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C.BIRD™ | An Innovative 3D Cell Culture System for Tumor Spheroid

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Abstract

For drug screening and diversity, tumor spheroids offer an intermediate complexity between cancer cells grown in monolayers and in vivo tumors. Tumor spheroids can improve the reliability of preclinical research data and reduce the need for animal testing but require shorter experiment timelines in order to remain healthy and efficient. In this study, we demonstrated that spheroid growth and cell health maintenance were improved with the C.BIRD™ culture method. Our results show the C.BIRD method shortens the period of spheroid formation and increases the duration of cell health during the drug screening material preparation process.

Introduction

Spheroid culture, an in vitro 3D cell culture system, provides cell-cell and cell-extracellular matrix (ECM) interaction networks to retain cellular phenotype through signaling. It breaks restrictions in recapitulating in vivo microenvironments and is emerging as a powerful tool for therapeutic development, including drug screening and cancer research. In recent studies, gene and physiological expressions in 3D cell spheroids are much closer to clinical expression profiles than those seen in 2D cell monolayers. Additionally, the diffusion-limited distribution of drugs, nutrients, oxygen and metabolites in solid tumors is mimicked in spheroid culture, but not in 2D monolayer culture. Spheroid culture bridges information from traditional 2D culture to in vivo animal models, and is a reliable and widely used method in pharmaceutical and biological fields.

While spheroids are compatible for high-throughput screening and can be scaled up in a multi-well plate, there are still limitations in generating high quality

spheroids in large quantities. Here, we demonstrated an innovative culture method for spheroids by using the C.BIRD microbioreactor **(Figure 1A)**. The C.BIRD is composed of an autonomous control unit and a C.BIRD lid with fluidic 24 or 96 channels **(Figure 1B)**. In this study, human colon carcinoma cell line HCT116 was analyzed for spheroid formation in an ultra-low attachment (ULA) round bottom plate **(Figure 1C)**. After the plate was transferred to the C.BIRD

device, pneumatic connection of the lid provided low-shear adjustable mixing for culture homogenization **(Figure 1D)**. By using this specific culture system, we demonstrated remarkable improvement in spheroid formation and cell health maintenance compared to spheroids formed in static culture. Our new technique sheds light on optimizing spheroid culture environments and overcomes the barriers associated with generating high quality spheroids.
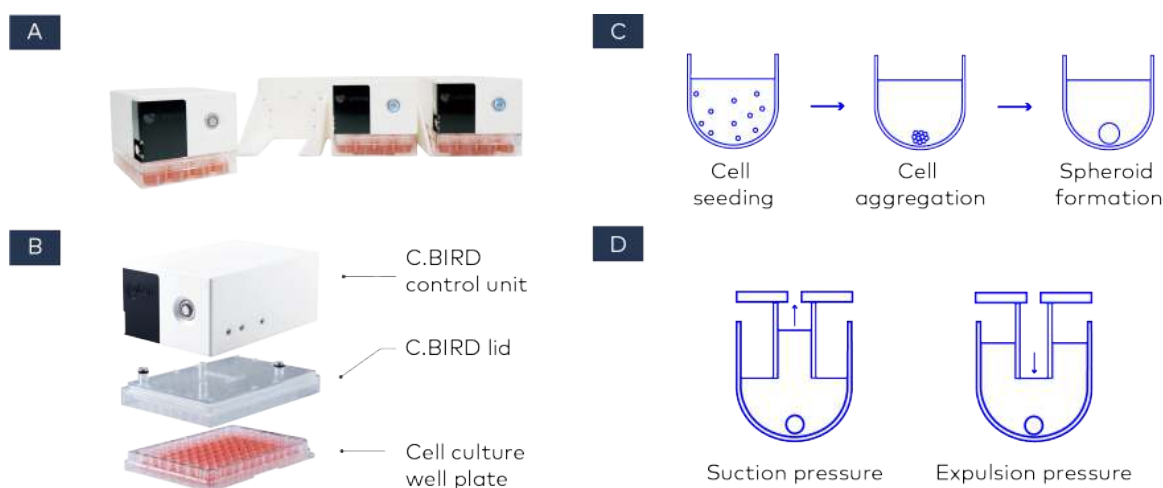

Figure 1. (A) *The C.BIRD device is composed of a docking station with three control units.* **(B)** *Each unit consists of a C.BIRD lid with fluidic 24/96 channels.* **(C)** *A spheroid is formed in the ULA 96-well plate.* **(D)** *Through pneumatic connection, the system provides reciprocal mixing in each well.*

Materials and methods

Human colon carcinoma cell line HCT116 (ATCC, #CCL-247) was used in this study. HCT116 cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) media (Corning, #10-092-CV) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Gibco, #A3160601), 100 units/mL of penicillin and 100 µg/mL of streptomycin (Thermo Fisher Scientific, Gibco, #15140122). For the spheroid culture, HCT116 cells were cultured in serum-free DMEM/F12 media containing B-27 plus supplement (Thermo Fisher Scientific, Gibco, #A3582801), 20 ng/mL Recombinant Human EGF Protein, CF (EGF, R&D Systems, #236-EG), 10 ng/mL Recombinant Human FGF basic/FGF2/bFGF (145 aa) Protein, CF (bFGF, R&D Systems, 3718-FB), 100 units/mL of penicillin and 100 µg/mL of streptomycin in round bottom Corning Costar ULA 96-well plate (Merck, #7007) at 37°C in a humidified 5% CO_2 atmosphere for 6 to 10 days.

Media for the spheroid culture were replaced on day 3 and day 6.

In order to generate the spheroid itself, 500 or 1,000 of the HCT116 cells were seeded with 200 µL medium per well in two 96-well round bottom ULA microplates and settled at the bottom of the wells by centrifuging the cells at 300 x g for 3 minutes. The spheroids were formed for 3 days in static culture conditions. Then, one plate was kept in static culture and the other was transferred to the C.BIRD culture with 25 s/cycle continuous mixing. Spheroid size was captured with a microscope image and diameter was measured using ImageJ image analysis software. Cell viability was measured on days 3, 6, 7, 8, 9 and 10, respectively. The spheroids were stained with live and dead dyes for image analysis on day 6.

PrestoBlue Cell Viability Reagent (Thermo Fisher Scientific, Invitrogen, #A13261) was used to assess spheroid cell health. Then, 20 µL of PrestoBlue

Cell Viability Reagent was added to each well and incubated at 37ºC and 5% CO_2 for 3 hours before being read on SpectraMax iD3 Multi-Mode Microplate Readers (Molecular Devices) using a fluorescence excitation wavelength of 560 nm and an emission of 590 nm. The spheroid cell viability was evaluated by Calcein, AM Dye (Thermo Fisher Scientific, Invitrogen, #C3099), NucBlue Live ReadyProbe Reagent (Thermo Fisher Scientific, Invitrogen, #R37605) and DRAQ7 Dye (Thermo Fisher Scientific, Invitrogen, #D15105). The images were taken and analyzed on an Opera Phenix High-Content Screening System (PerkinElmer, USA). Data were analyzed by an unpaired t test or one-way ANOVA test. The significance of P values is listed as the following: $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) and $P \leq 0.0001$ (****). Data were shown as mean ± SEM.

Results and discussion

The C.BIRD culture method was used to improve homogeneity of nutrients and oxygen. In previous application notes, we demonstrated that the C.BIRD method could improve cell proliferation of suspension cells, including Chinese Hamster Ovary (CHO) cells and Jurkat human T lymphocyte cells. The tumor spheroid was formed from cell aggregation and constituted a multicellular spheroid through self-assembly in a floating culture, which is another kind of suspension culture. In this study, we sought to verify that the C.BIRD culture method could also improve the 3D culture of tumor spheroids.

HCT116 cells form a round and floating colonosphere when cultured in serum-free media. To avoid mixing culture that affects spheroid formation in the early stages, the HCT116 cells were seeded and incubated in static culture for three days. After initial spheroid formation, the spheroids were transferred to the C.BIRD culture as cell growth and cell health were monitored from days 6 to 10.

Microscopic images showed that the diameter of the spheroid on day 3 was ~0.43 mm **(Figure 2A)**. In the static culture group, the average diameter of spheroids was 0.7738, 0.8505, 0.9075, 0.9587 and 0.9785 mm from days 6 to 10 **(Figure 2B)**. In the C.BIRD culture group, the average diameter of spheroids was 0.8344, 0.9515, 1.0455, 1.1158 and 1.1616 mm from days 6 to 10, which was 1.1 to 1.2-folds higher than the static culture group in the same culture period **(Figure 2B)**. The roundness of spheroids in the two groups was not significantly different, whereas the circularity of the day 10 spheroids in the C.BIRD culture group was lower than that in the static culture group **(Figure 2B)**. We speculated that the cells started to die on day 10 and the surface structure of the spheroid was broken by the mixing flow in the C.BIRD culture. Spheroid cell health was then assessed using PrestoBlue Cell Viability Reagent. The intensity of the PrestoBlue fluorescence signal was dependent on cell number. Cell health was also indicated by normalized cell size (diameter); healthier spheroids had a higher fluorescence/diameter ratio. The results showed that fluorescence signals in the C.BIRD culture group were significantly higher than in the static culture group on days 6, 7 and 10 **(Figure 2C)**, and that the C.BIRD culture generated healthier and larger spheroids overall compared to the static culture **(Figure 2D)**.

Since the spheroid size on day 7 in the C.BIRD culture was similar to the size of the spheroid on day 9 in the static culture group **(Figure 2B)**, we further explored the effect of different seeding densities on spheroid formation in both culture methods. The HCT116 cells were seeded at a density of 500 and 1,000 cells/well. After incubating in static culture for 3 days, the spheroids were subsequently incubated in static culture or the C.BIRD culture. Spheroid morphology, cell health and cell viability were analyzed in three groups: 1,000 cells/well in static culture (Static_1000); 1,000 cells/well in C.BIRD culture (C.BIRD_1000) and 500 cells/well in C.BIRD culture (C.BIRD_500).
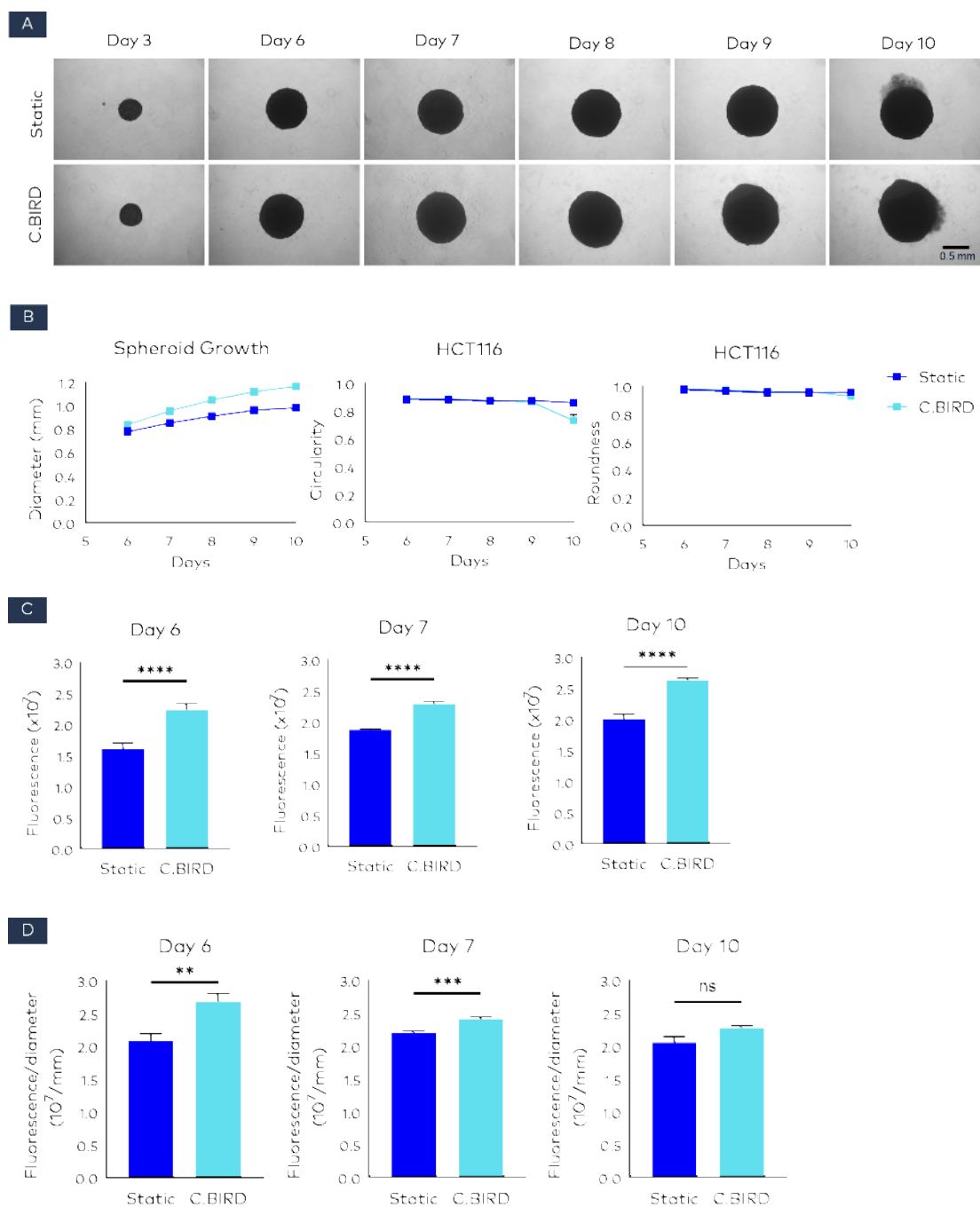
Figure 2. *Comparison of spheroid growth and cell health of colon cancer HCT116 spheroids in 96-well, ultra-low attachment plates in static and C.BIRD culture. **(A)** Spheroid morphology of HCT116 tumor cell lines formed from 3 to 10 days was observed under microscopy. Scale bar = 0.5 mm. **(B)** Growth kinetics, circularity and roundness of spheroids were evaluated over a period of 10 days. The analysis was performed using ImageJ software, and data represent mean ± SEM of six replicates for each group. **(C and D)** Spheroid cell health was assessed using PrestoBlue Cell Viability Reagent. After 6, 7 and 10 days of spheroid culture, 20 µL of PrestoBlue Cell Viability Reagent was added to each well, which were then incubated at 37°C and 5% CO_2 for an additional 3 hours before being read on a fluorescence-based microplate reader (Ex/Em ~560/590 nm). Cell viability was measured as fluorescence signals in each group with six replicates. Fluorescence signals were normalized by spheroid diameter; a higher ratio (fluorescence/diameter) indicates healthier spheroids. The significance of P values is listed as: P ≤ 0.01 (**), P ≤ 0.001 (***) and P ≤ 0.0001 (****). Data are shown as mean ± SEM.*

The spheroid diameter formed from 500 cells was ~0.35 mm on day 3, which was smaller than the spheroids formed from 1,000 cells in both the static and C.BIRD culture groups **(Figure 3A)**. However, the day 6 diameter, circularity and roundness of the C.BIRD_500 spheroids (0.7634 mm) were similar to the Static_1000 spheroids (0.7569 mm) **(Figure 3A and 3B)**. Additionally, the day 6 cell health of the C.BIRD_500 spheroids were even better than the Static_1000 spheroids **(Figure 3C and D)**.

The cell viability of the day 6 spheroids was further evaluated by using Calcein AM, NucBlue Live ReadyProbes Reagent and DRAQ7 staining. Calcein AM and NucBlue Live ReadyProbes Reagent (Hoechst 33342) were used to label live cells. The DRAQ7 is an anthraquinone compound that stained nuclei in dead and permeabilized cells. For the positive control, spheroids were fixed by 4% paraformaldehyde as dead cells. The fluorescence of spheroids was imaged with the Opera Phenix High-Content Screening System using confocal mode **(Figure 4A, 4B and 4C)**. The image is a maximum intensity projection of 31 images taken at 10 μm intervals along the Z-axis over 300 μm and under a 10× objective lens. The fluorescence in each cell was measured and quantified as an average intensity. The average fluorescent intensity of Calcein AM/NucBlue Live (live cells) was not significantly different between the static and C.BIRD cultures. Even the spheroid size of C.BIRD_1000 was larger than Static_1000, and the average intensity of DRAQ7 (dead cells) was also not significantly higher in the C.BIRD_1000 group **(Figure 4D)**. These results indicated that the C.BIRD culture method generated spheroids of similar quality and quantity as the static culture, even at a lower cell seeding density.
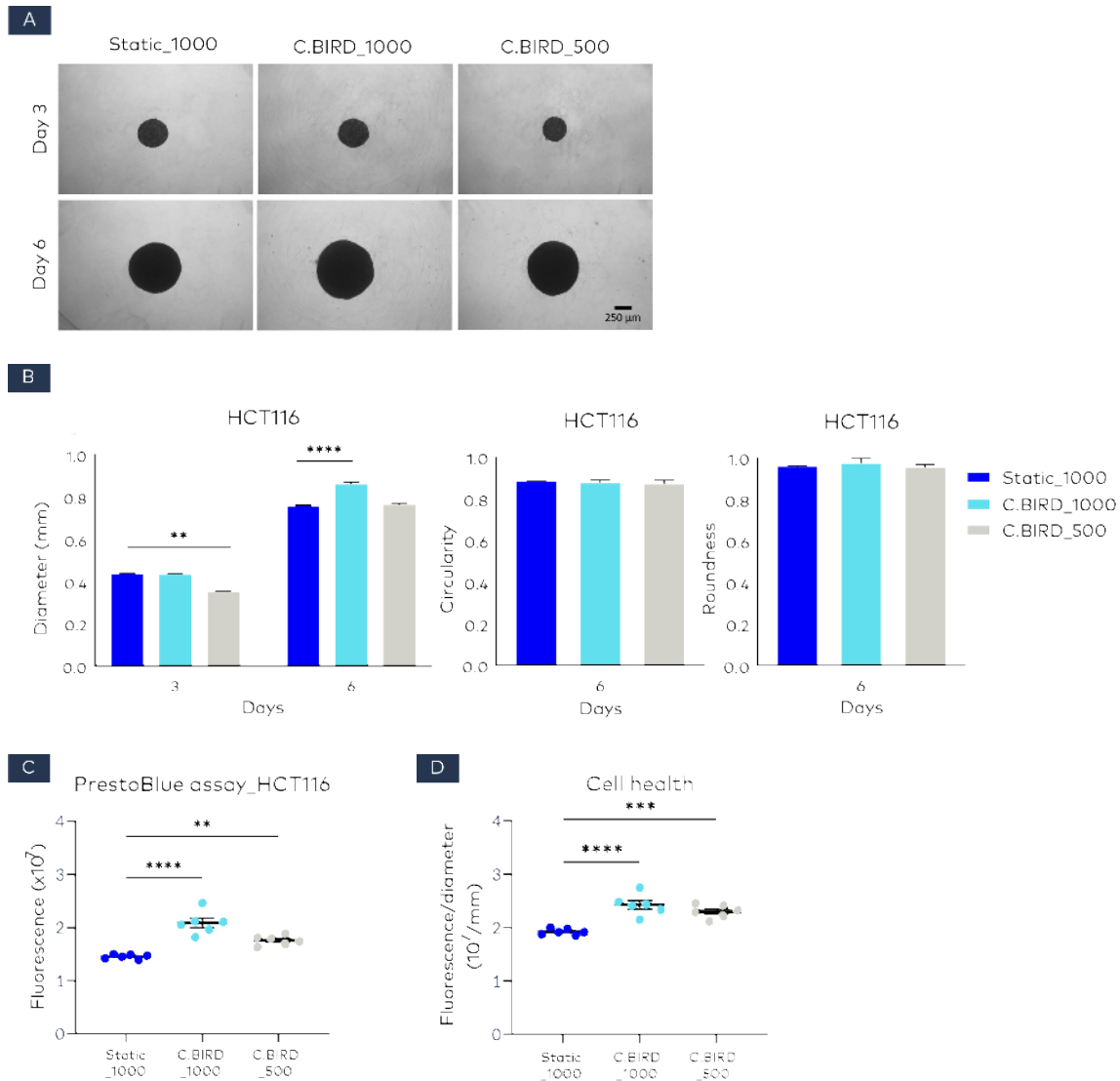
Figure 3. *Spheroid morphology and cell health were evaluated with different cell seeding density: 1,000 cells/well in static culture (Static_1000); 1,000 cells/well in C.BIRD culture (C.BIRD_1000) and 500 cells/well in C.BIRD culture (C.BIRD_500).* **(A)** *Cell morphology of the spheroid was observed under microscopy on day 3 and day 6; scale bar = 250 µm.* **(B)** *Spheroid growth kinetics were measured on day 3 and day 6. The circularity and roundness of spheroids were evaluated over a period of 6 days. Data were analyzed using ImageJ software and expressed as the mean ± SEM of six replicates for each group.* **(C and D)** *Spheroid cell health was assessed by using PrestoBlue Cell Viability Reagent on day 6. Then 20 µL of PrestoBlue Cell Viability Reagent was added to each well. Spheroids were subsequently incubated at 37°C and 5% CO$_2$ for 3 hours and the fluorescence was read on a fluorescence-based microplate reader (Ex/Em ~560/590 nm). Cell viability was measured as fluorescence signals in each group with six replicates. The fluorescence signals were normalized by spheroid diameter; a higher ratio (fluorescence/diameter) indicates healthier spheroids. The significance of P values is listed as the following: P ≤ 0.01 (**), P ≤ 0.001 (***) and P ≤ 0.0001 (****). Data are shown as mean ± SEM.*
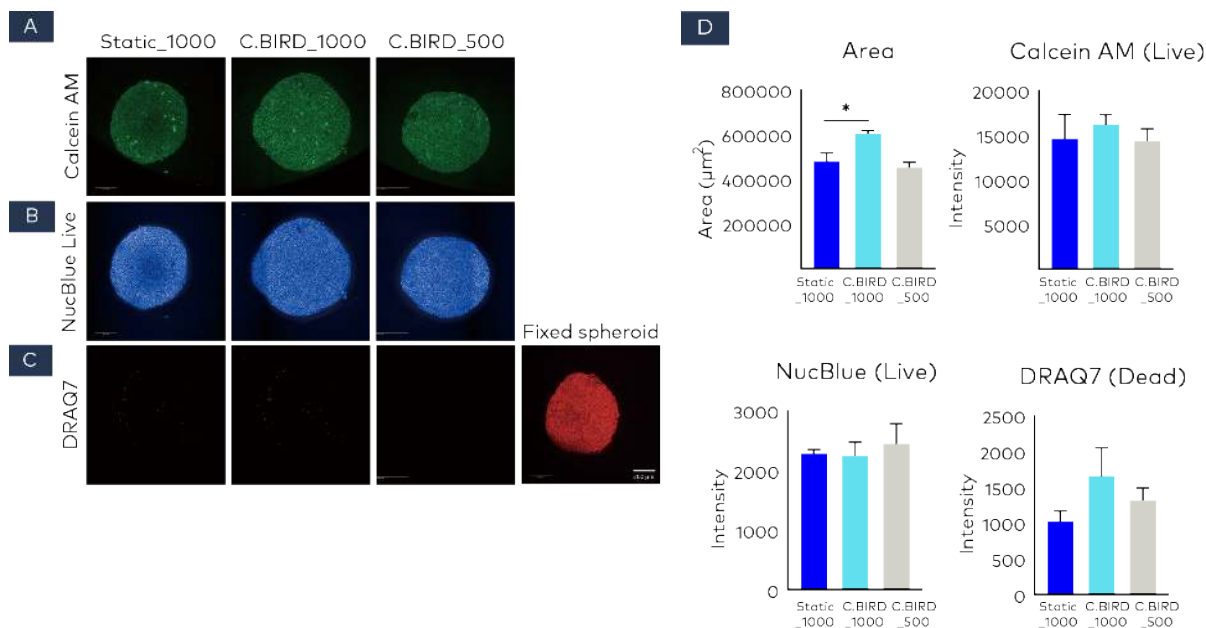
Figure 4. *Spheroid cell viability was evaluated using the Calcein AM Dye, NucBlue Live ReadyProbe Reagent and DRAQ7 Dye. After 6 days of spheroid culture, the Calcein AM and NucBlue Live ReadyProbe Reagents were added to plates, which were then incubated for 30 minutes and rinsed at least three times with a half-volume change of Dulbecco's phosphate-buffered saline (DPBS). Then DRAQ7 Dye was added to the plate and incubated for 10 minutes at 37°C, rinsed with DPBS and imaged using Opera Phenix High-Content Screening System. A 4% paraformaldehyde-fixed spheroid was stained as positive control of dead cell.* **(A-C)** *Live cells with staining emitted green fluorescence in cytoplasm* **(A)** *and blue fluorescence in nucleus* **(B)**. *Dead cells with staining emitted red fluorescence* **(C)**. *Scale bar = 200 µm.* **(D)** *Scanning area and intensity were analyzed using software from the Opera Phenix High-Content Screening System. Data are shown as mean ± SEM with three replicates. *P < 0.05 vs. Static_1000 spheroid.*

Conclusions

New cancer drug approval rates are lower than 5% despite tremendous investments in drug discovery and development. Due to tumor complexity, 3D culture techniques of tumor spheroids are widely used to study drug activity and have been proven to more closely resemble the pathophysiological features of clinical tumors than monolayer cell cultures, approaching the level of in vivo models with lower costs and reduced animal testing. One strategy to improve the success rate of new drug discovery includes using tumor spheroids for high-throughput drug screening. The optimization of spheroid cultures is essential for generating high-quality spheroids for use as a resource in medical fields. In our study, the growth rate of spheroids in the C.BIRD culture was higher than the static-cultured spheroids in the ULA plate **(Figure 2)**. The cell health in days 6 and 7 of the C.BIRD-cultured spheroids was also better than static-cultured spheroids **(Figure 2)**. Additionally, the size of the spheroids in the C.BIRD culture on day 7 were approximately the same size as spheroids in the static culture on day 9, even at the same seeding density **(Figure 2)**. In addition, the spheroids formed from half of the cells cultured in the C.BIRD grew to the same size in the static culture on day 6, and cell health of spheroids in the C.BIRD culture was better than spheroids in the static culture **(Figure 3)**.

In conclusion, our results have two main implications: (1) the C.BIRD culture method improves tumor spheroid growth in serum-free media by using a ULA plate, and (2) the duration of spheroid viability is prolonged by the C.BIRD mixing culture. This means the C.BIRD culture method is a better option to optimize the tumor spheroid 3D culture and can improve drug screening workflows to obtain more accurate results in viability/cytotoxicity assays.

References

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