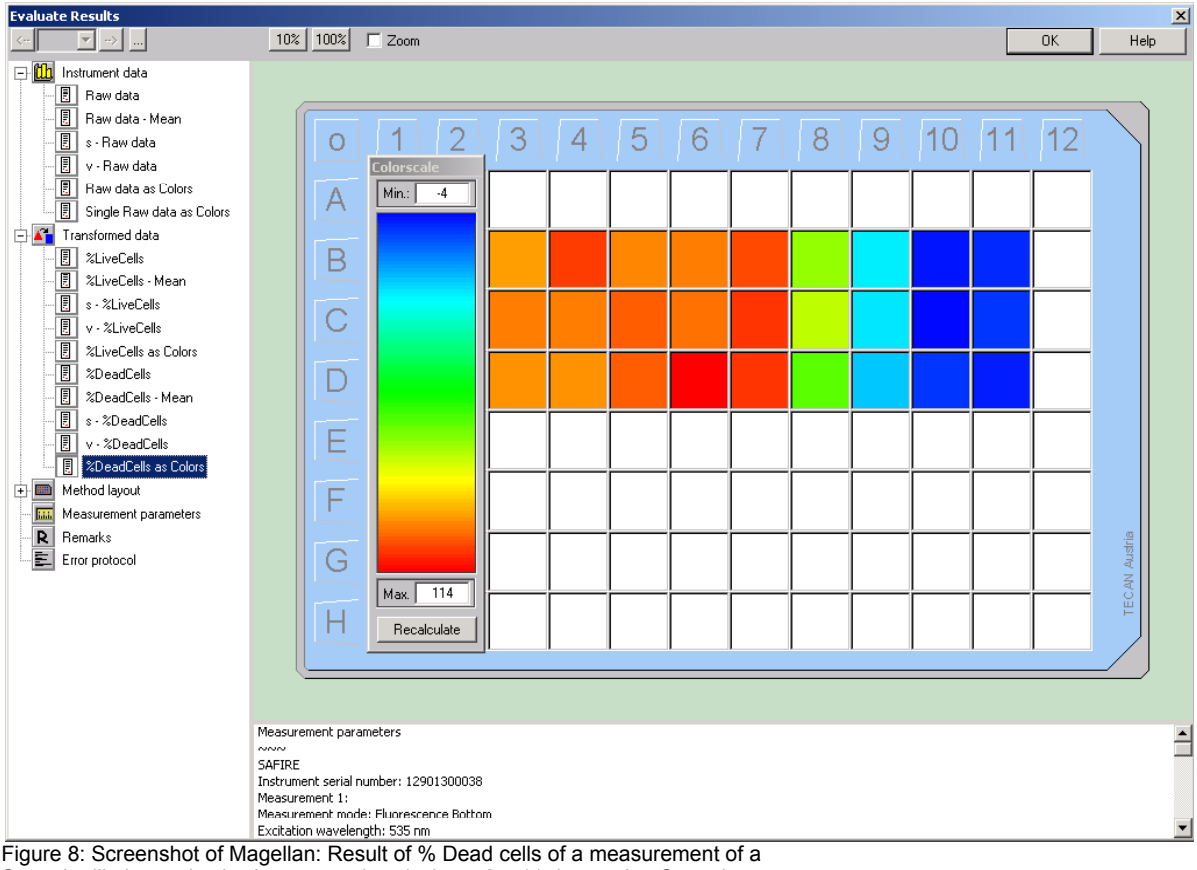


Figure 8: Screenshot of Magellan: Result of % Dead cells of a measurement of a Saponin dilution series is shown as colors (column 2 – 11 decreasing Saponin gradient, column 11: 100 % live cells).

Figure 8 shows a screenshot of Magellan displaying the results of a live-dead measurement. The displayed result is from a measurement of a Saponin dilution series taken with a Tecan Safire. %Dead Cells was calculated as stated in Equation 2 and visualized as colors.

Magellan is also able to visualize the single measurement points of a mrpw measurement within one well. Figure 9 shows the result of a sample at the switching point live to dead after applying different Saponin concentrations. The colors indicate the measured fluorescence intensity; the darker the color the higher is the measured RFU value.

The picture also gives an impression about the importance of the reading mode *multiple reads per well (mrpw)* for this application. The distribution of live and dead cells within one well might not be equal and therefore a single measurement could give a false live-dead switching point (see also figure 1 for graphical view of distribution of measurement points/well).

Discussion

The data above clearly show the ability of Tecan Ultra Evolution, Safire and GENios Pro to detect Calcein and EthD-1 and therefore to distinguish between live and dead cells with the bottom reading option of the instruments.

We recommend the use of the multiple read per well (mrpw) function when performing LIVE/DEAD® measurements. Figure 3 shows the effects when using single measurement points per well versus multiple reads per well. With single measurement points per well it is not possible to determine the lethal dose of EtOH and Saponin because of the unequal distribution of live and dead cells in a well (Figure 9). With the mrpw function a switching point of 19 % EtOH (Figure 4) and 14 µg/ml Saponin (Figure 5) was determined.

Magellan turned out to be a practical tool for automated evaluation of results. The software allows you to calculate the %live and %dead cells automatically and display them either as colors or percentage values. The visualization of results as colors makes it possible to easily determine the switching point (Figure 8) and the distribution of live and dead cells within one well (Figure 9).

Acknowledgement

We would like to thank Prof. Barbara Krammer (University of Salzburg, Institute of Biophysics) for the good cooperation. We also would 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

(1) www.probes.com; LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes: L-3224)

Glossary

AM	Acetoxymethyl
Cr	Chrome
DMEM	Dulbecco's Modified Eagle's Medium
EM	Emission
EthD-1	Ethidium homodimer
EtOH	Ethanol
EX	Excitation
FCS	Fetal calf serum
FWHM	Full width at half maximum, bandwidth
mrpw	Multiple reads per well
PBS	Phosphate buffered saline
Pen	Penicillin
RFU	Relative fluorescence unit
Strep	Streptomycin
TNF	Tumor necrosis factor
TTX	Tetrodotoxin

Tecan Asia (Pte) Ltd., 80, Marine Parade, #13-04, Singapore 449269, Singapore, T +65 644 41 886, F +65 644 41 836
Tecan Sales Austria GmbH, Untersbergstrasse 1a, A-5082 Grödig / Salzburg, Austria, T +43 62 46 89 33, F +43 62 46 72 770
Tecan Sales International GmbH, Untersbergstrasse 1a, A-5082 Grödig / Salzburg, Austria, T +43 62 46 89 33, F +43 62 46 72 770
Tecan Benelux B.V.B.A., Vaartdijk 55, B-2800 Mechelen, Belgium, T +32 15 42 13 19, F +32 15 42 16 12
Tecan Benelux B.V.B.A., Industrieweg 30, NL-4283 GZ Giessen, Netherlands, T +31 18 34 48 17 4, F +31 18 34 48 06 7
Tecan Deutschland GmbH, Theodor-Storm-Straße 17, D-74564 Crailsheim, Germany, T +49 79 51 94 170, F +49 79 51 50 38
Tecan France S.A., Parc d'Activités de Pissaloup, Bâtiment Hermes II, Rue Edouard Branly, F-78190 Trappes, France, T +33 1 30 68 81 50, F +33 1 30 68 98 13
Tecan Italia S.r.l., Via F.lli Cervi, Palazzo Bernini, Centro Direzionale Milano 2, I-20090 Segrate (Mi), Italy, T +39 02 215 21 28, F +39 02 215 97 441
Tecan Japan Co. Ltd, Meiji Seimei Fuchu Building 10F, 1-40 Miyamachi, Fuchu City, Tokyo, Japan, T +81 42 334 88 55, F +81 42 334 04 01
Tecan Nordic AB, Box 208, SE-431 23 Mölndal, Sweden, T +46 31 75 44 000, F +46 31 75 44 010
Tecan Portugal, Quinta da Fonte - Edificio Pedro I, 2780-730 Paço d'Arcos, Portugal, T +351 21 000 82 16, F +351 21 000 16 75
Tecan Sales Switzerland AG, Seestrasse 103, CH-8708 Männedorf, Switzerland, T +41 1 922 89 22, F +41 1 922 89 23
Tecan Spain, Sabino de Arana, 32, E-08028 Barcelona, Spain, T +34 93 490 01 74, F +34 93 411 24 07
Tecan UK, Theale Court, 11-13 High Street, Theale, UK-Reading RG7 5AH, United Kingdom, T +44 11 89 300 300, F +44 11 89 305 671
Tecan US, P.O. Box 13953, Research Triangle Park, NC 27709, USA, T +1 919 361 5200, F +1 919 361 5201

www.tecan.com