

Bioprocessing

Massively Expanding Stem Cell Suspensions

Achieving Optimal Cultivation and Maintaining Pluripotency and Differentiation Potential Counts

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Human pluripotent stem cells (hPSCs) and their derivatives have gained increased importance for industrial applications in recent years. They have a great potential for therapeutic applications as well.

In vitro assays and novel regenerative therapies will require large cell quantities produced under defined conditions.

In conventional mammalian cell culture, the utilization of bioreactors is well established, e.g., for the production of recombinant therapeutic proteins, vaccines, and antibodies. Established protocols are used for process development and manufacturing of mammalian cells in 100–1,000-L scale and beyond.

Cell cultivation in stirred tank bioreactors allows for tight control and online monitoring of all relevant process parameters such as temperature, agitation, pH or dissolved oxygen, and scaleup.

This know-how can serve as a basis for creating processes to cover the demand on hPSCs, including human induced pluripotent stem cells (hiP-

SCs) and human embryonic stem cells (hESCs).

There already have been investigations on cultivating adherence-dependent stem cells in suspension culture. These studies mainly focused on the modification of matrix-attached hESC cultivation on microcarriers, a method that is widely used in conventional mammalian cell culture.

However, culture heterogeneity due to the preference of undifferentiated hPSCs to stick to each other rather than to prescreened types of microcarriers might be challenging. Recent studies in our labs and elsewhere have now demonstrated the potential of cultivating undifferentiated human ESCs and iPSCs as cell-only-aggregates in suspension.

More recently, we have demonstrated the feasibility of translating this approach into stirred tank reactors, paving the way for the envisioned mass production of pluripotent stem cells and their derivatives. Key features of this technology include utilization of a fully defined serum-free culture medium, single cell-based inoculation, and significant long-term expansion of hPSCs in easy-to-scaleup suspension independent of extracellular matrices

or scaffolds.

The method was successfully applied to several human hPSC lines and cynomolgus monkey ES cells as well. In a previous step, transfer from static suspension in culture dishes to stirred spinner and shaken Erlenmeyer flasks was also enabled.

Such dynamic cultivation of cell-only-aggregates turned out to be robust regarding the reproducibility of cell expansion, karyotype stability, and overall preservation of the stem cells' pluripotency. Somewhat lower expansion rates in dynamic conditions further suggested a high potential for culture optimization by applying a more controlled environment.

To improve culture monitoring and control capabilities and to pave the way for larger-scale cultivation, the method was transferred to a stirred tank bioreactor system.

Studies were carried out in a **Dasgip** parallel bioreactor system consisting of four 250 mL cultivation vessels

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(100–250 mL working volume) including an integrated Dasgip control unit and software. Allowance was made for independent monitoring and control of temperature, pH, oxygen tension, and stirring conditions.

To test and ensure reproducibility, all approaches were performed in four independent experimental repeats in a culture volume of 125 mL

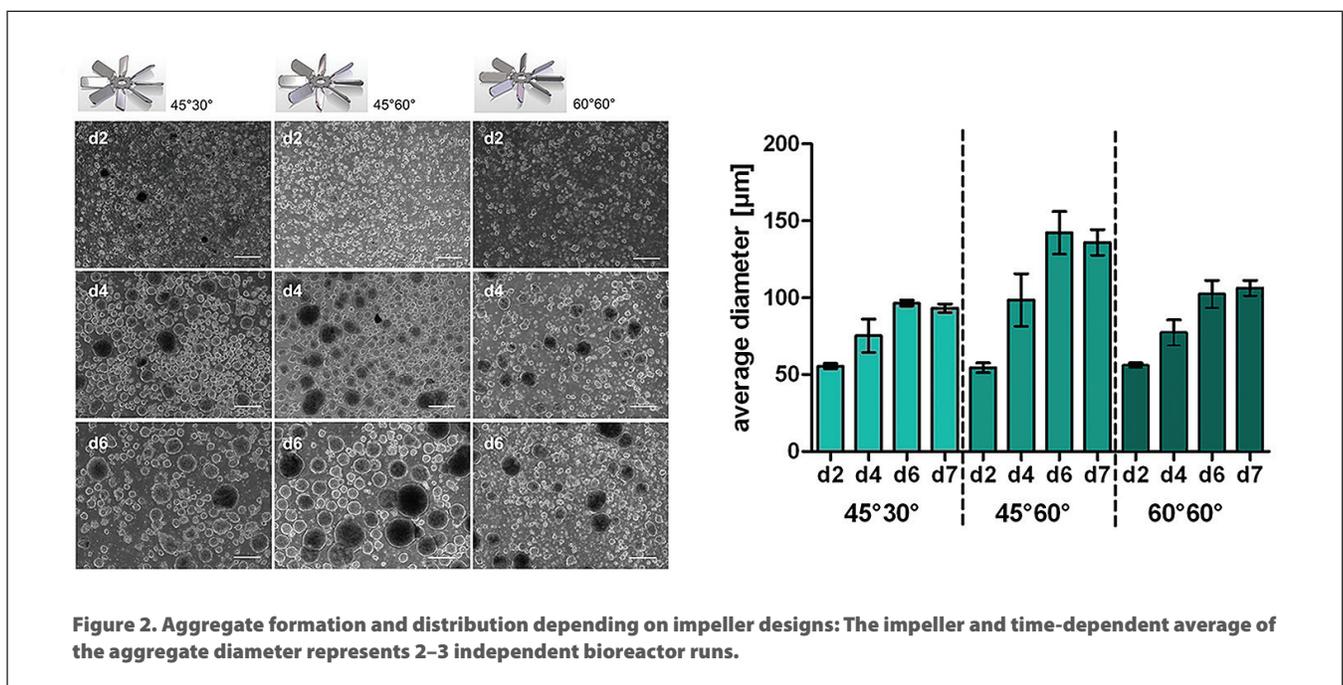
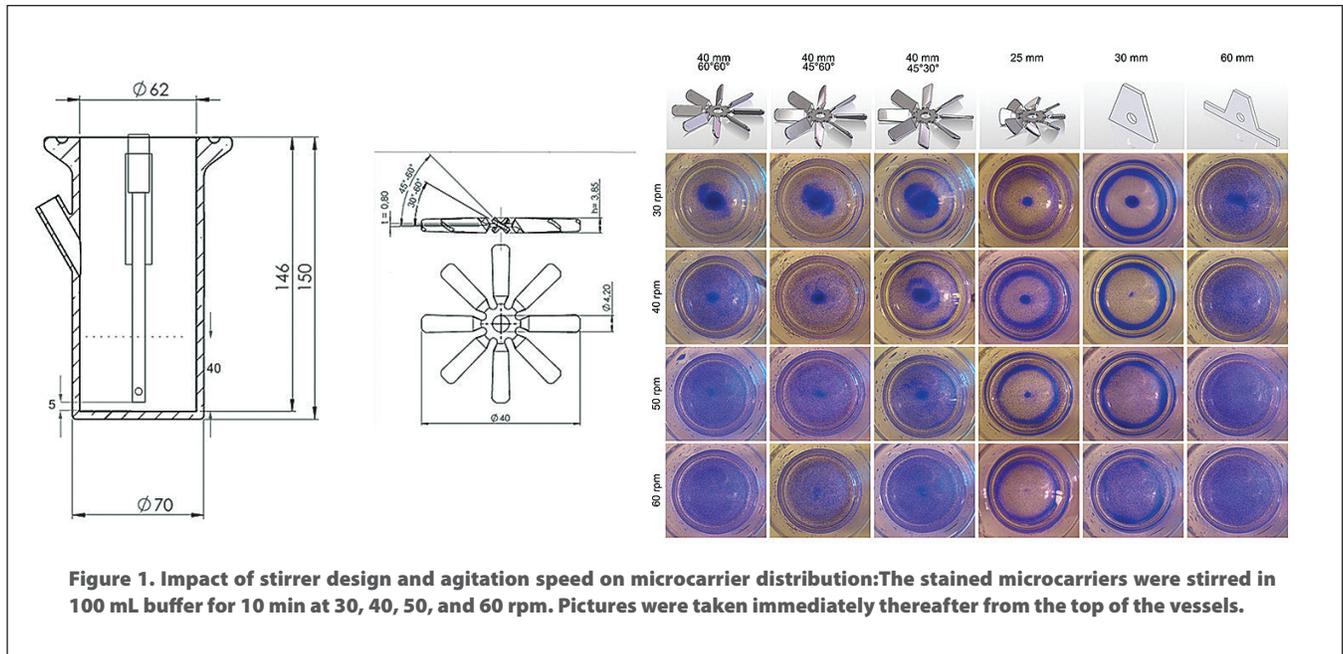
each. Utilizing cord blood endothelial cell-derived hiPSCs, preliminary experiments showed that the inoculation density and the agitation mode were highly critical for successful process initiation in impeller-stirred bioreactors.

Experiments revealed that 5×10^5 hPSCs/mL was an efficient inoculation density in the stirred bioreactor

setting. Subsequently, experiments were carried out to compare impeller designs and stirring speed modulation aiming at optimal control of cell aggregation and homogeneity of aggregate size distribution.

Impeller Design

Axial 8-blade pitched impellers and modified stirring bars were designed



and tested for comparative agitation studies (Figure 1). The basic design of the pitched blade impeller was previously developed and successfully applied to control single cell-inoculated mouse ESC aggregate formation in a 2-L bioreactor scale.

In the context of the 250 mL vessels inoculated with 125 mL culture volume, impeller dimensions were down-scaled to ensure similar geometries of the reactor-impeller design. Impeller variants also differed in blade size and angle as outlined in Figure 1.

To initially evaluate suitability of the agitation system, cell-free assessment of microcarrier distribution in the bioreactor was tested providing a meaningful and cost-efficient substitute of cell ag-

gregates. All impeller designs and stirring bars were analyzed at agitation speeds varying between 30–60 revolutions per minute (rpm; Figure 1). These experiments revealed that all three 8-blade 40 mm impeller variants as well as a 60 mm stirring bar resulted in homogeneous carrier distribution at 40 or 60 rpm, respectively. Having the blade impellers' applicability for the common use with bioreactors in mind, only these devices were chosen for the subsequent cell culture experiments.

Undifferentiated hiPSC cultures (expanded in conventional 2D culture) were dissociated and inoculated as single cell suspensions at $4\text{--}5 \times 10^5$ cells/mL in a total volume of 125 mL and stirred at 60 rpm. To visualize the im-

part of the three different 8-blade impeller design variants, aggregate diameter analysis and size distribution was carried out by light microscopy.

Notably, all approaches resulted in successful aggregate formation from single cells whereby minor variability between the three impeller designs was detected (Figure 2). Growth kinetics and metabolic activity revealed robust and reproducible hiPSC expansion in the stirred bioreactor system.

Evaluation of viable cell numbers in eight independent bioreactor runs over the time revealed, on average, a robust, about four-fold expansion of the inoculated hiPSCs after seven days of cultivation (Figure 3). Monitoring the metabolic activity by determining glucose, lactate, and amino acid concentrations as well as pH and dissolved oxygen levels confirmed efficiency and high reproducibility of the culture system.

Importantly, further analysis proved maintenance of pluripotency-associated marker expression of suspension culture expanded cells, and functional assessment confirmed their multilineage differentiation in vitro.

Conclusions

In summary, advanced parallel bioreactor systems are highly suitable for the transfer of single cell inoculated human pluripotent stem cell suspension to fully controlled cultivation. The precise process control, detailed online monitoring as well as evaluation and optimization of complex, multifactorial culture parameters will further allow significant process optimization in ongoing studies.

Capable of operating with even small working volumes of 125 mL (while the functionality and geometry is similar to larger-scale stirred bioreactors), these reactor systems are matching demands of process development and optimization for human stem cell cultivation. The yield of 2×10^8 pluripotent cells in a single process run in the 125 mL scale shown in our previous study has proven the technology to be suitable to enable the mass expansion of human pluripotent stem cells. **GEN**

