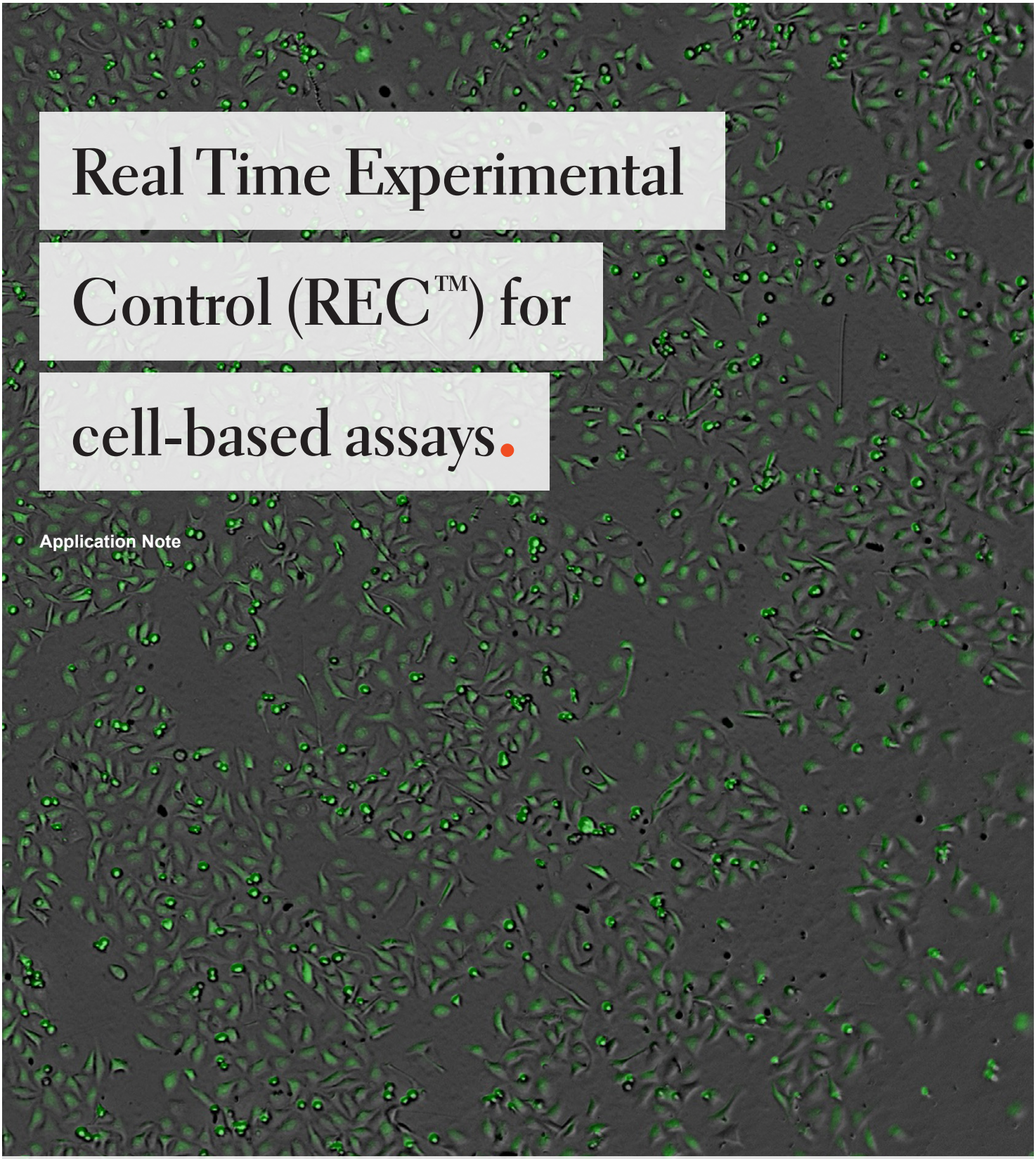
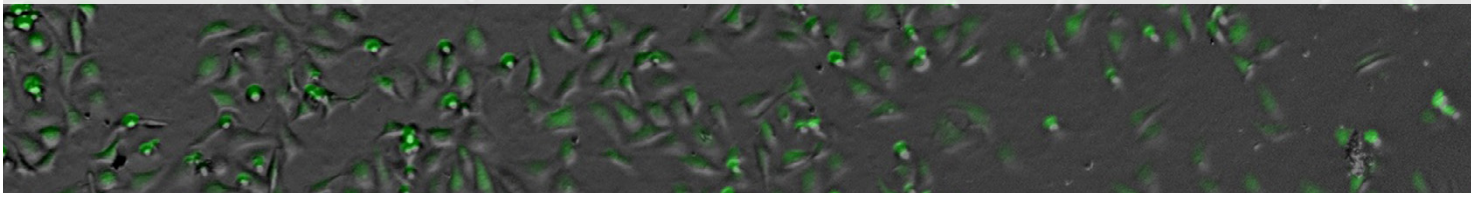


Real Time Experimental Control (REC™) for cell-based assays.

Application Note

WORKFLOW AUTOMATION WITH THE SPARK® CYTO MULTIMODE READER



INTRODUCTION

The Spark Cyto enables semi-automation of workflows for cell culture experiments. The combination of multimode detection and fluorescence imaging capabilities makes it possible to perform visual and functional examination of cell samples in a single experiment.

The Spark Cyto is equipped with a high-tech imaging module, incorporating three user-selectable objectives, four fluorescence channels, LED-based illumination and a rapid and highly reliable LED-based autofocusing system. Operated via the intuitive SparkControl™ software, the system offers advanced features – such as full environmental control and kinetic conditioning software– providing a walkaway solution for long-term cell-based assays and complex experimental set-ups.

Many live cell kinetic experiments require specific actions at different points in the experiment – for example, the addition of a compound once a certain confluence is reached. **Real Time Experimental Control (REC)** allows certain actions – such as injection of reagents – to be triggered automatically as part of a kinetic experiment, and offers real-time data and image analysis for complete confidence in your results.

This application note demonstrates how REC can be used as part of a compound screening workflow, showing how it is set up within SparkControl, and the valuable information that can be obtained from a single experiment.

MATERIALS AND METHODS

GFP-transfected HeLa cells (ATCC® Number: CCL-2™) were cultured in RPMI 1640 medium (Gibco, #31870074; supplemented with 10 % FBS) at 37 °C and 5 % CO_2 in a humidified atmosphere. Cells harvested with trypsin/EDTA were then counted to determine exact cell numbers, and 15,000 cells/well were seeded in 300 µl of medium in a 96-well, black tissue culture plate (TEC96fb_cell_clear). The cells were allowed to adhere before the unlidded plate was placed into the Spark Cyto's large Humidity Cassette filled with 5.2 ml of deionized water per reservoir. The reader's injectors were rinsed with 70 % EtOH and deionized water, then primed with 1 % saponin solution (Roth, #4185, diluted in growth medium).

Creating the method in SparkControl

The aim of this experiment was to monitor cell growth with the Spark Cyto, and determine the cytotoxic effects of different concentrations of saponin over time as part of a semi-automated workflow. In order to standardize the experiment, the injection of saponin was conditionally programmed to be carried out when the confluence of a reference well reached the chosen level.

In brief, the method consisted of gas, humidity and temperature control, and a kinetic loop consisting of fluorescence imaging, bottom reading fluorescence (FI bottom) measurements and conditional saponin injection.

The plate definition was chosen and the whole plate was selected for measurement within the predefined plate strip. In addition, the large Humidity Cassette and 'No lid' options were selected (see Figure 1).

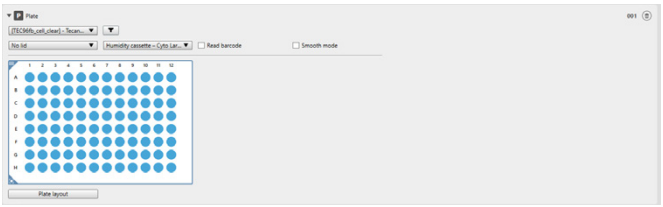


Figure 1: Plate strip highlighting wells to be imaged, and the use of the Humidity Cassette.

The large Humidity Cassette containing the test plate was placed in the instrument, and a kinetic measurement protocol was set up in Method Editor using the detection strips and settings shown in Figure 2 and Table 1, respectively.

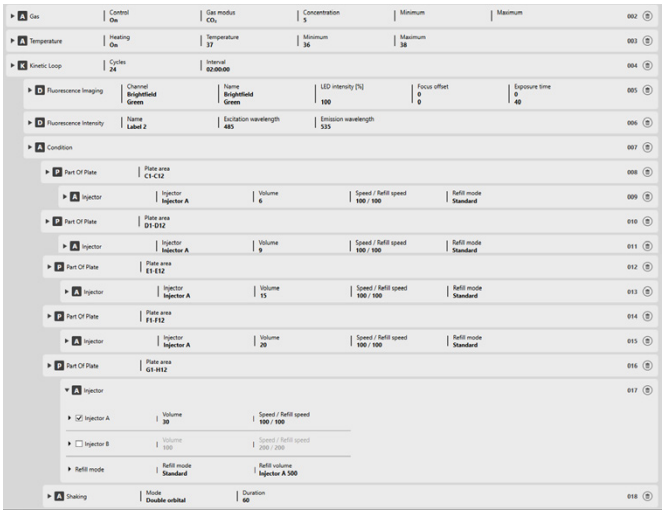


Figure 2: Collapse of all strips necessary for the kinetic experiment, including conditional injection.

Environmental control settings were defined outside of the kinetic loop, with CO_2 concentration set to 5 % and temperature to 37 °C. Within the kinetic loop strip, 24 cycles, at 2 hour intervals, were defined.

Parameter	**Setting**
Plate	TEC96fb_cell clear
Gas control	CO_2 5 %
Temperature	37 ˚C
Measurement mode	**Kinetic**
No. of cycles	24
Interval time	2 h
Measurement mode	**Fluorescence imaging**
Application	User defined
Objective	4x
Pattern	Whole-well
Border offset	50 µm
Bright field	
Focus offset	0 µm
Sensitivity	50 %
Object size	4-30 µm width, 4-40 µm length
Green channel	
LED intensity	100 %
Focus offset	0 µm
Exposure time	40 ms
Sensitivity	70 %
Object size	4-30 µm width, 4-40 µm length
Measurement mode	**FI bottom**
Excitation	485 nm
Emission	535 nm
Gain	Calculated from A1
Flash number	30
Integration time	40 µs

Table 1: Measurement settings.

FI bottom measurements were performed with the gain and Z-value calculated from well A1 (untreated control) using the Optimal Read function.

A Condition strip leads to the execution of all following (indented) strips. In this case, several conditions are set, for the injection of different volumes of the saponin working solution on reaching a specified confluence level of >89 % in a reference well (B5). Within each condition the box ‘Executed once’ was checked (see Figure 3).

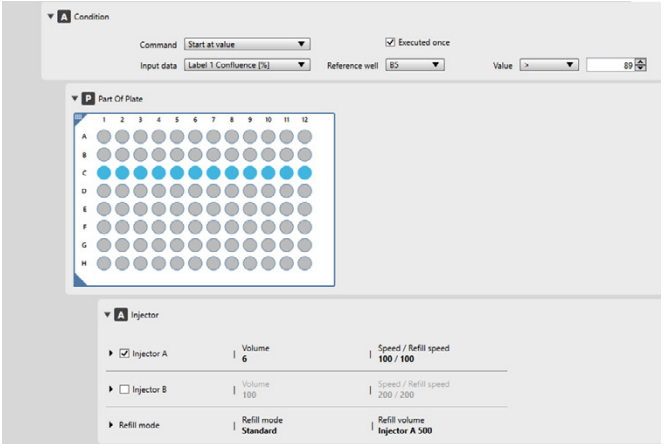


Figure 3: Condition and Part of Plate strips, including settings for a single saponin injection.

Injection was then performed via an Injector strip. A separate Condition, Part Of Plate and Injector strip was created for each injection volume – 6, 9, 15, 20 and 30 µl – resulting in final concentrations of 0.02, 0.03, 0.05, 0.06 and 0.1 % saponin.

Table 2 shows the final plate layout for the test plate indicating the injection volumes of 1 % saponin solution and the final saponin concentrations in the wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Untreated (no saponin injected)											
B												
C	0.02 % (6 µl of 1 % saponin solution injected)											
D	0.03 % (9 µl of 1 % saponin solution injected)											
E	0.05 % (15 µl of 1 % saponin solution injected)											
F	0.06 % (20 µl of 1 % saponin solution injected)											
G	0.1 % (30 µl of 1 % saponin solution injected)											
H												

Table 2: Plate layout indicating the final saponin concentrations (%) after injection.

The Shaking strip was used to ensure even distribution of saponin within each wells. Shaking was performed in situ for 60 seconds, using a double orbital mode, an amplitude of 2.5 mm, and a frequency of 108 rpm (see Figure 4).

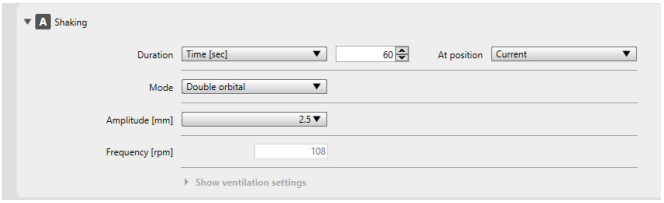


Figure 4: Shaking strip displaying settings for REC experiment.

The method was paused after 10 cycles, the wells were replenished with 100 µl of fresh, prewarmed medium, and the reservoirs of the Humidity Cassette were refilled with 5.2 ml of deionized water to ensure optimal experimental conditions.

RESULTS

While the default sensitivity settings were chosen for method execution (as shown in Table 1) post-acquisition optimization to 83 % sensitivity for the green fluorescence channel was performed using Image Analyzer.

The results show that the green object count steadily increases over time, until the confluence condition was fulfilled at cycle 12 (Figure 5).

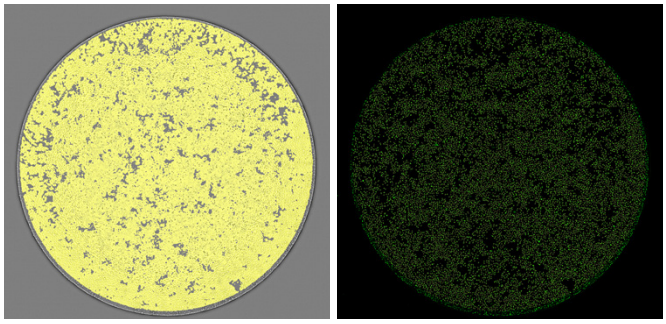


Figure 5: Confluency mask and fluorescent image of B5 reference well at cycle 12.

Following saponin injection, the green object count steadily decreased in a concentration-dependent manner in all treated wells, whereas untreated controls showed an increase in green object count until a plateau was reached at cycle 17 (see Figure 6).

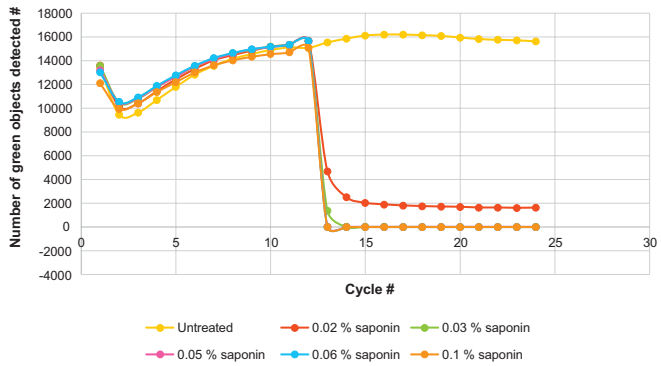


Figure 6: Number of green objects detected, plotted against cycle number (total of 48 h, 2 h interval time, injection of saponin at cycle 12).

All saponin concentrations led to a large decrease in FI bottom signal (up to a max. signal of ~33,000 RFU, see Figure 7), confluence level (13 % for 0.06 % saponin, see Figure 8) and almost complete loss of green object count (Figure 6).

For 0.02 % saponin, the curve's course was less steep, meaning the cells lost viability more slowly, demonstrating the concentration dependency of saponin-induced cell death.

The slight increase of FI bottom signal for samples treated with 0.03, 0.05, 0.06 and 0.1 % saponin can be explained by the background signal of additional GFP being released from dead or dying cells, which have lost their membrane integrity.

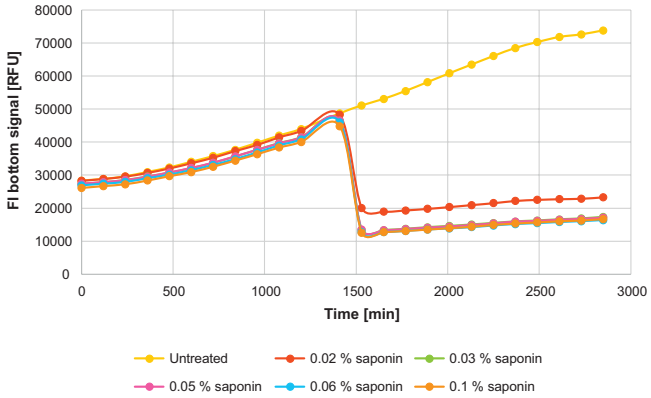


Figure 7: GFP signal of HeLa GFP cells plotted against cycle number (total of 48 h, 2 h interval time, injection of saponin at cycle 12).

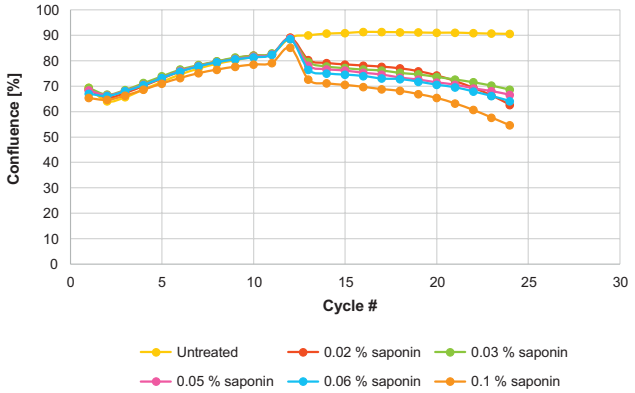


Figure 8: Confluence level of HeLa GFP cells plotted against cycle number (total of 48 h, 2 h interval time, injection of saponin at cycle 12).

Complementary detection modes, combining the area algorithm for bright field imaging and the segmentation algorithm for the green fluorescence channel, provide corroborative results within a single experiment.

CONCLUSIONS

The Spark Cyto multimode reader is ideally suited for semi-automated workflows combining live cell imaging with other detection modes. A single experiment approach allows efficient generation of results from multiple detection modes, under the same standard conditions and time points, eliminating the need and expense of performing multiple cellular assays in parallel. The result-dependent Condition strip enables detection (eg. absorbance) or actions (eg. injection) to be carried out at the right time, without the user needing to be present for subjective assessments, reducing restrictions on when experiments can be performed and enhancing the reproducibility of results. Using the Spark Cyto's advanced features, procedures can be standardized and productivity enhanced, allowing high quality reproducible results to be obtained every time.

ABBREVIATIONS

GFP	green fluorescent protein
FI	fluorescence intensity
LED	light-emitting diode
REC	real-time experimental control
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
CO_2	carbon dioxide
RFU	relative fluorescence units

About the authors

Dr. Nicole Eggenhofer is an application specialist at Tecan Austria. She studied genetics at the University of Salzburg and focused on cell biology and microbiology during her Ph.D. Nicole gained further experience in the field of molecular biology before joining Tecan in 2017. She has been involved in the development of Spark Cyto.

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Dr. Lynda O'Leary is an application specialist at Tecan Austria. She received her PhD in Biochemistry at the National University of Ireland, Galway, working in the field of apoptosis and cell signaling. Following this, she joined the Institute of Cancer Research in London as a postdoctoral fellow focusing on metastatic dormancy in breast cancer. Lynda has extensive experience in a wide range of molecular cell biology techniques, cell culture and imaging. In 2018, she joined the sales and marketing department with a primary focus on the Spark Cyto multimode microplate reader.

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Tecan has not independently validated this method with all possible sample types or analytical uses and is providing this example method as a convenience to users. The user must independently evaluate and validate: (a) the suitability of the method for their use, (b) their ability to process samples of their choosing following the method; and (c) their ability to proficiently perform the method in their facility with their personnel.

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