CYTENA BPS
A BICO COMPANY

S.NEST | A modern screening platform with real-time dissolved oxygen and pH monitoring as novel cytotoxicity assay for drug screening

Shih-Pei (Betty) Lin, PhD
CYTENA Bioprocess Solutions, Taipei City, Taiwan

Abstract

Drug discovery requires screening a large number of drug candidates for their efficacy, cytotoxicity, and possible side effects. This screening process is labor- and time-consuming. In this application note, we propose a new high-throughput drug screening platform by leveraging S.NEST, which is a microbioreactor capable of using real-time monitoring of dissolved oxygen (DO) and pH to determine oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) throughout cell culture and treatment. This platform eliminates traditional endpoint cell viability assays and provide prompt indications of the cell growth index, significantly improving the efficiency of the drug screening process. By monitoring cellular metabolic activity and viability in real-time, we can accurately and rapidly evaluate drug candidates without the need for pre-testing to determine treatment and sampling timepoints. This platform therefore has the potential to streamline drug screening, enhance efficiency, and advance the field of drug discovery.

Introduction

In vitro cell culture systems play a crucial role in preclinical drug screening by providing insights into cellular response to drugs and treatments. However, it can be time-consuming and labor-intensive to evaluate cellular response in cell culture systems. To overcome these challenges, real-time monitoring has emerged as a promising solution. Real-time monitoring reduces time and labor requirements by eliminating frequent sampling for end-point assays. Moreover, real-time monitoring could improve the chances of obtaining useful data via multiple means. For instance, real-time data improves precision in controlling the cell culture conditions and "in plate" measurement reduces the risk of contamination. Taken together, real-time monitoring delivers better results with less time and resources, significantly improving the efficiency and throughput of evaluating the efficacy and toxicity of candidate drugs. [2,3]

When evaluating cellular response, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) are two commonly used indicators for core energy metabolism. Mammalian cells rely on both oxidative and glycolytic metabolism to generate ATP and biosynthetic precursors. OCR quantifies the oxygen depletion in oxidative metabolism, while ECAR reflects the accumulation of metabolic byproducts (e.g. lactate) in glycolytic metabolism and the generation of CO_2 from oxidative metabolism. In the case of cancer therapy, for example, many drugs are designed to inhibit key enzymes in core metabolism or to induce apoptosis. Therefore, changes in metabolism and proliferation activity resulting from drug treatment can be detected by observing alterations in OCR and ECAR.

To evaluate cellular response in real-time via OCR and ECAR, we propose a new drug screening platform based on the S.NEST microbioreactor. S.NEST is an advanced microbioreactor for high-throughput cell culture. It consists of four independent chambers, where each chamber can hold a 24-well culture plate under precisely controlled conditions (**Figure 1A**). Each well in the 24-well plate incorporates two sensors for real-time monitoring of dissolved oxygen (DO) and pH in the cell culture medium (**Figure 1B**), from which OCR and ECAR are typically derived. The accuracy of these sensor measurements is supported by homogenizing the culture medium (**Figure 1C**) with pneumatic mixing.

Our proposed drug screening platform can greatly accelerate early-stage drug screening for drug developers and oncologists. As previously mentioned, the ability to continuously monitor cellular behavior in real-time eliminates the need for laborious and time-consuming endpoint assays, thereby expediting the screening process and reducing operational costs. More specifically to drug screening, by monitoring OCR and ECAR through non-invasive real-time measurements of DO and pH, researchers can gain valuable insights into the impact of candidate drugs on cellular metabolism and proliferation activity. Such insights allow the researchers to infer information on efficacy, cytotoxic effect, and optimal dosage in real-time prior to the end of the experiments. [4]

In this application note, we demonstrate the use of our proposed drug screening platform with A549 cells, a widely used model for the study of lung cancer [5]. A549 cells (human lung carcinoma) are an epithelial cell line derived from lung adenocarcinoma. In this study, A549 cells were treated with either apoptosis activator or chemotherapy drug in the S.NEST system with real-time DO and pH monitoring. By comparing real-time data against end-point cell viability measurements, we demonstrated that the real-time DO and pH level changes can assess cytotoxicity in early stage drug treatment.

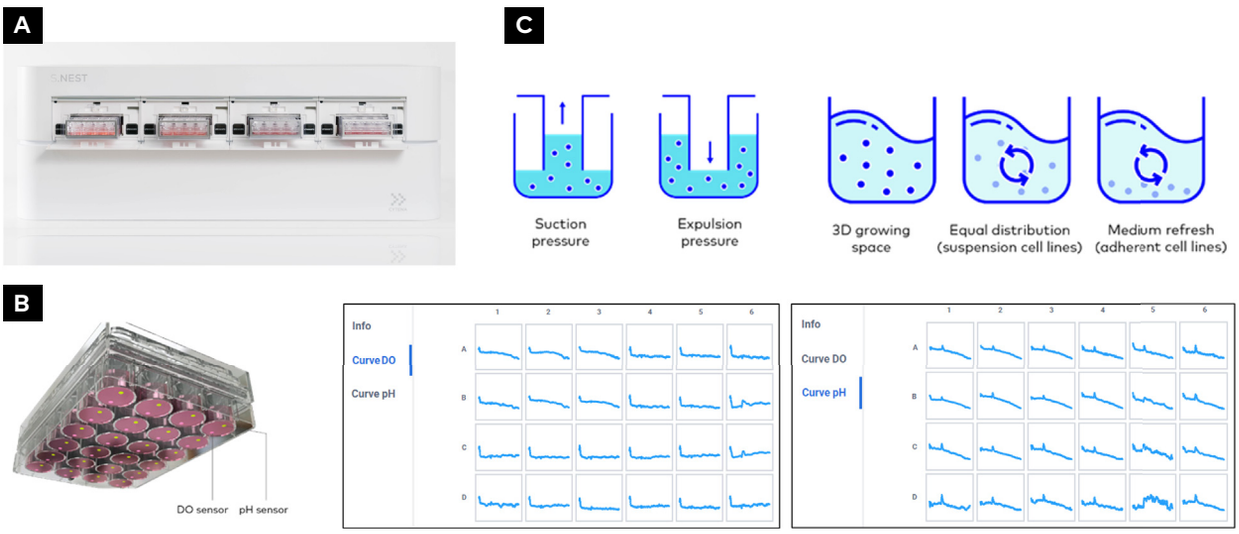

Figure 1. (A) *The S.NEST culture system contains 4 independent chambers for cell culture.* ***(B)*** *To provide real-time monitoring of DO and pH in the S.NEST system, sensor spots were attached to the bottom of the microplates for detection.* ***(C)*** *The S.NEST lid allows for a mixing culture mode to make medium more homogenous and DO and pH level more consistent across the medium.*

Materials and methods

Materials

Methotrexate (MTX) (#GC47649) was purchased from GLPBIO (Montclair, CA, USA). Staurosporine (SSP) (#A10864) was purchased from AdooQ BioScience (Irvine, CA, USA). The final concentrations of the drug vehicle (DMSO) added to the cell cultures were all <0.1%. All other chemicals used in the present study were of the highest purity commercially available for cell culture.

Cell culture

The human-derived alveolar adenocarcinoma cell line A549 was cultured in DMEM (Thermo Fisher Scientific, Gibco, #11960-044) supplemented with 10% fetal bovine serum (ThermoFisher, Gibco, #A31606-01), 4 mM L-glutamine (Corning, #25- 005-CI), 1 mM sodium pyruvate (Corning, #25- 000-CI), 100 µg/mL streptomycin and 100 U/ mL penicillin (Thermo Fisher Scientific, Gibco, #5140122), in a humidified 5% CO_2 atmosphere at 37°C. A549 cells were passaged every 3 to 4 days when ~80% confluent.

Drug treatment and DO/pH monitoring in S.NEST

A549 cells were seeded at a density of 5 × 10^4 cells/ well in a 24-well S.NEST sensor plate (CYTENA BPS, #PX24A006) for real-time DO/ pH monitoring. 18 mL of Dulbecco's phosphate-buffered saline (DPBS) (Corning, #21-031-CM) was added to the inter-well spacing to reduce evaporation. Cells were cultured in the CO_2 incubator for 6-8 h. Then the S.NEST lid was placed onto the sensor plate and transferred to S.NEST with 50 secs/cycle mixing rate and DO/ pH monitoring. The fluorescent signals of the pH and DO sensor spots in the microplate were detected by the camera module equipped in the S.NEST system, with which data was collected every 10 min. After 20 h, the culture process was paused using the pause function of S.NEST, which temporarily suspends the DO/pH monitoring to prevent discontinuity in the data. The cells were treated with DMSO (vehicle control), MTX (50 nM, 100 nM) and 100 nM SSP for 48 h. The treated cells were further incubated in S.NEST with 50 secs/ cycle mixing rate and DO/pH monitoring. After 48 h, cell viability was analyzed with PrestoBlue assay. The OCR and ECAR values were calculated from real-time DO/pH values.

Cell culture

The PrestoBlue™ Cell Viability Reagent (#A13261) was purchased from Thermo Fisher Scientific Inc (Waltham, MA, USA). The PrestoBlue assay was used for detecting metabolic activity of mammalian cells and hence only live cells could be detected. All viability assays were performed on A549 cells with the PrestoBlue reagent according to the manufacturer's protocols. Briefly, after 48 h exposure of A549 cells to SSP or MTX, 120 µL of PrestoBlue solution (10% in culture medium) was added into the wells containing 1.2 mL medium and the plate was incubated in darkness for 60 min at 37 °C. After incubation, 100 µL of the PrestoBlue/medium solution was transferred to the assay plate. Final cell viability was determined

by measuring the resulting fluorescent signal using the microplate reader (SpectraMax iD3, SoftMax® Pro Software, Molecular Devices, San Jose, CA, USA) with the excitation/emission wavelengths set at 550/600 nm.

Statistical analysis

The data points were expressed as the mean ±standard deviation (SD). Statistical analyses were performed using one-way ANOVA followed by Tukey's test for multiple comparisons test. A p value of less than 0.05 was considered a statistically significant difference. The P values are indicated listed as the following: $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) and $P \leq 0.0001$ (****).

Results and discussion

Establishment of drug screening procedure with adherent cell in S.NEST

In traditional drug screening processes, A549 cells were seeded in microplate overnight for attachment and then treated with different therapeutic drugs at different concentrations. After 48-72 h of treatment, the cytotoxicity and efficacy of the drug was assessed by end-point cell viability assay. The end-point assays of multiple time points were needed to determine the response time of various drugs and dosages. This procedure was very laborious.

In this application study, we chose two reagents to mimic the process of drug screening. Staurosporine (SSP), a nonselective protein kinase inhibitor, has been shown to induce apoptosis in several nonneuronal cell types. [6] Methotrexate (MTX) is known to induce apoptosis in A549 cells. [7] To measure cell response time using cell viability assay under the traditional workflow, A549 cells were seeded with $5x10^4$ cells/well (~2.6x104/ cm^2) in a 24-well plate and treated with DMSO (control), 100 nM SSP and 100 nM MTX for 48 h. The cell viability was measured at 24 h and 48 h after drug treatment. The results showed that the cell viability was significantly reduced at 24 and 48 h after treatment by 100 nM SSP, but not by 100 nM MTX (**Figure 2A**). The difference in cell viability between different treatments was significant at 24 h but not at 48 h after treatment. Under this traditional workflow, it was difficult to define specific reaction times for different drugs. Therefore, we proposed using a novel drug screening platform with real-time monitoring to validate the drug response during the 48 h after drug treatment.

For cell culturing and DO/pH monitoring in S.NEST, A549 cells were also seeded with $5x10^4$ cells/well (~2.6x104/cm^2) but in a 24-well sensor plate. DMSO was added as a solvent control. The positions of different treatments in the 24-well sensor plate is shown in **Figure 2B**. After cell seeding, A549 cells were cultured statically in a CO_2 incubator for 6-8 h until the cells were fully attached to the well plate (**Figure 2C, micrograph**). After cell attachment, a functional lid for mixing culture was placed on the sensor plate and the lid/plate system was cultured in S.NEST with DO/pH monitoring. After 20 h of DO/pH monitoring, the A549 cells were treated with 50 nM MTX, 100 nM MTX or 100 nM SSP. After drug treatment for 48 h, the cell viability was measured with the Prestoblue cell viability assay. This full procedure is shown in **Figure 2C**.

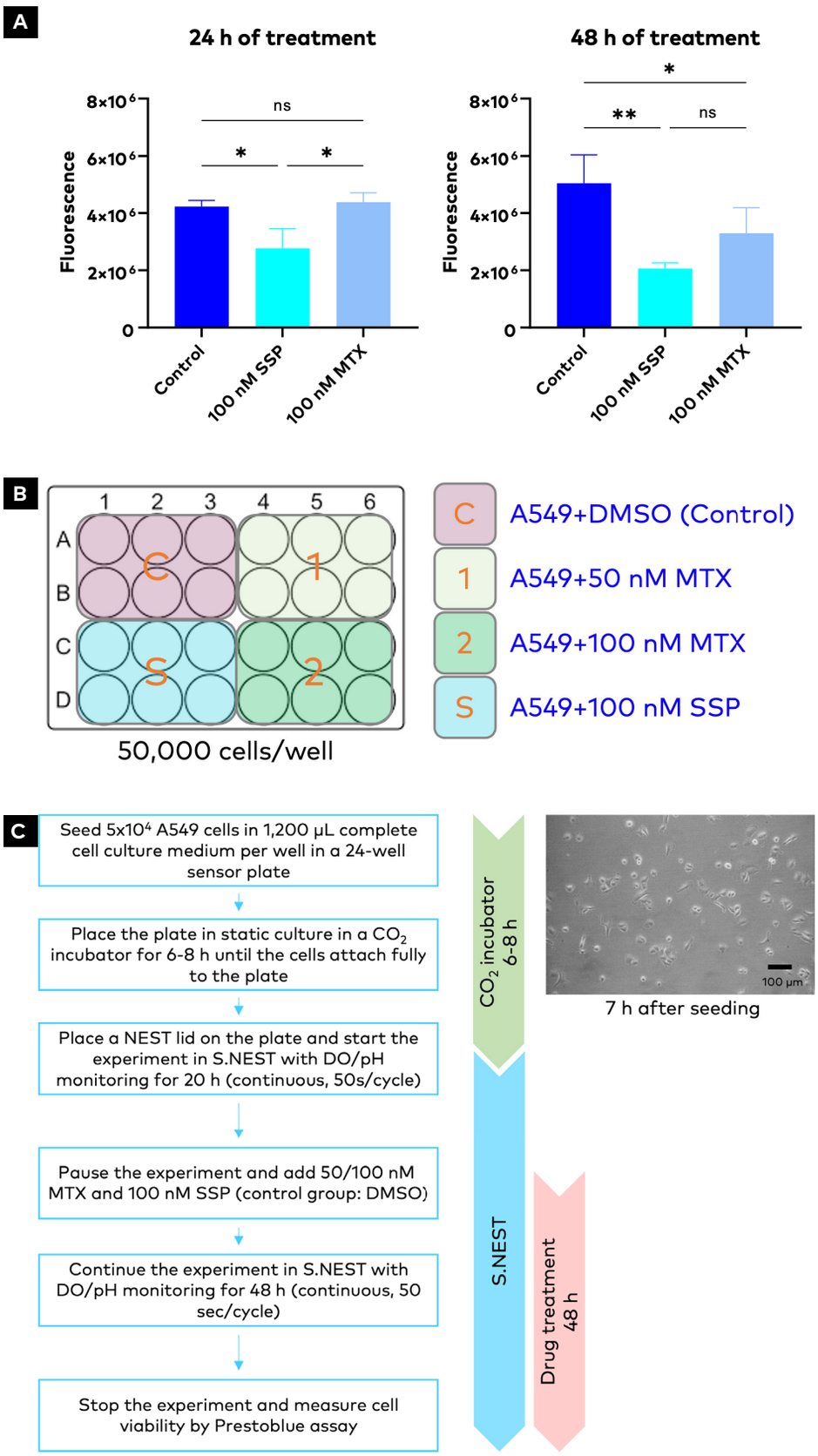

Figure 2. (A) *Prestoblue cell viability assay at 24 h and 48 h after drug treatment. The P values are indicated as: P ≤ 0.01 (**), P ≤ 0.001 (***) and P ≤ 0.0001 (****). Data were shown as mean ± SD.* ***(B)*** *Schematic diagram of different drug treatment conditions in the microplate.* ***(C)*** *Procedure for drug screening using A549 cells in the S.NEST culture platform.*

Using real-time DO/pH monitoring and OCR/ ECAR measurement to evaluate cytotoxicity after drug treatment

DO and pH play a pivotal role in reflecting cell physiological status, as we have already demonstrated in our previous application study. [8] In this study, all group of A549 cells presented almost identical changes in DO and pH values in the first 20 h of S.NEST cultivation (**Figure 3A, 3B**). This alignment means cell growth and metabolism of all group had the same growth profile before drug treatment. After the drug treatment, DO and pH values in different groups started showing different trends over time. The DO curve of all treated groups started to separate from the control group at 8 h after treatment. At 24 h after treatment, the treatment groups have widened their differences from the control groups. The decrease in DO (8 to 24 h after treatment) for control, 50 nM MTX, 100 nM MTX and 100 nM SSP groups were 4.6%, 1.7%, -3.3% and -0.04%. At the end of the experiment, the decrease in DO (8 to 48 h after treatment) for control, 50 nM MTX, 100 nM MTX and 100 nM SSP groups were 20.7%, 3.2%, -0.2% and -3.8% (**Figure 3A**).

OCR was calculated from DO values and used to evaluate cell growth and metabolism activity. After 20 h of culture in S.NEST, the initial OCR of all group before treatment was 0.301-0.373 µm/ min. At 12 h after treatment, the OCR of treated groups were maintained at 0.355-0.391 µm/min and the OCR of control group raised to 0.542 µm/ min, indicating that the cell growth continued in control but not in treated groups. After another 12 h, the OCR of 100 nM MTX was 0.170 µm/ min, significantly lower than that of the 50 nM MTX and 100 nM SSP treated groups (0.321 and 0.353 µm/ min). At the same time point, the OCR of the control group was 0.813 µm/min, which is 4.78-folds of 100 nM MTX, 2.53-folds of 50 nM MTX and 2.30-folds of 100 nM SSP treated groups (**Figure 3C**). These results indicate that cells in control group were still proliferating while the drug treatment limited cell growth and induced cell death.

The lowering of pH values followed the decrease in DO. Decreasing oxygen indicates increasing biomass and metabolism activity. This metabolic activity results in by-products that lowered the pH of the media. In this study, the pH curve of treated groups showed a mild decline (compared against the control group) from 8 to 24 h after drug treatment. The decrease in pH of 50 nM MTX, 100 nM MTX and 100 nM SSP groups were 0.037, 0.037, 0.050, respectively, while the decrease in the control group was 0.09, which was more than in treated groups. At the end of the experiment, the decrease in pH (8 to 48 h after treatment) of control, 50 nM MTX, 100 nM MTX and 100 nM SSP groups were 0.31, 0.13, 0.13 and 0.15 (**Figure 3B**). The differences between these values indicated that cell growth was affected by drug treatment.

ECAR was calculated from pH values. After 20 h of S.NEST cultivation, the initial ECAR of all group before treatment was 0.020-0.054 mpH/ min. At 12 h after treatment, the ECAR of treated groups were 0.069-0.074 mpH/min and the ECAR of control group was around 0.099 mpH/min, indicating that the cellular metabolic activity of control group was higher than of treated groups. After another 12 h, the ECAR of treated groups decreased to 0.040-0.060 mpH/min and the ECAR of control group raised to 0.105 mpH/min. The difference in the ECAR of control and treated groups has increased from 1.75-fold to 2.63-fold, indicating that the drug treatment significantly decreased cellular metabolic activity (**Figure 3D**).

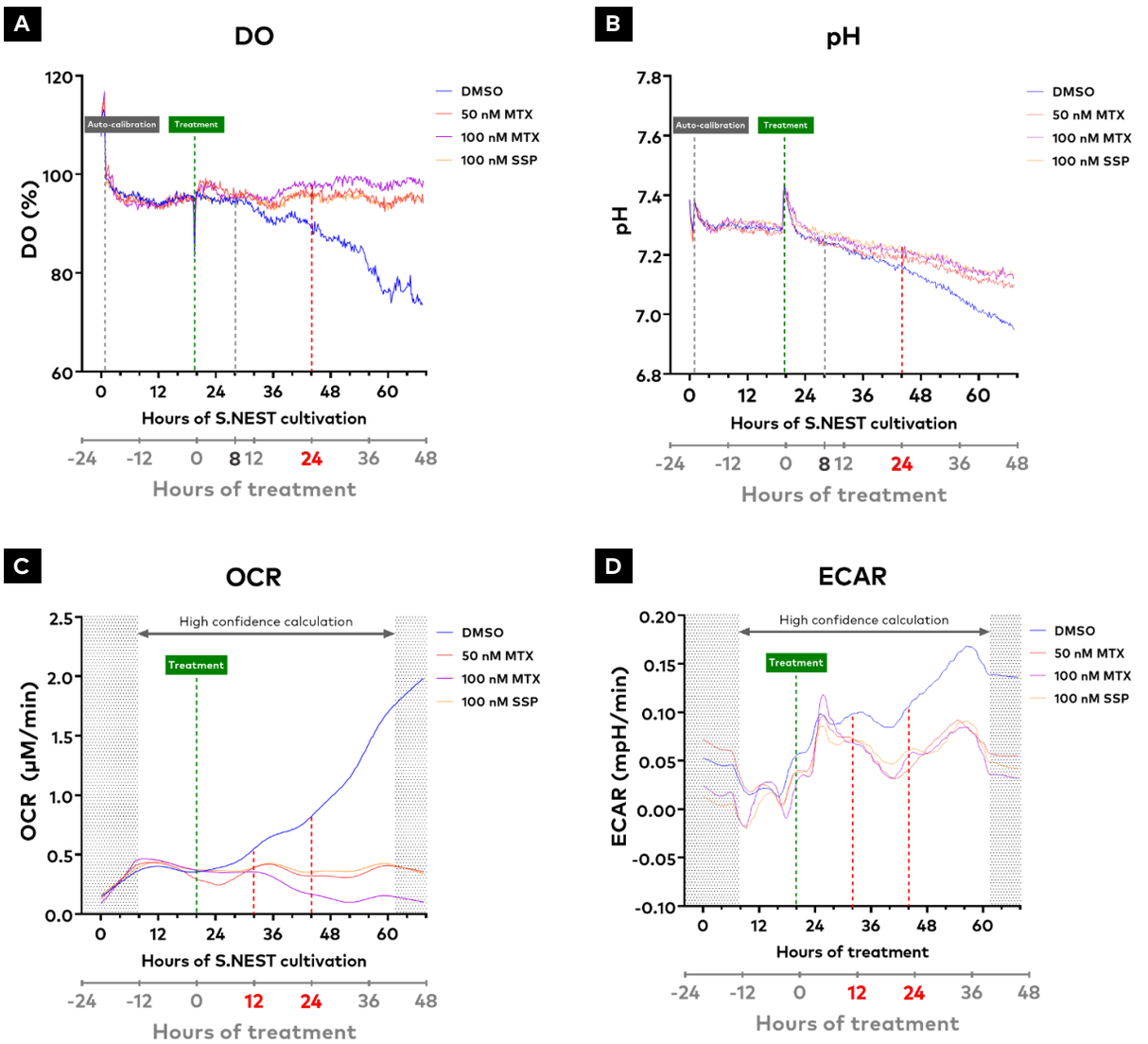

*Figure 3. The DO **(A)** and pH **(B)** data of different drug treatment conditions. The DO and pH values were measured for each experimental group of six replicates. All experimental groups had similar DO and pH value trends in the first 20 h of S.NEST cultivation. After drug treatment, the DO and pH values of all treated groups deviated significantly from the control group after 24 h. OCR and ECAR values were calculated from DO and pH values using the S.NEST software after completion of the experiment. This OCR/ECAR calculation feature will be available in the next S.NEST software version. Due to limitations in the calculation method, OCR and ECAR values in this experiment can only be calculated with high confidence between 7 and 61 hours after the initiation of DO/pH measurement (indicated by the arrow). After drug treatment, the OCR and ECAR values of all treated groups deviated significantly from the control group after 11 h. After 24 h, the difference in OCR and ECAR between the control and the treated group has more than doubled. The differences in OCR and ECAR between control and treated groups indicated that the changes in cell metabolism were due to drug treatment.*

Using conventional method to verify cell growth and cytotoxicity after drug treatment

To examine if DO and pH (OCR and ECAR) were related to cell growth and cytotoxicity, changes in cell morphology were validated by microscopy and Prestoblue cell viability assay. In the control group, A549 cells were short, spindle-shaped or triangle-shaped and resembled epithelial cells. As for A549 cells treated with 50 nM MTX, 100 nM MTX and 100 nM SSP for 48 h, some of the cells were long spindle-shaped and exhibited slender protrusions. Small cavities were visible in the cytoplasm of some cells. Some cells retracted, became round-shaped, and shed off (**Figure 4A**). The cell viability was measured by Prestoblue assay after treatment for 48 h to determine the viable cell number and cell activity. The Prestoblue assay results showed that MTX and SSP significantly affected cell growth and cell viability in A549 cells.

The Prestoblue fluorescence signals of 100 nM SSP, 50 nM MTX and 100 nM MTX treated cells decreased to 32%, 35% and 25% of the control group. Moreover, the Prestoblue assay showed dosage-dependent difference in cell viability between the two MTX treatment groups (**Figure 4B**). When we compared real-time DO (OCR)/pH (ECAR) data against end-point cell viability assay data, we confirmed that we could observe the effects of drug treatment earlier with real-time DO/pH monitoring. Significant differences in DO and pH curves between control and treated groups was observable within 24 h of treatment. The OCR and ECAR values were also discernable within 12 h of treatment. Therefore, we confirmed that the cytotoxic potential of an added reagent could be reflected by real-time decreases in DO and pH levels, which were more efficient to measure than end-point cell viability using traditional workflows.

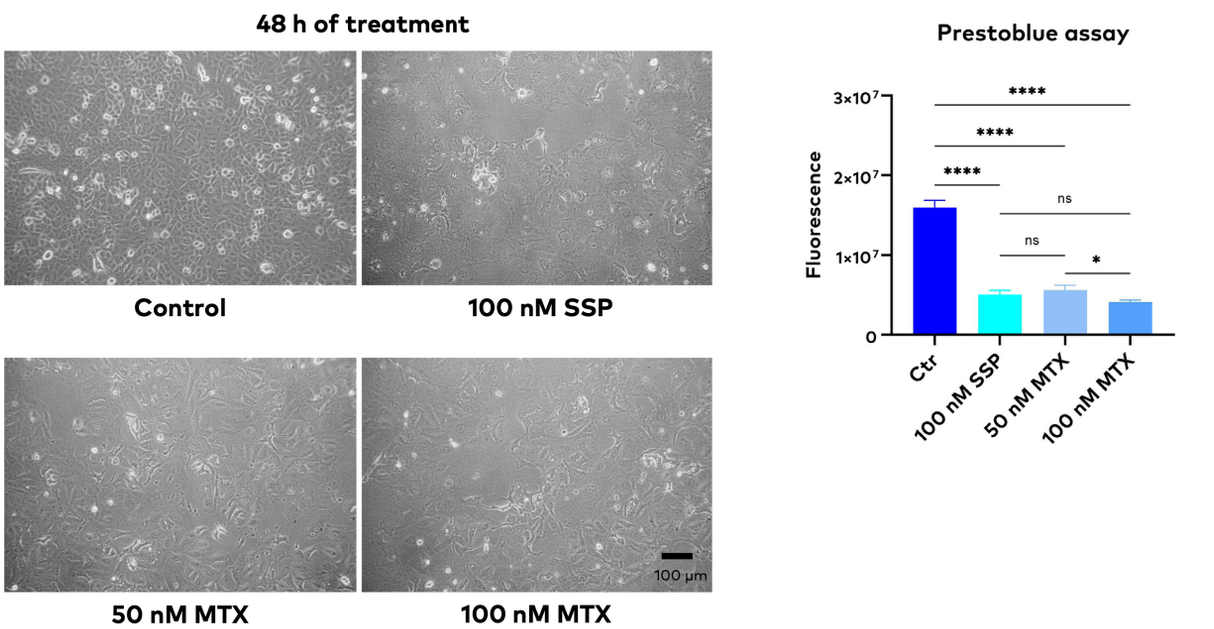

Figure 4. Effect of staurosporine and MTX on the A549 cell morphology and viability. ***(A)*** *The cell morphology under different treatment conditions was observed by microscopy 48 h after drug treatment.* ***(B)*** *Cell viability was measured by the Prestoblue assay as fluorescence signals in groups of four replicates. The P values are indicated as: P ≤ 0.01 (**), P ≤ 0.001 (***) and P ≤ 0.0001 (****). Data were shown as mean ± SD.*

Conclusion

In this study, we presented a rapid and non-invasive drug screening procedure for assessing cytotoxicity using the human-derived alveolar adenocarcinoma cell line A549 as the in vitro model. Our DO/pH detection method was fluorescence based and specifically detected dissolved extracellular O_2 and H^+ molecules in the samples. Importantly, this method was dye-free, eliminating the need for additional reagents and sampling timepoint pre-determination using sacrificial samples. Our approach allowed for real-time tracking of in vitro cell growth, which facilitated the identification of the time frame in which cytotoxic processes occur and enabled the assessment of time-dependent effects. The S.NEST system, which combines small-scale mixing culture with efficient measurement tools, can evaluate numerous samples or drugs within a short timeframe, making it suitable for high-throughput drug screening. In conclusion, CYTENA BPS presents S.NEST as an alternative high-throughput drug screening platform that utilizes real-time cytotoxicity evaluation instead of standard cell viability assay kits. This method promises saving in time and labor for high-throughput screening while providing reliable results.

Reference

1. Naciri M, Kuystermans D, Al-Rubeai M. Monitoring pH and dissolved oxygen in mammalian cell culture using optical sensors. Cytotechnology. 2008; 57(3):245-50. DOI: 10.1007/s10616-008-9160-1
2. Weltin A, Slotwinski K, Kieninger J, Moser I, Jobst G, Wego M, Ehret R, Urban GA. Cell culture monitoring for drug screening and cancer research: a transparent, microfluidic, multi-sensor microsystem. Lab Chip. 2014; 14(1):138-46. DOI: 10.1039/c3lc50759a
3. Alborzinia, H. Real-Time Monitoring of Cancer Cell Metabolism for Drug Testing. 2015. DOI:10.1007/978-3-658-10161-9
4. Shi H, Kwok RT, Liu J, Xing B, Tang BZ, Liu B. Real-time monitoring of cell apoptosis and drug screening using fluorescent light-up probe with aggregation-induced emission characteristics. JAm Chem Soc. 2012; 134(43):17972-81. DOI: 10.1021/ja3064588
5. Garcia-de-Alba C. Repurposing A549 Adenocarcinoma Cells: New Options for Drug Discovery. Am J Respir Cell Mol Biol. 2021; 64(4):405-406. DOI: 10.1165/rcmb.2021-0048ED
6. Wang Y, Yang H, Liu H, Huang J, Song X. Effect of staurosporine on the mobility and invasiveness of lung adenocarcinoma A549 cells: an in vitro study. BMC Cancer. 2009; 9:174. DOI: 10.1186/1471-2407-9-174
7. Sung JM, Cho HJ, Yi H, Lee CH, Kim HS, Kim DK, Abd El-Aty AM, Kim JS, Landowski CP, Hediger MA, Shin HC. Characterization of a stem cell population in lung cancer A549 cells. Biochem Biophys Res Commun. 2008; 371(1):163-7. DOI: 10.1016/j.bbrc.2008.04.038
8. Chiu HJ., Yu YN., and Lin SP. S.NEST: An intelligent tool with real-time dissolved oxygen and pH monitoring on batch cultures. CYTENA BPS. 2022. (CBSAPP16)

CYTENA BPS
A BICO COMPANY

Edited version: September 2023 | CBS_PUB_SNEST_App-Note-17_Digital

info@cytena-bps.com | +886 2 27206135 | www.cytena-bps.com