



>> Quantifying chemotoxicity on cancer cell colony formation using live-cell imaging

Key findings:

- Live-cell imaging allows for kinetic measurement and analysis of mammalian colony formation.
- Continuous monitoring ensures optimal assay timing.
- Doxorubicin has a dose-dependent effect on the formation of colonies by CHO-K1 cells.

Abstract

Doxorubicin (Dox) is one of the chemotherapy substituents used to inhibit the growth of tumors. The effectiveness of cancer treatment is often evaluated using *in vitro* cell survival assays like the clonogenic assay. In this study, we examined the effect of a range of Dox concentrations on the growth of single CHO-K1 cells into colonies. Hereby, an automated label-free method was used to analyze the size, number, and circularity of colonies. Dox was shown to have a significant dose-dependent effect on both the number and growth of CHO-K1 colonies.

Introduction

The clonogenic assay, also known as colony formation assay is an *in vitro* cell survival assay¹. It assesses the ability of single cells to survive and reproduce to form colonies. This assay was first described in the 1950s, where it was used to study the effects of radiation on cancer cell survival and growth and has subsequently played an essential role in cancer research, allowing for relatively easy drug screening and radiation dosing². In order to measure clonogenicity in traditional clonogenic assays, cells need to be seeded at very low densities and left for a period of 1-3 weeks for colonies to form. Colonies are then fixed, stained with crystal violet to make them visible, and counted. Today, clonogenic assays are used to answer a variety of experimental questions, especially in cancer biology.

Although this manual assay is the current golden standard, it has a few disadvantages. The quantification and scoring of the colonies are laborious and only results in one parameter, specifically, colony count. This method does not give any insight into the size of the colonies and their growth over time. It is also prone to intra-operator variation, as a colony is defined to consist of at least 50 cells, making the output fully dependent on the ability of the operator to visualize and count colonies³. The assay is mostly performed in a 6-well plate to ensure that cells have sufficient time to form proper colonies while not overlapping, which would create erroneous results. This hinders the ability to scale up to larger plate formats, limiting the testing of multiple drug concentrations or radiation doses with sufficient biological repeats⁴. In addition, crystal violet is extremely toxic, and some colonies may be lost during washing and staining steps.



These challenges make it difficult to ensure a proper quantifiable and repeatable assay. Therefore, we propose a label-free and fully automated live-cell imaging method to quantify mammalian colony formation and growth over time.

In this study, a clonogenic assay was performed in a 24-well plate format, after pre-treating CHO-K1 cells with a range of concentrations of the common chemotherapy drug, doxorubicin (Dox). We quantified colony growth and number over time using the CytoSMART Omni, providing a complete overview of all the samples without disturbing the cells. Live-cell imaging, enabled easy and accurate determination of the tipping point when colonies started to merge, allowing to set well-defined end points for this specific assay.

Materials and Methods

Cell culture and drug treatment

CHO-K1 cells were seeded in a 6-well plate at a concentration of 50,000 cells per well in F12 Nutrient mixture medium (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS; Gibco) and 1% penicillin-streptomycin (PS; Gibco). Hourly scans were performed on the CytoSMART Omni in an incubator. After the cells reached 70% confluency, the cells were rinsed twice with phosphate-buffered saline (PBS; Gibco) and treated with (except for the control) 0.2, 0.5, 1.0, 2.0, or 5.0 μM Dox (D1515-10MG, Sigma Aldrich), respectively. After 1 hour, the cells were rinsed twice with PBS, trypsinized, and counted using the CytoSMART Exact. Treated cells were seeded in a 24-well plate, 100 cells per well ($N=4$), and incubated at 37°C and 5% CO_2 .

Colony detection using the CytoSMART Omni

Following treatment with Dox, newly formed CHO-K1 colonies were analyzed using the CytoSMART Omni (Fig.1). A full-plate scan was generated every 12 hours. The Clonogenic Assay module automatically determined the number, size, and circularity of all the colonies over time.

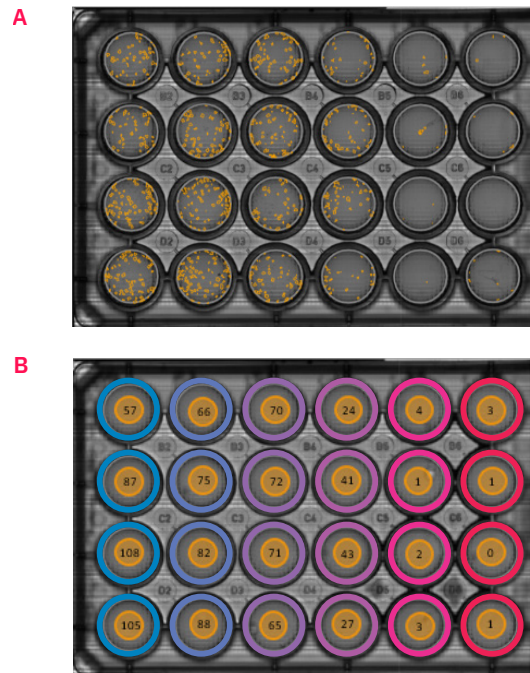


Figure 1: Summary of the Clonogenic Assay module output. (A) A high-resolution scan of the 24-well plate, including the colony overlay generated by the Clonogenic Assay module, and (B) colony count with the grouping of samples.

Colony growth analysis

The number of colonies seeded in each well and the number of colonies at $T = 120$ were used to determine the plating efficiency (eq.1) and the surviving fraction (eq.2). To calculate the IC_{50} , a dose-response curve was made by fitting a nonlinear curve of the normalized survival fraction on a logarithmic scale. The concentration that results in a 50% decrease of the survival fraction is described as the IC_{50} value.

The statistical differences in colony count were analyzed using two-way ANOVA followed by a Tukey's multiple comparison test in Graphpad Prism (V8.02). Values were considered significant at $p < 0.05$. All data is reported as mean \pm standard error of mean.

Eq. 1

$$PE = \frac{\text{no. of colonies formed}}{\text{no. of cells seeded}} * 100\%$$

Eq. 2

$$SF = \frac{\text{no. of colonies formed after treatment}}{\text{no. of cells seeded} * PE}$$



Results

Real-time tracking identifies assay tipping point for optimal timing

Determining the tipping point in a clonogenic assay enables the researcher to determine the effect of the treatment on colony growth before clustering occurs. The tipping point was measured using the colony count graph. After 120 hours the number of colonies starts to decrease, while the colony size starts to increase for the control group, indicating overlapping and merging of colonies. Therefore, 120 hours was selected to be the ideal time point to calculate the plating efficiency, surviving fraction, and IC_{50} . In the traditional end point clonogenic assays, the end point is predefined. Clustering of colonies can occur before pre-defined end points, decreasing the accuracy of the number of colonies. The use of live-cell imaging makes it easier to make decisions during a running experiment.

Doxorubicin decreases the surviving fraction of CHO-K1 colony formation in a dose-dependent manner

The cell survival curve was made to quantify the chemotoxic effect on CHO-K1 cells. The surviving fraction and plating efficiency (PE) were calculated as described in the Methods. Here, PE was calculated to be 89.25%. The surviving fraction (SF) is the number of colonies that arises after treatment, normalized to the PE of the untreated control. The SF for the control group was $79.7\% \pm 20.9\%$, while the SF for the cells treated with 0.2 and 0.5 μM Dox was $69.4\% \pm 8.4\%$ and $62.0\% \pm 2.8\%$, respectively. For cells treated with the 1.0 ($30.1\% \pm 8.6\%$), 2.0 ($2.2\% \pm 1.2\%$) and 5.0 ($1.1\% \pm 0.9\%$) μM of Dox, the SF was significantly lower compared to the control group ($p < 0.0001$) (Fig. 3). Higher concentrations of Dox contributed to a lower surviving fraction of the colonies, especially for concentrations above 1.0 μM .

To calculate the IC_{50} , a dose-response curve was made by fitting a nonlinear curve of the normalized survival fraction (Fig. 4, $R^2 = 0.92$). The IC_{50} was determined to be 0.78 μM Dox. These results support the inhibitory

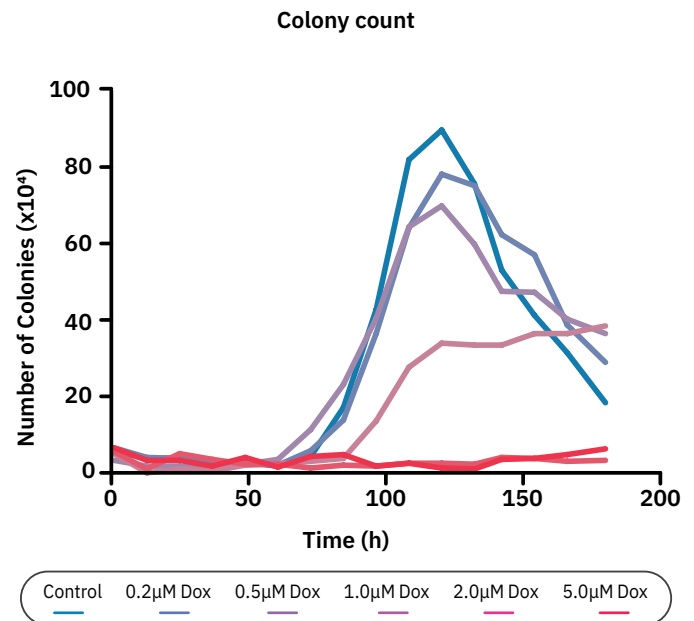


Figure 2: The number of CHO-K1 colonies in the first 170 hours of the clonogenic assay is dependent on the Dox concentration. The colonies start to merge in the control (CTRL), 0.2 μM Dox, and the 0.5 μM Dox group at 120 hours indicating the tipping point.

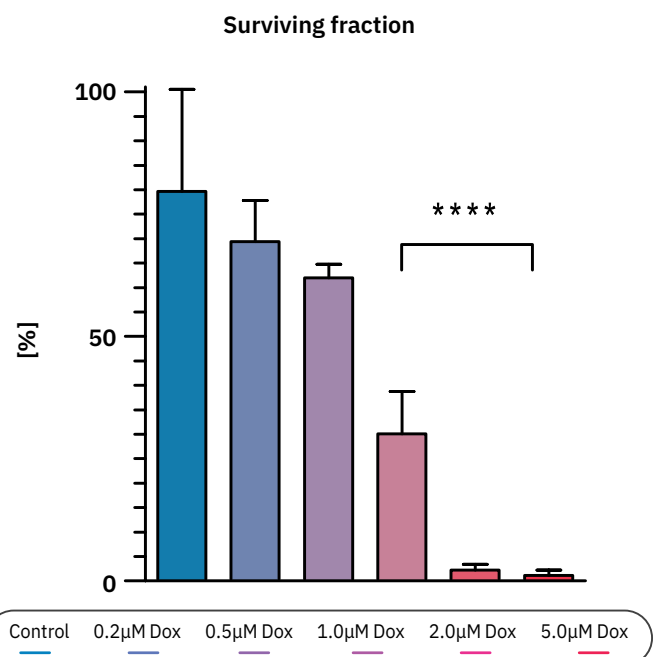


Figure 3: Dox reduces the surviving fraction of CHO-K1 colony formation. Surviving fraction of all treatments vs. control (mean \pm standard error of mean).

effect of Dox on colony formation, but also the effectiveness of the CytoSMART Omni to automatically analyze the colony size and number and define the tipping point.



Conclusion

The CytoSMART Omni allows for automated, low-laborious, and label-free imaging and analysis of mammalian colony formation over time directly inside the incubator. This method can be used to accurately define the number of colonies, surviving fraction, and IC_{50} values for different cancer cell lines and drugs through identification of optimal assay timing. In this study, we demonstrated that Dox showed chemotoxic effects that inhibit colony formation of CHO-K1 cells in a dose-dependent manner.

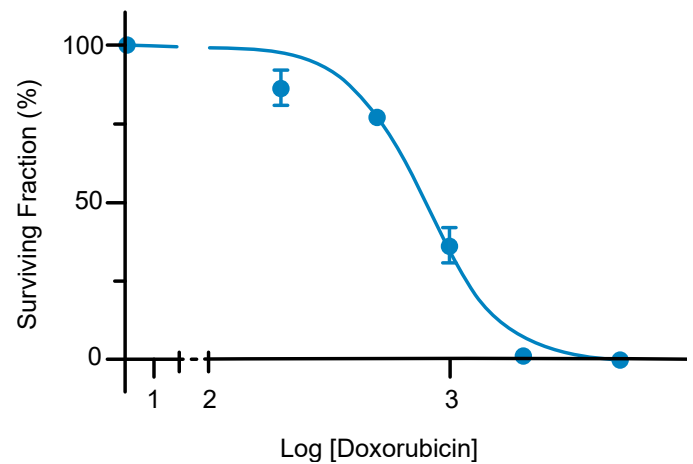


Figure 4: Dox dose-response curve. The curve was used to measure the IC_{50} of Dox against CHO-K1 cells.

References

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