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Culture of 3D Cell Aggregates in Perfusion in a DASbox® Mini Bioreactor System

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Abstract

Three-dimensional (3D) cell aggregates are of great interest for many applications, including disease modeling, drug toxicity assessment, and manufacturing of stem cell-based products. Stirred-tank bioreactors are promising culture systems for 3D cell aggregates, as they allow efficient establishment and maintenance of cell aggregates, process monitoring and control, and process scale-up to larger volumes. Furthermore, they can be operated in perfusion mode, which allows 3D cell aggregates to be sustained longer than in traditional batch cultures.

Researchers at the Instituto de Biologia Experimental e Tecnológica (iBET) in Portugal have tested the suitability of the Eppendorf DASbox Mini Bioreactor System for the cultivation of the human tumor cell line H157 as 3D cell aggregates. First, they evaluated the impact of impeller geometry on cell growth and aggregate formation. After identifying the best impeller option, they cultivated H157 cell aggregates in perfusion mode for up to 15 days. They used a stainless steel sparger with a pore size of 10 – 20 µm to retain 3D cell aggregates in culture while ensuring the continuous flow of fresh medium and the removal of metabolic waste products.

The results demonstrate the suitability of the DASbox Mini Bioreactor System for bioprocess development and the intensification of 3D cell aggregate cultures.

Introduction

3D culture systems provide cell-cell and cell-extracellular interactions that reproduce the cellular microenvironment *in vivo* better than typical two-dimensional monolayers. This property is of paramount importance in many applications, including disease modeling, drug toxicity assessment or manufacturing of stem cell-based products [1].

Cultivation in stirred-tank bioreactors using perfusion mode opens up new possibilities in the cultivation of 3D cell aggregates. Perfusion allows the removal of detrimental metabolites, cell debris, and proteases from the culture, and the addition of fresh nutrients. This facilitates longer cultivation periods than the traditional batch system [2]. Notably, perfusion systems allow for establishing concentration gradients and smooth transitions between growth factor concentrations in the culture medium. This is particularly relevant for the bioprocessing of stem cells or 3D cell models, since it can provide a more physiologically accurate *in vitro* reproduction of *in vivo* tissue development,

ultimately resulting in increased cell yields and/or improved cell functionality and quality [2, 4, 5]. One of the significant challenges in perfusion systems is the choice of the retention device used to maintain cells within the bioreactor while ensuring the continuous flow of fresh medium and the removal of metabolic waste products.

Stirred-tank bioreactor hydrodynamics plays a critical role in cell survival, growth, and aggregation in 3D spheroids [3]. By fine-tuning process parameters such as agitation speed and impeller geometry, it is possible to control the macro-environment and therefore modulate the behavior and fate of cells.

The objective of the study was to evaluate the impact of impeller geometry on cell growth and aggregate formation. Furthermore, it was intended to determine whether an Eppendorf stainless-steel sparger with a pore size of 10 – 20 µm could be used as retention device for 3D spheroids in perfusion culture.

Material and Methods

Cell lines

The researchers used two human tumor cell lines derived from Non-Small Cell Lung Carcinoma: H1650 (adenocarcinoma; CRL-5883, ATCC®) and H157 (squamous mucosa, CRL-5802, modified from ATCC). The researchers had demonstrated previously, that both lines are capable of forming tumor cell spheroids in a stirred-tank bioreactor system [7].

Preculture under static, two-dimensional conditions

The researchers expanded both tumor cell lines in T-flasks. They used RPMI Medium 1640 with GlutaMAX™, supplemented with 10 % (v/v) FBS, 1.25 % (v/v) HEPES, 1 % (v/v) sodium pyruvate, 1 % (v/v) Pen/Strep, and 1 % (v/v) non-essential amino acids. The cells were cultivated at 37 °C in an incubator with a humidified atmosphere of 5 % CO_2 and 95 % air. Cells were subcultured twice a week. To subculture them, cells were trypsinized using TrypLE™ Select reagent for 3 minutes at 37 °C.

Cell culture in bioreactors

Culture medium and cell inoculation

The researchers cultivated the tumor cell lines in bioreactors in RPMI Medium 1640 plus GlutaMAX supplemented with 10 % (v/v) FBS and 1 % (v/v) Pen/Strep. They inoculated the cultures as single cells at a density of 0.3 x 10^6 cell/mL.

Process parameters common to all bioreactor cultures

The bioreactor cultures were aerated with 5 % CO_2 and 95 % air, at 0.1 vessel volumes per minute (vvm) via the vessel headspace. The researchers monitored dissolved oxygen and pH of the medium over the duration of the culture. The temperature was controlled at 37 °C.

Fig. 1: DASbox Mini Bioreactor System.

DASGIP® control 4 software (now DASware® control 5) was used for data acquisition and process control.

Evaluation of impeller geometries

To identify the most suitable impeller geometry for the cultivation of 3D tumor cell aggregates in the DASbox Mini Bioreactor System, the researchers cultivated 3D

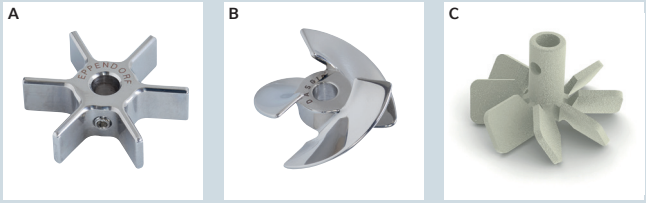

Fig. 2: Different impeller geometries used with the DASbox Mini Bioreactor System.
A. 6-blade impeller, Rushton-type. **B.** 3-blade impeller with 30 ° pitch. **C.** 8-blade impeller with 60 ° pitch.

spheroids in vessels equipped with a 3-blade impeller with 30 ° pitch, an 8-blade impeller with 60 ° pitch, and a 6-blade Rushton-type impeller, respectively (Figure 2). They compared process performance in these vessels with process performance in a 125 mL spinner flask equipped with a flat-blade impeller (Corning®, #4500-125) and a DASGIP® Bioblock Stirrer Vessel equipped with a trapezoid-shaped paddle impeller. They had successfully used these two culture systems for the cultivation of 3D cell aggregates in previous experiments [5]. Agitation speeds from 75 rpm to 220 rpm were tested. The researchers defined them based on engineering correlations [3, 6]. The rationale was to have the specific power input or the tip speed in the DASbox Mini Bioreactor System similar to control cultures in the DASGIP Bioblock or spinner flasks, for which optimal conditions (e.g. for spheroid formation, compaction, limiting spheroid clumping and fusion) had been set previously [5, 7]. The parameters for the different impeller types in the DASbox Mini Bioreactor System are listed in Table 1.

To assess the suitability of the different impeller types for the cultivation of 3D cell aggregates, the researchers monitored their diameter, number, and sedimentation in the bioreactor.

Perfusion culture

The researchers performed bioprocesses in perfusion mode in a DASbox Mini Bioreactor System. The vessels were equipped with an 8-blade impeller with 60 ° pitch. As cell retention device, an Eppendorf stainless-steel sparger (Figure 3) was connected to one of the bioprocess control system's pumps with a silicon tubing. The filter's pore size of 10 – 20 µm retained cell aggregates in the culture, while allowing the removal of culture medium, single cells, and cell debris. The setup of a comparable bioreactor system for perfusion is described in detail in [5].

The researchers started perfusion at day 1 or at day 3 post-inoculation with a dilution rate of 0.167 per day. Perfusion was controlled using automated gravimetric control as described before [5].

Analytics

Cell concentration and viability

The researchers determined cell concentrations and viability in two-dimensional monolayer cultures using a Cedex® HiRes Analyzer (Roche Diagnostics®, Germany). They determined the cell concentration in 3D cultures using crystal violet staining. Briefly, cells/aggregates were collected, centrifuged (300 x *g*, 5 min), incubated with lysis solution (1 % (w/v) Triton™ X-100 in 0.1 M citric acid) at 37 °C for a minimum time-period of 24 h, and then stained with 0.1 % (v/v) crystal violet. Violet-stained nuclei were then counted in a Fuchs-Rosenthal hemocytometer chamber using a phase contrast microscope.

Cell membrane integrity assay

The researchers evaluated cell viability using two fluorescent dyes. The cell-permeant esterase substrate fluorescein diacetate (FDA) labels live cells and TO-PRO®-3 iodide (Thermo Fisher Scientific®, USA), a cell-impermeable nucleic acid stain, identifies dead cells. The cultures were incubated with 10 µg/mL FDA in DPBD and 1 µM TO-PRO-3 iodide in DPBS for 5 min at room temperature (22 – 25 °C) and then analyzed by fluorescence microscopy.

Fig. 3: Stainless-steel sparger used as cell retention device. The device consists of a metal tube and a sintered metal filter with a pore size of 10 – 20 µm. The sparger is inserted into the vessel headplate using a compression fitting.

Spheroid size and concentration

The researchers inspected suspensions of tumor cell spheroids in a phase contrast microscope. They used the open source ImageJ software to determine spheroid concentration (number of aggregates/mL) and the aggregate diameters. The Feret's statistical diameters of a minimum of 60 aggregates were considered.

Metabolite analysis

Glucose and lactate concentrations in the culture medium were assessed using a YSI® 7100MBS system (YSI, USA).

Results

Impact of impeller geometry on 3D cell aggregate formation

To identify the most suitable impeller for the cultivation of human tumor cell aggregates in the Eppendorf DASbox Mini Bioreactor System, the researchers tested the different impeller types described in the Material and Methods section. They monitored the number and size of 3D cell aggregates, and the sedimentation of cells in the bioreactor.

The results are summarized in Table 1. They demonstrate that the 3-blade impeller and the Rushton-type impeller induced the generation of few and small (≈ 50 µm) 3D cell aggregates, and more importantly led to cell sedimentation in the bioreactor. The 8-blade impeller induced the generation of 3D cell aggregates in number and size similar to control cultures, particularly at low agitation speeds of 75 – 90 rpm. The cells did not sediment to the vessel bottom (Figure 4, Table 1)

Based on these results, the researchers selected the 8-blade impeller for the following perfusion experiments in the Eppendorf DASbox Mini Bioreactor System.

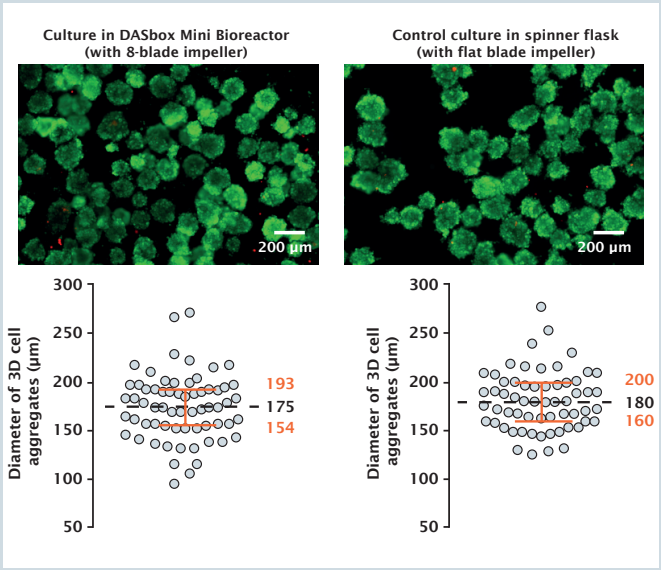

Fig. 4: Impact of impeller geometry on 3D cell aggregate formation.
Morphology (upper panel) and diameter (lower panel) of 3D aggregates of H157 cancer cell line at day 2 post-inoculation in DASbox Mini Bioreactor equipped with 8-blade impeller (at 75 – 90 rpm) and in 125 mL spinner flasks. Upper panel: H157 cells were stained with fluorescein diacetate (green, viable cells) and TO-PRO-3 iodide (red, non-viable cells). Lower panel: Lines show the median and interquartile (25th – 75th) range. Unpaired t-test (two-tailed) was used to calculate the statistical significance (* p-value=0.05). There is not a significant difference in the aggregate diameter for the DASbox Mini Bioreactor System and the control culture (t=1.213, df=122, p-value = 0.2274).

Culture of 3D cell aggregates in perfusion in DASbox Mini Bioreactor System

To assess the suitability of the DASbox Mini Bioreactor System for culture of 3D cell aggregates in perfusion, the researchers inoculated four DASbox Mini Bioreactors with the human H157 cancer cell line and cultivated the cells as 3D aggregates in perfusion mode for up to 15 days. Eppendorf stainless-steel spargers were used for cell retention. The scientists started perfusion at day 1 post-inoculation in two bioreactors (replicates) and at day 3 post-inoculation in the other two bioreactors (replicates). They

Table 1: Culture of human tumor cells as 3D aggregates in a batch process in the Eppendorf DASbox Mini Bioreactor System. Impact of impeller geometry on the number and size of 3D cell aggregates and on cell sedimentation in the bioreactor vessel (pi: post inoculation).

Culture conditions			Culture outcome		
Impeller type	**Tumor cell line**	**Agitation speed (rpm)**	**Number of 3D cell aggregates**	**Size of 3D cell aggregate**	**Cell sedimentation**
3-blade, 30 ° pitch	H1650	180 – 220	low	≈ 50 µm	yes (at day 1 pi)
6-blade, Rushton-type	H1650	180 – 220	low	≈ 50 µm	yes (at day 2 pi)
6-blade, Rushton-type	H1650	115 – 140	low	≈ 50 µm	yes (at day 2 pi)
8-blade, 60 ° pitch	H157	75 – 90	high	150 – 175 µm	no
8-blade, 60 ° pitch	H157	85 – 100	high	80 – 90 µm	no
8-blade, 60 ° pitch	H157	120 – 135	high	≈ 75 µm	no

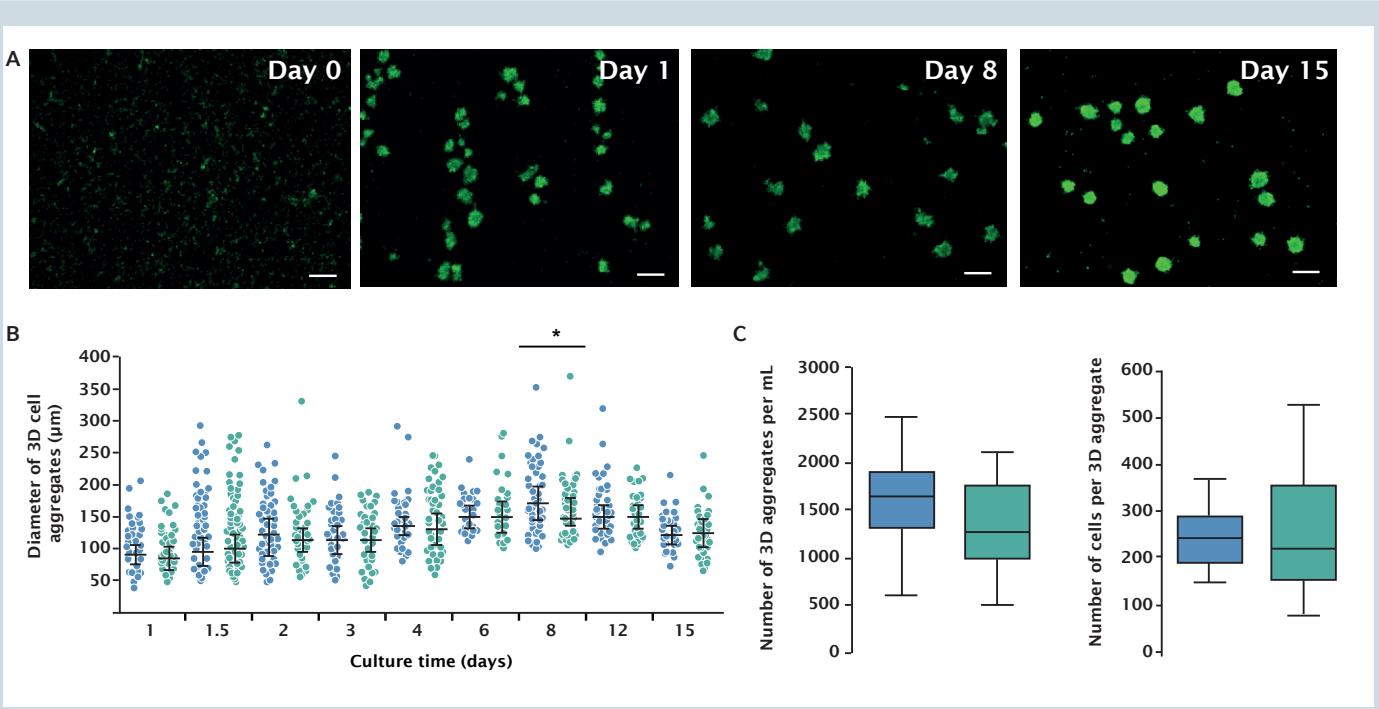

Fig. 5: Culture of 3D cell aggregates in perfusion in DASbox Mini Bioreactor System.
A. Fluorescence microscopy of 3D cell aggregates. Perfusion started at day 1 post-inoculation. Cells were stained with fluorescein diacetate (green, live cells) and TO-PRO-3 iodide (red, non-viable cells). Scale bars: 200 µm. **B.** The diameter of 3D aggregates throughout culture time for bioreactors operated under perfusion from day 1 (n=2, blue circles) and from day 3 post-inoculation (n=2, green circles) is shown in a scatter dot plot diagram. Lines show the median and interquartile (25th - 75th) percentile. One-way ANOVA and Holm-Sidak multiple comparisons test was used to calculate the statistical significance (* p-value=0.05). With the exception of day 8 post-inoculation (t=2.775, df=1762, Adjusted P value = 0.0491), there is no significant difference between bioreactors operated under perfusion from day 1 and from day 3 post-inoculation. **C.** The number of 3D aggregates per mL and the number of cells per 3D aggregates obtained throughout the culture is shown in box plots. Perfusion started at day 1 (blue bars) or at day 3 (green bars) post-inoculation. Number of bioreactors per perfusion experiment: n=2. Boxes show the median, 25th – 75th percentiles, and error bars show maximum and minimum values. Unpaired t-test (two-tailed) was used to calculate the statistical significance (* p-value=0.05). There is no significant difference in the number of 3D aggregates per mL (t=1.979, df=34, p-value = 0.056) or in the number of cells per 3D aggregate (t=0.467, df=34, p-value = 0.6435) for bioreactors operated under perfusion from day 1 and from day 3 post-inoculation.

considered the perfusion culture to be successful if the following criteria were met:

1. The cell retention device did not clog and the cells did not sediment.
2. The cells in the aggregates were viable throughout the culture and the aggregate morphology resembled the one reported previously by the group [7].
3. The concentration and diameter of 3D aggregates throughout the culture were similar to the values reported previously by the group [7].
4. The ratio of lactate production to glucose consumption was similar to literature values for mammalian cells [8].

The perfusion cultures in the DASbox Mini Bioreactor System met these criteria. The cell retention device did not clog and the cell aggregates stayed in suspension. Cells formed irregularly shaped 3D aggregates, with typical cell spreading and branching on the edges as reported by the group [7]. The cells remained viable throughout the

entire culture period (Figure 5A). The diameter of 3D cell aggregates increased from around 80 µm at day 1 up to 160 – 180 µm at day 8 – 12 (Figure 5B). The concentration of 3D aggregates varied mostly between 1000 and 2000 aggregates/mL (Figure 5C). These values are comparable to those reported in literature for the same cells cultured as 3D aggregates in spinner flasks equipped with a flat blade impeller (141 ± 21 µm at day 3 post inoculation and 1000 – 1500 aggregate/mL) [7]. The number of cells per 3D aggregate obtained throughout the culture varied mostly between 150 and 350 cells per aggregate (Figure 5C).

The estimated ratios of lactate production to glucose consumption were 1.73 ± 0.05 (perfusion started at day 1) and 1.64 ± 0.10 (perfusion started at day 3), which are similar to those reported in literature for mammalian cells [8].

Comparable results were obtained when perfusion was started at day 1 and day 3.

These results clearly demonstrate the suitability of the Eppendorf DASbox Mini Bioreactor System for cultivation of 3D cell aggregates in perfusion mode.

Conclusion

Researchers at the Instituto de Biologia Experimental e Tecnológica (iBET) in Portugal have demonstrated the suitability of the Eppendorf DASbox Mini Bioreactor System for the cultivation of the human tumor cell line H157 as 3D cell aggregates. The concentration, morphology, and diameter of the spheroids, and the metabolic profile of the cells resembled values that have been reported in literature [7, 8].

The scientists found that among the tested impeller types, the 8-blade impeller with 60 ° pitch was best suited to keeping the 3D cell aggregates in suspension. This result is in line with data obtained from cultures of human induced pluripotent stem cells as cell-only aggregates in stirred-tank bioreactors. Agitation using similar 8-blade impeller types kept these aggregates in suspension, as well as supporting their formation in the first place [9].

Using a stainless-steel filter with a pore size of 10 – 20 µm as cell retention device, the researchers successfully cultivated the aggregates in perfusion mode up to 15 days. The results confirm the general suitability of the culture system for bioprocess development and intensification.

Acknowledgements

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Ordering information

Description	Order no.
DASbox® Mini Bioreactor System for Cell Culture, max. 5 sL/h gassing, 4-fold system	76DX04CC
DASbox® Mini Bioreactor, for Cell Culture	76DS0250ODSS
Impellers	
3-Blade Impeller, 30 ° pitch, stainless steel, O.D. 30 mm, I.D. 5 mm, marine impeller	78107325
Pitched-Blade Impeller, 60 ° pitch, O.D. 34 mm, I.D. 5 mm, PA12	78107377
Holding Sleeve, for 8-blade impeller, stainless steel with set screw, I.D. 8 mm, for shaft with O.D. 5 mm (DASbox®)	78100595
6-Blade Rushton-Type Impeller, stainless steel, O.D. 30 mm, I.D. 5 mm	78107304
Micro Sparger, stainless steel, pore size 10 µm, O.D.6 mm, with O.D. 4 mm pipe, L 245 mm	78530511
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