Validation of CellRev's continuous technology using C2C12 cells

Summary of case study

This case study validates CellRev's proprietary media supplement (Continuase™) as a strategy to continuously manufacture adherent cells at a steady state. Using C2C12 as a model cell line, CellRev demonstrated that continuous cell detachment can be achieved for at least 60 days for adherent cells). Validation was performed firstly at small scale and subsequently in a proprietary bioprocess design at 2L scale (Figure 1). In the 2L bioreactor, the integration of a collection system demonstrated successful continuous collection of the detached cells. Overall, the case study highlights CellRev' capabilities in allowing for continuous manufacturing of adherent cells with maintained phenotypic properties.

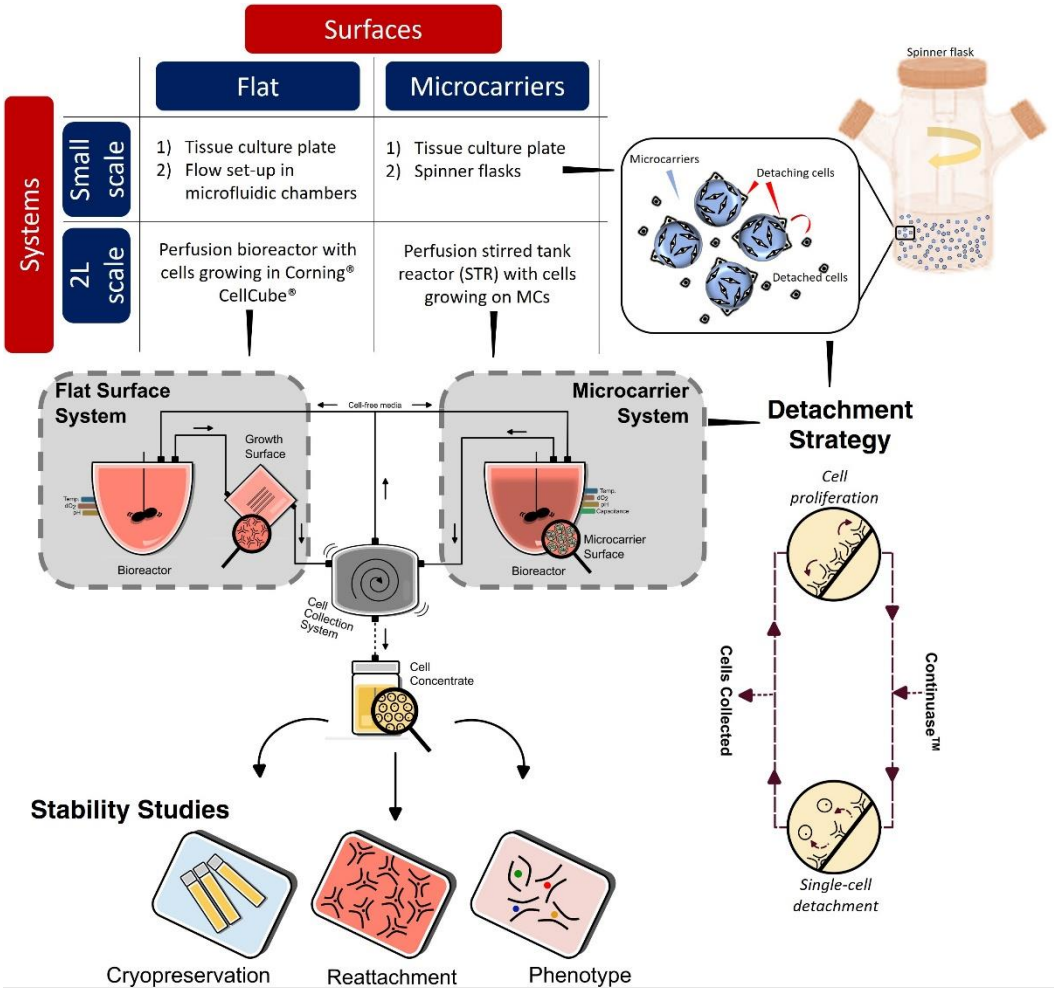

Figure 1. Systems and conditions used in the study. The technology was firstly validated at small scale on flat surfaces and on microcarriers (MCs) both in tissue culture plastic and in flow conditions. Subsequently, both flat and MCs were successfully scaled-up to a 2L proprietary bioprocess and stability tests evaluating cell reattachment, phenotype and suitability to cryopreservation, were performed throughout the process. For all systems and conditions, our proprietary detachment strategy with Continuase™ was utilised to continuously grow, detach and collect C2C12 cells.

Continuase™ allows for continuous manufacture of C2C12 growing on flat surfaces.

A murine myoblast cell line (C2C12) was selected as a model cell line due to its short doubling time and suitability for long-term continuous culture. For studies at small-scale, C2C12 cells were grown on a 1cm^2 flat surface in a well plate with Continuase™ supplemented to serum-free media (SFM). The detached cells were harvested every 24 hours, with continuous cell growth and detachment successfully demonstrated over 3 weeks. Confluency of growing cells was maintained (Figure 2A) whilst cells continuously detached (Figure 2B). The respective count of attached and detached cell numbers (Figure 2C) indicated a steady pattern of cell proliferation and yield was achieved. No changes in cell phenotype were observed during the process. Moreover, to verify that flow would not negatively impact the continuous cell detachment, experiments were performed in a small-scale set-up under flow growing C2C12 in microfluidic "Ibidi" chamber slides. Consistently with previous data, a comparable number of proliferating cells were maintained on the originally seeded surface and the continuously detached cells were viable, capable to reattach and maintained the initial undifferentiated phenotype (Figure 2D).

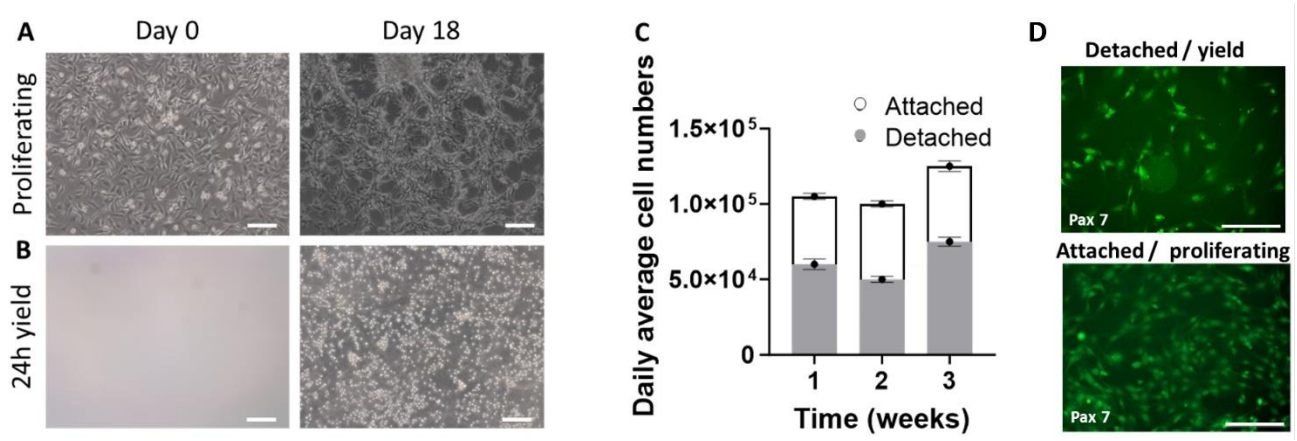

Figure 2. Continuous detachment in small-scale flat systems. (A) Representative images showing a similar number of attached cells over 3 weeks. (B) No detached cells were observed on day 0 before adding Continuase™, whilst after Continuase™ addition cells started to detach as exemplified in a 24h yield from day 17 to 18. (C) Quantification of attached and detached cells expressed as daily average cell numbers cultured for over 3 weeks, suggesting steady state achievement with a consistent number of proliferating cells (attached) and yielded cells (detached). (D) Representative images showing expression of undifferentiated cell marker Pax7 in both detached and attached cells at the end of the experiment, suggesting maintenance of cell phenotype over the time in culture. Scale bars: 200μM.

Subsequently, experiments were scaled-up to a 2L perfusion reactor with C2C12 cells growing on the surface of a Corning® CellCube® (surface can range from 8500cm^2 to 85000cm^2) with a continuous bioprocess designed and patented by CellRev. Such bioprocess was initially validated with three 14-day runs where C2C12 cells were continuously grown, detached, and collected demonstrating that continuous production of adherent cells can be achieved in an automated system allowing high cell yield and viability. Through iterative process refinement, both the duration of the runs and the overall quality of the process were improved. Run duration was successfully extended to 60 days.

Stability studies were performed to ensure C2C12 cell characteristics were unaffected by the process by assessing the capacity of the collected cells to re-adhere, maintain their characteristic growth rate, phenotype, and differentiation potential. The obtained results from a 60-day continuous

run showed positive outcomes. Indeed, the collected C2C12 demonstrated their ability to re-adhere, maintain their typical doubling time, phenotype, and differentiation potential (Figure 3).

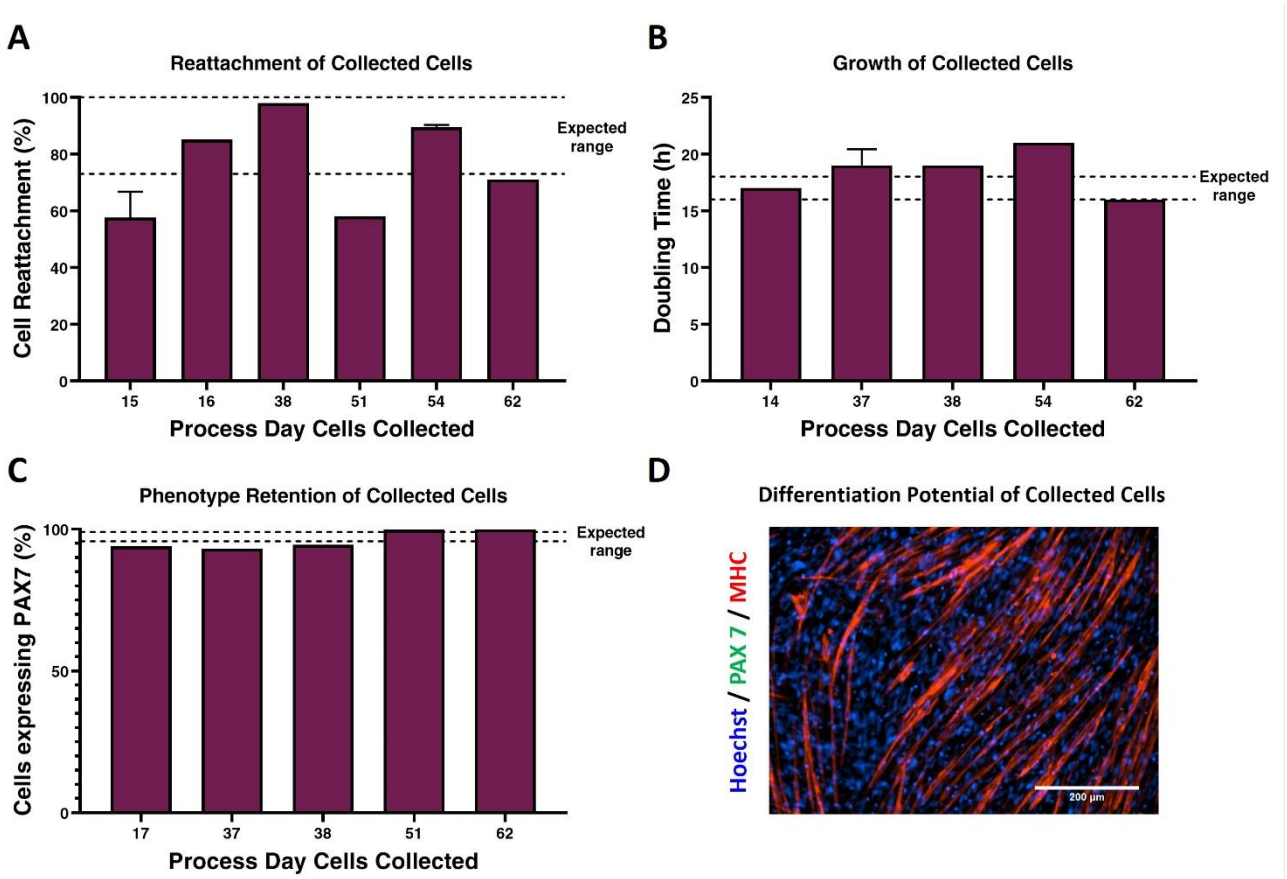

Figure 3. Stability studies. Cells collected during the 60 days continuous culture in the 2L bioreactor were routinely assessed for reattachment (A); proliferation rate (B); phenotype retention (C) and differentiation potential after inducing differentiation (D). For cell phenotype evaluation, expression of undifferentiation cell marker Pax 7, (green stain) and differentiation cell marker MHC, (red stain) were analysed from collected cells. Scale bars: 200µM.

Although the scalability of a flat system is limited, it can achieve a productivity rate of 2 tons/m^3/year, which is equivalent to 167g/L/month, hence relevant for the cell & gene therapy and biopharmaceuticals markets. Such productivity rates are three times greater than running the identical system in a batch process.

Continuase™ allows for continuous manufacture of C2C12 growing on microcarriers .

CellRev technology was also validated on microcarriers (MCs), and continuous cell detachment was demonstrated for at least 40 days at small scale (Figure 4) and at 2L. Whether in well-plates or in spinner flasks, C2C12 were grown on MCs in SFM supplemented with Continuase™ and the detached cells were manually collected every 24 hours. C2C12 confluency on the MCs was successfully maintained during the time in culture with continuous cell detachment occurring in both systems (Figure 4A, 4B). Importantly, cell functionality was maintained during the process: i) the continuously detached cells were capable of reattaching to a new surface and maintain their phenotype (Figure 4C); ii) the cells harvested from the respective surfaces at the end of the experiment were also capable of reattaching and display an undifferentiated phenotype (Figure 4D).

Interestingly, due to CellRev's supplement ability to maintain the system at a steady state with a stable cell confluency on the MCs, the typical MCs aggregation phenomenon did not occur over the long time in culture (Figure 4F).

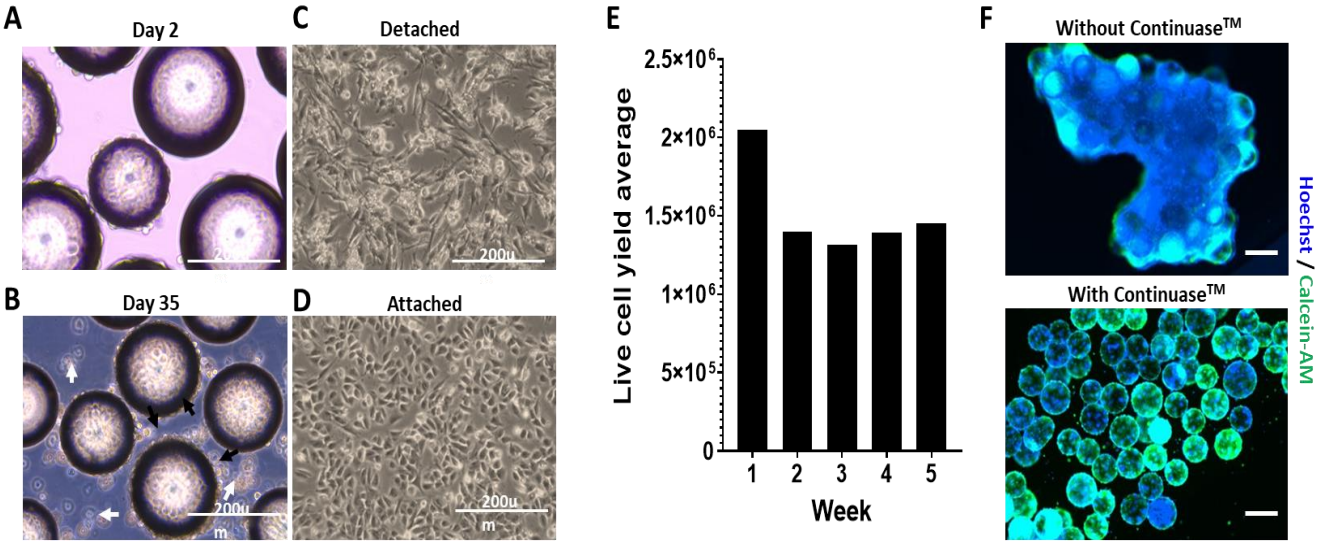

Figure 4. Continuous cell detachment from MCs in small-scale MCs systems. Representative image of C2C12 cell confluency after seeding (A) and after 35 days in culture with Continuase™ (B) where the detached can be seen at the bottom of the well (white arrows). (C) Images showing re-attachment capabilities of the continuously detached cells and (D) of the harvested cells from MCs at the end of the experiment. (E) Graph showing the daily average number of detached cells over 5 weeks using Continuase™. (F) Images showing *the effect of Continuase™ on MCs aggregation using Hoechst and Calcein-AM staining. Scale bars: 200μM.*

The detachment strategy was further validated in our proprietary 2L bioprocess in a stirred-tank reactor (STR) growing C2C12 on MCs in SFM. The technology was validated at this scale with a 1% MCs loading and automated collection in reactor runs lasting at least 16 days (2 days process start-up and 14 days total bioprocess time). The advantage of scaling the technology from spinner flask to a STR system allows for process control and offline/online monitoring systems as well as larger biomass to perform downstream studies for assessing cell re-adherence, growth rate, phenotype maintenance, and differentiation potential. The whole bioprocess was monitored (**Figure 5A**) measured with an integrated Futura biomass probe (Aber Instruments Ltd). Addition of Continuase™ on day 2 (**Figure 5A**) resulted in continuous detachment of cells from the surface of microcarriers (**Figure 5C**). Continuously collected cells (**Figure 5B**) as well as cells harvested at the end of the bioprocess (**Figure 5D**) were tested for adherence and morphology.

The current 2L system can achieve a theoretical productivity of 4 tons/m^3/year with a 15% v/v MCs loading (corresponding to 333g/L/month) and 480g/L/month if the MCs loading is 40% v/v. CellRev is currently working on process intensification by increasing the MCs loading to maximise reactor productivity. Overall results highlighted the feasibility of CellRev's continuous processing for scalable, continuous adherent cell production.

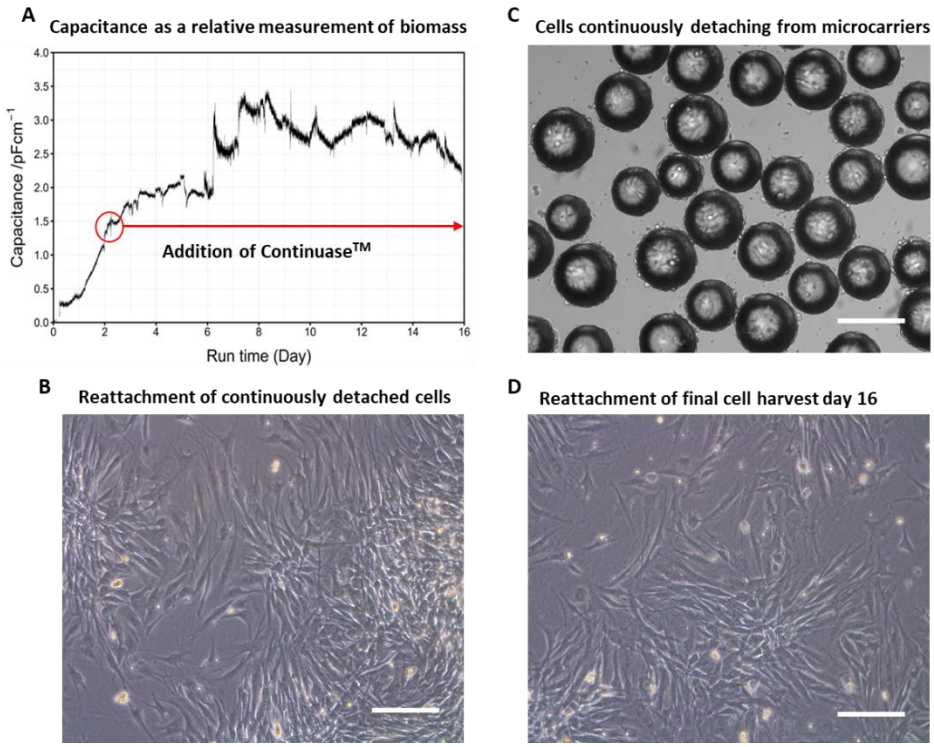

Figure 5. Continuous cell detachment from MCs in small-scale MCs systems. Representative image of C2C12 cell confluency after seeding (A) and after 35 days in culture with Continuase™ (B) where the detached can be seen at the bottom of the well (white arrows). (C) Images showing re-attachment capabilities of the continuously detached cells and (D) of the harvested cells from MCs at the end of the experiment. (E) Graph showing the daily average number of detached cells over 5 weeks using Continuase™. (F) Images showing *the effect of Continuase™ on MCs aggregation using Hoechst and Calcein-AM staining. Scale bars: 200µM.*

Conclusion

CellRev have demonstrated the ability to continuously manufacture adherent cells in a bioreactor system. CellRev's Continuase™ proves its efficiency by matching the cells' proliferation rate with their detachment rate, hence maintaining the system at steady state, across various conditions and scales. CellRev's patented bioprocess allows to continuously manufacture adherent cells outperforming traditional batch processes in terms of cell yield, while maintaining cell functionality.

Overall, CellRev's offering facilitates processing of adherent cells in a continuous manner providing a scalable and more efficient platform compared to batch processes for research and manufacturing purposes.

Please email enquiry@cellrev.co.uk to discuss your processing needs.

CellRev

Scalable adherent cell processing for research and manufacturing.
The company's patented manufacturing platform is an industry-first, facilitating faster, cheaper, and more sustainable production of cellular products. CellRev's platform offers seamless translation from research to market with superior automation, control, and stability versus existing technologies.