**An innovative fixed bed bioreactor platform for linearly scalable, biomass predictable, single-use adherent cell biomanufacturing**

Vasiliy N. Goral\*, Yulong Hong\*, Jeffery J. Scibek\*, Yujian Sun\*, Lori E. Romeo, Abhijit Rao, Daniel Manning, Yue Zhou, Joel A. Schultes, Vinalia Tjong, Dragan Pikula, Kathleen A. Krebs, Ann M. Ferrie, Stefan Kramel, Jennifer L. Weber, Zara Melkoumian, Todd M. Upton, Ye Fang\*\*

*Corning Life Sciences, Corning Incorporated, NY 14831, USA*

\* Equal contributions.

\*\* Corresponding author. Email: fangy2@corning.com.

**Key words**: Fixed bed bioreactor; computational fluid dynamics; adherent cell biomanufacturing; adeno-associated virus vector; oxygen uptake rate; biomass

**Abbreviations**

AAV, adeno-associated virus; CFD, computational fluid dynamics; CGT, cell and gene therapy; DPBS, Dulbecco's phosphate buffered saline; DO, dissolved oxygen; FBR, fixed bed bioreactor; FBS, fetal bovine serum; GFP, green fluorescence protein; GMP, good manufacturing practice; MCV, medium conditioned vessel; OUR, oxygen uptake rate; PET, polyethylene terephthalate; qPCR, quantitative polymerase chain reaction; RTD, residence time distribution; VG, viral genome

**Abstract**

Scalable single use adherent cell-based biomanufacturing platforms are part of solutions to realize the full potential of cell and gene therapies. Here, we reported the development of an innovative fixed bed bioreactor platform for the scale-up of adherent cell culture. The bioreactor platform is centered on a packed bed of woven polyethylene terephthalate mesh discs that are vertically stacked and sandwiched between two fluid guide plates. Computational fluid dynamics modeling was used to direct the design and development of bioreactor series, targeting uniform flow with minimal shear stress. Residence time distribution measurements revealed that a pulse injected dye tracer solution passed through the bioreactors with great uniformity and narrow distribution of residence time, mimicking plug flow. Periodic media sampling with an offline analyzer showed that there was minimal gradient of four important metabolites (glucose, glutamine, lactate, and ammonia) across the bioreactor throughout cell growth. The bioreactor platform was further validated in automated cell harvesting with ~96% efficiency and ~98% viability, as well as linear scalability, in terms of both operational parameters and performance, for cell culture and adeno-associated virus vector production. Finally, mathematic models based on oxygen uptake rates were developed and proven effective to model cell growth curves and estimate biomass in real-time. This study shows that this innovative fixed bed bioreactor platform enables linearly scalable adherent cell-based biomanufacturing with high productivity.

**Introduction**

The past decade has witnessed increasing demand for scalable single-use adherent cell-based biomanufacturing solutions, mostly driven by the accelerating development of cell and gene therapies (CGT).1-3 Scalability is essential to advance a process from development to production. For instance, an ideal viral vector biomanufacturing platform must be able to scale to at least 1018 viral genome (VG) per batch for certain therapeutic applications.4 However, scaling-out using conventional 2-dimensional (2D) flask-based systems is highly costly, labor-intensive, and impractical for commercial scale production of both therapeutic cells5 and gene delivery vehicles such as viral vectors or viral like particles6 for CGT applications.

Packed or fixed bed bioreactors (FBRs) have been emerging as a promising platform to address the unmet needs in CGT biomanufacturing, especially when higher cell numbers or virus titers are needed. The iCELLis® bioreactor systems from Cytiva Life Sciences uses a disposable vessel packed with polyethylene terephthalate (PET) matrix of varied sizes at two different densities (144 or 96 g/L), providing up to 500m2 effective surface area for cell culture and viral vector production.7-11 However, this platform suffers two drawbacks – difficulty in cell harvesting and relatively poor uniformity in cell distribution and fluid flow arising from varied compaction of the PET matrices. The Scale-X™ bioreactor systems from UniverCells Technologies employs a single-use bioreactor vessel consisting of non-woven spiral-wound, double-layer PET with a spacer netting between the layers, enabling up to 600m2 surface area for cell culture.12,13 Similar to iCELLis, Scale-X systems use magnetic stirring to drive media recirculation and mixing inside the vessel. This platform has shown some improvements in cell distribution and fluid flow, enabling linear scalability from development to production. However, this platform seems to have limited harvest capability which requires vibration and only works for the small-scale reactors. The BioBLU® bioreactors from Eppendorf are pre-loaded with Fibra-Cel® Disks, providing a single ready-to-use disposable vessel with a total of 18m2 surface area for cell culture.14 Fibra-Cel disks are round-shaped and made of non-woven polyester mesh with polypropylene support. Other higher scale systems from Eppendorf are not ready-to-use; these systems also do not support effective cell harvesting.

Beside bioreactor design and fluid dynamics, scalability is also stemmed from the automated systems that monitor and control all relevant parameters. The ability to monitor all relevant parameters with the same measurement type at each process scale is highly desired to keep the product quality and quantity high and within Good Manufacturing Practice (GMP) compliance.15-17 Biomass is an essential metric in cell culture and is one of the most important indicators for productivity and product quality in large scale mammalian cell culture and biomanufacturing.18,19 Real-time biomass monitoring is critical to assess process quality, increase productivity, and develop model-based process control such as feed schedule, perfusion rate control, and timing of transfection and harvest. Capacitance sensors have been used in selective small iCELLis systems for biomass monitoring. However, these biosensors have clear limitation for large scale bioreactors, since they only permit detection of biomass within a short sensing distance (<40mm)20.

Herein, we employed modeling to guide the development of a novel fixed bed bioreactor platform that is centered on the vertical stacking of woven PET mesh discs within a vessel. The platform allows for improved mixing options by having a medium conditioned vessel (MCV) separated from the bioreactor, and improved recirculation rate in both range of and precision via recirc pump. The platform also has capability to direct measure both inlet and outlet oxygen level, thus enabling optimized control of flow rate, oxygenation levels, and modeling of cell growth curves. The bioreactor platform combines the benefits of adherent cell-based bioproduction with the scale and automation of suspension systems.

**Results**

**Modeling directs the development of the FBR platform**

To meet the needs for various applications associated with adherent cell-based biomanufacturing, we developed a FBR platform consisting of bioreactors and control systems at three different scales, Process Development (PD, benchtop), medium, and large, each having three different cell growth surface areas (1, 2.5 and 5m2 for the benchtop; 20, 50 and 100 m2 for medium scale; 200, 500 and 1000 m2 for large scale) (**Fig.1a**, **Fig.S1**).

We applied computational fluid dynamics (CFD) modeling, a powerful tool for analyzing and visualizing fluid dynamics in reactors21, to guide and characterize the bioreactor design. A variety of CFD stimulations were performed to optimize the design including the diameter, height, and packing density of the packed bed to achieve linear scalability in operation and performance based on the desired flow uniformity and stress profiles under normal culture conditions. As a result, the packed beds of three bioreactors for each scale were finalized to have the same diameter, but distinct heights (**Fig.S1**). The packed bed was made via vertical stacking of a desired number of circular PET mesh discs (**Fig.S2a-c**) to achieve the same packing density, ensure uniform flow, and prevent nesting and compaction. The packed bed was sandwiched between the two fluid guide plates to further improve the flow uniformity.

Macro-scale CFD simulations were performed to verify the flow uniformity. The packed bed was treated as a porous medium. Results showed that the flow velocity was uniform across the entire packed bed of a 2.5m2 bioreactor, but not the entrance and exit regions of the housing vessels (**Fig.1b**). Similar pattern was found for other sized bioreactors (**Fig.S3**).

Micro-scale CFD simulations were also carried out to study local flow and shear characteristics within the packed bed. A flow rate of 2 L/min, which is much higher than those commonly used for cell growth (**Table S1**), was used to model the shear stress within a benchtop bioreactor. Results showed that the shear stress inside the liquid, calculated as strain rate magnitude multiplied by viscosity, was uniform and relatively small (<0.1 Pa) (**Fig.1c**); a similar pattern was observed for the wall shear stress on the PET mesh surfaces (**Fig.1d**, **Fig.S4**). These results suggest that the induced shear stress is much smaller than the limit that can be tolerated by cells (in the order of 1-10 Pa22-24) and is expected to have minimum impact on cell growth.

Together, CFD simulations have been applied to direct the bioreactor design in terms of the diameter, height, and packing density of the packed bed such that flow velocity is uniform, and both fluid and wall shear stress are acceptably small.

**The FBRs provide homogeneous environment for adherent cell culture**

To examine whether the bioreactors can provide homogeneous environment for cells, we first determined the residence time distribution (RTD) of a tracer molecule flowing through three different bioreactors, 2.5, 5 and 100m2. The RTD value was determined by rapidly injecting a tracer dye solution into the water flow at the inlet of a bioreactor and monitoring the temporal concentration profiles of the tracer at the outlet (**Fig.S5a**). Here, McCormick® Culinary Green food color was used as the tracer. In this pulse test, the tracer was injected into a bioreactor at time *t=*0, and the tracer concentration *C* in the effluent stream (the outlet) was measured by its absorbance at 630nm. To better visualize the RTD, the absorbance data were converted to a normalized, dimensionless function *E(θ)*, which is independent of reactor size and flow rate25. Results showed that the tracer solution passed through all three bioreactors as a narrow band (**Fig.S5b-d**) and tight distribution of the residence time (**Fig.1e**), suggesting that the fluid flow is uniform and independent of bioreactor volume, and all media components if not being consumed by cells will reside in the packed bed for the same amount of time.

Considering cell metabolism, we next examined the metabolite gradients across a 1m2 bioreactor during a 4-day culture of HEK293T cells. Here, media before and after the bioreactor were sampled and measured offline using an analyzer every 30min for 8 hours each day. Results showed that the four important metabolites (glucose, lactate, glutamine, and ammonia), together with pH, all displayed negligible gradient across the bioreactor throughout the culture (**Fig.1f** and **Fig.S6**), suggesting that the cell culture environment is quite homogeneous inside the bioreactor. However, the dissolved oxygen (DO) displayed a time dependent gradient across the bioreactor (**Fig.1g**). Considering that the DO at the outlet was set to be above 20% all the time, the increasing DO gradient over time is expected to have minimal impact on cell growth.

Together, both RTD and metabolite results suggest that the bioreactors provide homogeneous environment for cell attachment, growth, and transfection.

**The FBRs enable linear scalability of operation, cell attachment and growth**

To demonstrate the linear scalability of the bioreactor platform, we first optimized cell attachment and growth using 1m2 bioreactors with HEK293T cells as the model. Culture experiments identified optimal operational parameters for cell attachment and growth (**Table S1**). Among them, the flow rate for seeding was found to be critical to the uniformity of cell distribution within a packed bed, while the media volume during batching and inoculation determined the feeding frequency and cell health, and the starting flow rate for culture was important to ensure the environment uniform within the packed bed. We further found that these operational parameters can be linearly scaled up to achieve optimal or near optimal culture for larger size bioreactors (**Table S1**). Specifically, the flow rate for seeding was a function of bioreactor size or packed bed volume, while the flow rate for culture was a linear function of the unit area of the packed bed, and the same ratio between the medium volume during batching and inoculation and the reactor’s surface area was maintained among different bioreactors (**Fig.S7**).

Second, we examined the cell attachment kinetics. Here, cells were added into the MCV and sampled periodically until the cells in the MCV medium were depleted at least 90% under the optimal flow rate for seeding (**Table S1**). Results showed that all the cell attachments followed a one-phase association process, yielding a kinetics that is independent of reactor size (one-way ANOVA *p-*value of 0.5026) (**Fig.2a**, and **Fig.S8**).

Third, we assessed the cell distribution within a bioreactor. Cells were seeded at a density of 22,000 cells/cm2 and cultured for four days in three different 5m2 bioreactors. After culture, the bioreactors were dissembled. Each packed bed was divided into 23 segments; from each segment one disc was stained with crystal violet and imaged, while the rest of the discs were subjected to manual cell harvesting and counting. Results showed that for the three independent experiments the final cell density was found to be between 84k and 104k per cm2, all having a standard deviation of <30% within each bioreactor (**Fig.2b**). Optical imaging after staining also confirmed the uniformity of cell distribution within each mesh disc or within each reactor (**Fig.S2d-e,** and **Fig.S9**). A similar trend was observed in a 50m2 bioreactor (**Fig.S10**). These results confirmed that cells can attach uniformly within bioreactors using the optimal seeding protocol.

Fourth, we examined the efficiency of auto-harvesting. To do so, after culture five individual discs were removed, one from each pre-defined location of the packed bed (as exampled in **Fig.S9**), and subject to manual harvesting, while the remaining packed bed was subject to automated harvesting. The averaged cell densities were obtained and compared to determine harvesting efficiency. Results showed that the averaged cell harvesting efficiency was 96.7±2.7% (n=9) (**Fig.2c**) with a viability of 96.4±0.8% (n=9) for 5m2 bioreactors. In addition, as cell density increases, the auto-harvest efficiency decreases slightly, as evidenced by the slope of 0.932 (**Fig.2c**). Similar auto-harvesting efficiency was observed for other sized bioreactors (data not shown). These results suggest that the bioreactor platform permits automated harvesting with great efficiency and high cell viability.

Fifth, we examined the population doubling time of HEK293T cells. Here cells were seeded at ~22k/cm2 and cultured for 3 to 4 days for all bioreactors, except for the 100m2 bioreactors where cells were seeded at ~15k/cm2. Results showed that the cell doubling time was consistent across all bioreactors, except for the 100m2 bioreactor which gave rise to slightly longer doubling times (one-way ANOVA *p*-value of 0.0325 for all, 0.1554 excluding the 100m2 bioreactors), suggesting that the bioreactor systems enable consistent cell growth and the cell culture condition need to be further optimized for the 100m2 bioreactor.

Sixth, we evaluated the growth curve in 5m2 reactors. Here, cells were seeded at 22k/cm2 for 3- or 4-days culture, but at 15k/cm2 for 5-days culture to avoid very high density over long term culture. Results showed that there are increasing cell numbers over time, and the highest cell density achievable for HEK293T seems to be around 600k/cm2, equivalent to ~70 million/mL (**Fig.2e**).

Lastly, we compared glucose consumption. Results showed that the amount of glucose consumed per cell was comparable among different bioreactors (one-way ANOVA *p*-value of 0.1806) (**Fig.2f**), suggesting that cell metabolism is similar among different bioreactors.

Together, these results suggest that the FBR platform enables process simplicity in terms of scale-up operation and automated harvesting and provides a linearly scalable solution for cell attachment and growth.

**The FBRs permit uniform transfection and high titer for AAV production**

Since adeno-associated viruses (AAVs) have high prominence in gene delivery,26 we examined the transfection efficiency and viral vector titers using AAV2-GFP as the model. Here classical triple transfection protocol was used to transfect cells after 3-days culture, followed by 2-days production. Afterwards, the bioreactor was dissembled, representative PET discs at defined locations were used to harvest cells, followed by flow cytometry analysis. The remaining discs were subject to cell lysis to collect viral vectors. Results showed that the transfection efficiency was high and uniform at different locations within a bioreactor with >90% GFP positive cells and >2000 mean fluorescence intensity (**Fig.3a**), indicating an even distribution and uptake of transfection complexes which, in turn, resulted in uniform transfection across the 2.5m2 packed bed.

qPCR was used to determine AAV titers. Results showed that the total genome copies obtained displayed a linear function of surface areas (**Fig.3b**), and the productivity per cell showed little difference among the bioreactors and 2D controls (**Fig.3c**). The averaged titer per cell was found to be ~65000 for the bioreactors. One-way ANOVA analysis confirmed that there is no statistically significant difference among means obtained in different vessels (*p* = 0.5275). These results demonstrated that the bioreactor platform is linearly scalable for AAV production.

**DO profiles are indicative of biomass in real-time**

Oxygen consumption has been used to estimate viable cell density for microbial and mammalian cells in suspension culture27-31, but not for perfusion adherent cell culture. Thus, we explored the possibility to leverage real-time DO monitoring to model cell growth curves for AAV2-GFP production experiments. Here, a pair of oxygen biosensors were used to monitor DO level, one in MCV media (termed as MCV DO) which was maintained at ~100% DO (182µM) all the time, and another in the outlet media (termed as FBR DO) which was set to be above 20%. The HEK293T AAV2 bioproduction experiment in a 2.5m2 bioreactor consisted of four phases: cell seeding (~3 hours), growth (~3 days), transfection (~1 day), and production (~2 days). As expected, as the cells grew, the DO in the FBR media outlet decreased initially until it reached and then maintained at ~20% via increasing flow rate (**Fig.4a**).

Given that there is an increasing DO gradient within the bioreactor over time, a mass balance perfusion bioreactor model was used to first determine oxygen uptake rate (OUR):

(1)

Where *Cin*is the concentration of DO in the medium entering the bioreactor (the same as the MCV DO), *Cout* the concentration of the FBR DO, and *Q* the flow rate.

Assuming that the averaged oxygen consumption rate per cell is constant and independent of cell cycle and culture condition,32 the OUR is a direct function of cell number *N*:

(2)

Since cell attachment is a rapid process33,34, it is reasonable to assume that there is little cell growth during this short phase. Thus, the apparent obtained through dividing the OUR values by the cell seeding number were used to fit with the one-phase association model to obtain cell attachment kinetics. Results showed that initially increased from an apparent negative value and quickly reached to a steady state (**Fig.4b**). The initial negative values suggest that the oxygen consumption rate is different for cells suspended in media versus adhered onto the mesh surfaces. The steady level once cell adhesion completed suggests that the value is close to being constant, consistent with what’s reported in literature for cells cultured in stirred bank bioreactors35. Results also showed that the initial values fit well with the one-phase association model, yielding an apparent cell attachment kinetics of 0.51 hr-1, almost identical to that obtained using the offline cell counting approach (0.49 hr-1) (**Fig.4b**).

According to a cellular energy metabolism model, the OUR is related to both oxygen necessary for biomass maintenanceand oxygen necessary for growth36,37:

(3)

Where *N* is the cell number, the oxygen consumption coefficient for maintenance, and *Yxo* the yield of oxygen consumed for cell growth. According to a logistic growth model, the cell number *N* at a given time *t* after cell attachment completes is38:

(4)

Where *A* is the maximal fold expansion which equals to *Nmax/N0*, *Nmax* the maximal cell number, *N0* the cell seeding number, *µmax* the maximal cell growth rate, and *λ* the lag time.

The OUR value was found to increase over time but displayed several distinct phases for the AAV2 experiment (**Fig.4c**), suggesting that transfection and AAV2 production may have an impact on nutritional demand and cell growth. Furthermore, the overall OUR curve fitted well with the logistic model (**Fig.4c**), yielding a final cell number of 1.10E+10, close to the harvested value (9.63E+9). These results suggest that the OUR driven modeling is effective to model cell growth curve and predict biomass at the end of culture or bioproduction.

As expected, the apparent profile, obtained via dividing the OUR values by the cell numbers predicted with the logistic model, was relatively constant, except for the early adhesion and late transfection phases (**Fig.4d**). Results also showed that the cell growth curve predicted with the logistic model was comparable to those obtained using the historical normal value (termed -cell line) for HEK293T cells or the steady value (termed -steady) obtained within seven hours after cell seeding started (**Fig.4e**). The -cell line value was found to be 2.20±0.17E-13 (n=9) mole/hr/cell for HEK293T cells. The cell number predicted in the end of culture was 1.33E+10 and 1.19E+10 for the -cell line and -steady approaches, respectively.

Finally, we compared the accuracy of the OUR-driven biomass prediction for three independent AAV2 production experiments. Results showed that the logistics modeling led to a final cell number close to those being harvested (**Fig.4f**).

Together, we developed mathematical models to estimate cell attachment and growth kinetics and demonstrated the effectiveness of two different approaches based on the cell line- and experiment-specific values to predict biomass in real-time for cell culture and bioproduction experiments. This can be a viable solution to the industry wide problem of monitoring and estimation of cell growth and biomass accumulation in a fixed bed bioreactor.

**Discussion**

Here we described the development of a novel FBR platform and characterized the flow, cell attachment, growth and transfection, and biomass prediction with a focus on linear scalability for biomanufacturing. The bioreactor systems are designed to provide a range of surface areas to meet the unmet needs in CGT workflows including viral vectors, stem cells, extracellular vesicles, and other bioproduction applications.

The packed beds of the FBRs consist of controlled packaging of PET mesh discs within thermoplastic housing vessels. CFD simulations were used to guide the development of the bioreactor platform, targeting the linear scalability for bioproduction with minimal shear stress and uniform fluid flow (**Fig.1**).

RTD and metabolite measurements confirmed that there is minimal mixing within the bioreactor, while culture media flows uniformly and metabolites, except for oxygen, display minimal concentration gradient across the packed beds (**Fig.1**), leading to a homogeneous environment for cell attachment, growth, and transfection. These results suggest that the bioreactor systems closely mimic a plug flow reactor, in which all the molecules leaving the reactor would have the same residence time, if no cell metabolism occurs.

The bioreactor systems provide automated control and user-friendly operation, enabling reduced risk of human errors and requiring minimal set-up time. The systems are designed around single-use bioreactors, each supported by a MCV, disposable sensors, and other accessory consumables, which are monitored and controlled by an integrated software controller. These sensors are used to monitor key process parameters including temperature, flow rate, pressure, pH, and DO. All fluid-contacting components are pre-assembled, single-use, irradiated, and ready to use out of box.

Cell expansion experiments using HEK293T cells showed that operational parameters, cell attachment kinetics, cell distribution within packed beds, cell growth rate (doubling time), and glucose consumption per cell all displayed linear scalability (**Fig.2**). These results further suggest that minimal optimization is needed when scaling up from development to production once optimization is done with the benchtop system, thus saving process development time and cost.

Cell harvest experiments showed that the bioreactor systems permit automated harvesting of viable and functional cells with high efficiency (>90%) (**Fig.2c**), thus allowing the same platform to be used as the seed train to generate sufficient cells for medium and large-scale cell culture. Figure 5 shows an in-house used seed train process starting from frozen cells to 50m2 bioreactors. Using the bioreactor systems as the seed train can simplify the workflow for viral vector production and cell therapy by reducing the number of technologies required for cell scale-up during upstream process.

Using AAV2-GFP viral vector as the model, we have demonstrated that the bioreactor platform enables robust and reproducible viral vector production. GFP expression was found to be uniform within the packed bed, while viral genome was a linear relation to the surface area, and the viral genome productivity per cell remains to be similar when scaling up (**Fig.3**).

Given the importance of biomass monitoring for bioproduction using mammalian cell culture39, we had developed mathematic models based on OUR profiles to monitor and predict biomass in real-time. Currently, there are no robust tools available for global readouts of biomass inside FBRs. Molecular oxygen is a key regulator for cell proliferation and differentiation and a key substrate for cell metabolism,40 and is one of the most important variables and parameters for bioprocess development41. For the bioreactor systems, we applied two oxygen biosensors to monitor DO levels, one in the MCV and another at the bioreactor outlet, the latter of which is used as a control element to maintain oxygenation level above a preset threshold via automatic adjustment of perfusion flow rate as needed. We have developed OUR-driven mathematic models and demonstrated their effectiveness and accuracy to determine cell attachment kinetics and characterize cell growth (**Fig.4**). More importantly, we have shown that biomass can be predicted in real-time with acceptable accuracy using two different approaches (**Fig.4**). The real-time cell growth prediction using the -cell line value has an advantage in that it permits identification of potential abnormality in cell growth arising from nutrition shortage or other operational errors, while the biomass prediction using the -steady value is sensitive to the intrinsic biological properties of cells (e.g., passage) or environmental factors (e.g., medium composition, DO, pH, temperature) and thus specific to the culture experiment.

In conclusion, we have developed an innovative single-use FBR platform for adherent cell biomanufacturing and showed that the bioreactor systems provide minimal shear stress, uniform flow, and homogeneous environment to enable uniform cell distribution within the packed beds. Our unique bioreactor system design enables linear scalability of operational parameters, cell culture, bioproduction, real-time biomass monitoring, and automated harvesting. Furthermore, the same platform can be used as the seed train to simplify the workflow, reduce the number of technologies required to support large-scale production, and provide true scalability from development to production. The bioreactor platform holds great potential for adherent cell-based biomanufacturing.

**Materials and Methods**

**Materials**

McCormick® Culinary green food color solution was purchased from Amazon (Seattle, WA, USA). Gram crystal violet staining solution (BD Biosciences #212525) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Benzonase was obtained from Sigma (St. Louis, USA). HEK293T cell line (ATCC #CRL-3216) was obtained from American Type Cell Culture (Manassas, VA, USA). AAV2-GFP helper-free packaging system (Cell BioLabs #VPK-402) was purchased from Cell BioLabs (San Diego, CA, USA). PEIpro® (PolyPlus #115-010) was obtained from PolyPlus (Illkirch-Graffenstaden, France). All media, components, and culture vessels were obtained from Corning Incorporated (Corning, NY, USA), unless specified.

**Bioreactors systems and dissolved oxygen sensors**

All 1, 2.5, 5, 50, and 100-m2 Corning® Ascent® FBR bioreactors were obtained in house. Both Corning Ascent Benchtop and Polit systems were used to carry out all cell culture and AAV production experiments. All bioreactor consumables, harvest consumables, accessories, and disposable sensors were single use and obtained in house. Two dissolved oxygen (DO) sensors are located on the sensor tray.

Both the MCV and the FBR DO sensors are in-line sensors. The DO was measured in terms of % air saturation level (i.e., 100% air saturation indicates that the gases dissolved in the media are in equilibrium with air at normal atmospheric conditions). The MCV DO level is automatically maintained by the DO cascade control loop via three different output variables: O2 % in the sparging gas mixture, sparging gas flow rate, and agitator speed. Similarly, the user defines the FBR DO outlet level, causing the recirculation pump flow rate to increase when the DO at the bioreactor outlet drops below the setpoint.

**CFD modeling**

To perform CFD simulations, we first constructed a fully resolved 3-D geometry of PET mesh based on the three measured dimensions including yarn diameter, spacing, and thickness (**Fig.S2a**). The wavy yarn was created by sweeping a circle of diameter over a pathline that consists of tangent arcs and lines. The half periodic section was mirrored and multiplied in various directions to create the 3D geometry of a mesh (**Fig.S2b**). After layers of meshes were aligned, a periodic domain was cut out and used for simulations (**Fig.S2c**) . All simulations were carried out using ANSYS Fluent software (Ansys, Canonsburg, PA).

The mesh microscale CFD simulation was first used to model local flow pattern around the mesh yarns and open holes. Here, the simulations were carried out in ANSYS Fluent with a steady state laminar solver since the Reynolds number is small enough due to the low velocity and small scale. Symmetry boundary conditions were applied on all 4 traverse sides, while a periodic interface was needed to link the two sides in the axial or flow direction. The mass flow rate was set in the periodic property setting. Once the simulation was converged, the pressure gradient (pa/m) in the axial direction will reach a plateau and was used to calculate the permeability of the mesh substrate: , where is the liquid viscosity and the superficial velocity. The local flow and shear patterns were visualized in post processing. The wall shear stress along with face area was exported and analyzed to create distribution plot.

The reactor macroscale CFD simulations were used to predict global flow pattern in the bioreactor. Since it requires extremely large computational grid and is prohibitively expensive to resolve the detailed pack bed at the mesh scale, we modeled the packed bed as an isotropic porous media. The permeability derived from the mesh scale modeling and the porosity directly from the created geometry were used to analyze the global flow pattern and particle tracking inside the entire reactors.

**RTD measurements**

The RTD of a tracer dye molecule was measured according to the experimental setup in **Figure S5**. Specifically, the original green food color dye solution was first diluted 1:200 with Milli-Q water. A syringe was then attached to the tubing anterior to the bioreactor inlet to inject the dye solution (2.5mL for 2.5- and 5-m2 bioreactors, or 25mL for the 100m2 bioreactor) within a short period of time (<3sec) into a flow of Milli-Q water (**Fig.S5a**). The flow rate of water was 100 mL/min for both 2.5- and 5-m2 bioreactors, but 1000 mL/min for the 100m2 bioreactors. Immediately after the dye injection, the absorbance at 630nm was recorded at every 2 seconds using a 100µL UV-Vis flow cell attached to the tubing at the bioreactor outlet. For this pulse test, an *E-curve* was calculated from the recorded absorbance as:

(5)

(6)

Here, *∆t* = 2 s, and A is the absorbance. The space time, , is defined as being equal to , where is the volume of the bioreactor and the flow rate. In the absence of dispersion, for constant volumetric flow the space time, , should be equal to the mean residence time, . The mean residence time, , can be calculated from the *E-curve* by

(7)

To compare reactors with different volume or tests using different flow rates, a normalized, dimensionless RTD function was used instead of the function , while the quantity represents the number of reactor volumes of fluid that has flowed through the reactor in time , and is defined by . Thus, is independent of the reactor size or volumetric flow rate25:

(8)

**Cell maintenance culture**

HEK293T cells were routinely cultured in Dulbecco's modified eagle medium (DMEM; Corning #15-018-CM) supplemented with 6 mM L-glutamine (Corning #25-005-CI), 1X Pen/Strep (Corning #30-002-CI), and 10% fetal bovine serum (FBS) (Corning #35-010-CV). Before use, cells were passaged on CellBind® T-75 flaks in a humidified environment at 37°C and 5% CO2 and were generally maintained between passage 3 and 6 post-thaw.

**Cell culture in Corning Ascent bioreactors**

The bioreactor system setup and batching protocol for a 2.5m2 bioreactor is described as an example. Prior to cell seeding the PD benchtop system was set up and batched with complete cell culture media a minimum of 2 hours prior to seeding. Specifically, one set of the main consumables (Corning #6972) was installed on the controller which consists of two parts, one is the bioreactor and the other is a 3.5L MCV with all tubing and in-line sensors already assembled and attached. Connection of MCV, bioreactor, and all accessory reagent bottles was done in an open lab environment using AseptiQuik® Sterile connectors (#AQG17004/ #AQS17002) supplied with the consumable set. Afterwards, 1.1L cell culture media was added to the MCV, circulated from the MCV through the bioreactor and then returned to the MCV at a flow rate of 100 mL/min for the remainder of the batching process. The controller was programmed to automatically control the conditions in the MCV to maintain the media at 21% O2, 5% CO2, and 37°C. After 2 hours of batching, the in-line optical pH and DO sensors were calibrated. Once complete, the system was set to maintain the media and allowed to equilibrate overnight.

On the day of cell seeding, cells cultured in the HYPER*Stack®*-12 were harvested using Accutase® (Corning #25-058-CI). The cells were pelleted by centrifugation and then resuspended in complete cell culture media. Inoculation phase was initiated on the bioreactor system, and once prompted, 140 mL of complete media containing 5.5 x 108 cells, targeting seeding density of 22,000 cells/cm2, was inoculated into the MCV using a storage bottle with dip tube and aseptic connector (Corning #6980). The cells in the suspension were then circulated from the MCV and through the bioreactor at a flow rate of 100 mL/min to allow cell attachment complete.

After seeding, the cells were cultured using the Media Maintenance Phase for a certain duration under controlled conditions. Glucose and L-glutamine, together with the accumulation of lactate and ammonia, were monitored daily via media sampling from the MCV and measured on the BioProfile® FLEX2™ automated cell culture analyzer (Nova Biochemical, Waltham, MA, USA). 2L of fresh media was added to the MCV between 24 to 48 hours to support cell growth. If necessary, additional fresh media was used to replenish the spent media in the MCV when glucose dropped below ~0.5g/L.

Corning CellBIND T-75 flask and CellBIND HYPER*Stack*-12 were used as 2D controls, with a seeding density of 10,000 and 12,000 cells/cm2, respectively.

**Triple transfection and AAV2 production**

The AAV2-GFP helper-free packaging system was used to transfect HEK-293T cells. Transfection complexes were formed with PEIpro® and 3 plasmid DNAs at a ratio of 2:1 (PEIpro:DNA) using a mass ratio of the three plasmid DNAs of 1:1:1 (pAAV-GFP: pHelper: pAAV-RC2). The total amount of plasmid DNA for each vessel was calculated based on the total surface area with a pre-defined DNA density of 0.18 μg/cm2.

Two hours prior to transfection, spent media from the 3-day culture was removed using the automated Media Removal phase and exchanged with the same volume of fresh media using the Media Addition phase and the pH was adjusted from 7.2 to 7.0. Once the system stabilized, the transfection complex was added aseptically using the Transfection phase. 24 hours post-transfection, the transfection media was replaced with the same volume of fresh media using the Media Removal and Addition phases. The transfected cells were then cultured for an additional 2 days where metabolites were monitored offline daily, and fresh media were added as necessary to support cell growth. Samples of cell culture medium was collected at 48- and 72-hours post-transfection and stored at -80°C for viral titer analysis.

At 72 hours post-transfection, AAV vectors were harvested from the cells by using an *in-situ* lysis method. Briefly, cell culture media was first drained completely from the system to allow manual sampling of the mesh substrates. The FBR was dissembled, and several pieces of substrate samples were removed from the packed beds for manual harvest and analysis. Once the FBR was reassembled, 1L of pre-warmed 37°C lysis buffer (10 mM Tris-HCl pH8.0, 150 mM NaCl, 2 mM MgCl2, 1% Tween® 20, and 25 units/mL Benzonase) was added to the MCV via one of the auxiliary ports. pH was set to pH 8.0 and the lysis buffer was circulated through the FBR continuously for 3 hours at 400-500mL/min. The lysis buffer was completely drained from the system and collected followed by sequential wash of the FBR with Dulbecco's phosphate buffered saline (DPBS) for 30 minutes and with high salt solution (500 mM NaCl) for another 30 minutes. Each wash collected separately for analysis. All volumes collected were measured and an aliquot of lysis buffer, DPBS wash, and high salt wash were collected and stored at -80°C for later viral titer analysis.

For 2D controls, all transfected cells were first harvested from the vessel using Accutase solution. Then an aliquot of cell harvest solution containing 1 x 107 cells was pelleted by centrifugation, resuspended in 1 mL lysis buffer, and incubated at 37°C for 1 hour. The resulting cell lysate was centrifuged to remove cell debris and the supernatant was stored at -80°C for viral titer analysis.

**Crystal violet staining**

Crystal Violet staining was used to visualize cells on the bioreactor substrates. Substrate samples removed from the bioreactors were submerged into 1:10 dilution of Gram Crystal Violet staining solution in DPBS in a container and incubated for 5 minutes. The stained substrate samples were then rinsed with deionized water to remove excess dye, air-dried, and imaged using a HP ScanJet Scanner.

**Manual and automated cell harvesting**

The cell harvesting protocol for a 5m2 bioreactor is described as an example. For manual harvesting, representative mesh discs for specific locations of the packed bed were removed from the bioreactor after culture and placed into a 100mm petri dish containing 20mL Accutase solution and incubated at room temperature for 40 minutes under agitation using an orbital shaker. The cell harvest solutions were finally collected for cell enumeration using Beckman Coulter Vi-CELL™ XR cell viability analyzer.

For automated harvesting, the protocol described in the PD benchtop system user manual was used. Briefly, prior to harvest, the Maintenance phase was stopped, and Harvest phase was initiated. Following the prompts on the screen, the MCV tubing was removed from the recirculation pump and replaced with the harvest pump tubing. Color coded tubing lines from the 2L Harvest Consumables (Corning #6987), which are connected each harvest vessel, were installed into the matching color-coded pinch valves to automatically manage flow during the harvest phases. The medium in the bioreactor was fully drained, followed by washing with 500mL DPBS buffer once, followed by recirculation of 800mL 1x Accutase® solution for about 40 minutes. To prevent cells forming clumps and inactivate the enzymatic activity of Accutase, 100mL of FBS was added to Cell Harvest Bottle (Corning #6985) prior to harvest. When final cell density was high (>400,000 cells/cm2), an extra recirculation with 800mL 25unit/mL Benzonase (EMD Millipore Corporation #71205-3) in DPBS was used to maximize harvest yield. Finally, cells were flushed out the bioreactor and into the collection bottle by pressurizing the system via the pressure control valve inside the instrument. Air pressure was set at approximately 10 to 15 psi.

**Flow Cytometry**

Harvested cells were first passed through a 40 µm cell strainer (Corning #431750) to remove any large cell clumps, were then pelleted by centrifugation and resuspended in DPBS to a final concentration of 0.5 x 106 cells/mL. Samples were run on a BD FACSCalibur™ Flow Cytometer using pre-determined settings to count 30,000 events for each sample. Mock-transfected cells were used as a negative control to determine percentage of cells expressing GFP (GFP+ cells).

**AAV titer quantitation**

Cell culture media collected at 48- and 72-hours post-transfection, cell lysate, PBS wash, and high salt wash samples were sent to Welgen, Inc. (Worcester, MA, USA) for AAV titer analysis. Welgen used real-time quantitative polymerase chain reaction (qPCR) to measure copy numbers of GFP gene in AAV. Specifically, the samples were briefly treated with DNase I prior to being isolated using a DNA extraction kit (Applied Biosystems #4403319). qPCR assays were then performed on the StepOne™ PCR system (Applied Biosystems #4376357) by adding the diluted extracted samples to a master mix solution consisting of water, PrimeTime® Gene Expression master Mix (IDT #1055770), TaqMan® probe, and forward and reverse primers against the GFP insert (F: GAACCGCATCGAGCTGAA, R: TGCTTGTCGGCCATGATATAG, Probe: ATCGACTTCAAGGAGGACGGCAAC). A standard curve was constructed using the purified pAAV-CMV GFP plasmid DNA and the results given as genome copies (GC)/mL.

**Statistical analysis**

The statistical significance reported was obtained using One-way ANOVA with Prism 7.0.

**Acknowledgements**

The authors would like to acknowledge Steve Caracci, Christine Cecala, Jennifer Curtis, Anthony Frutos, Tom Garvey, Tyler Lawton, Danielle Loscig, Chris Timmons, and Andreas Weiss for their support in the development of Corning Ascent FBR systems.

**Conflict of interest**

All authors, except for A.R., K.A.K, S.K. who are former employee, are employee of Corning Incorporated. Corning Ascent FBR systems are commercial products from Corning Incorporated.

**Ethical approval**

This study does not contain any studies with human or animal subjects performed by any of the authors.

**Authorship**

V.N.G., J.A.S., D. M., Y.Z., A.F., and S.K. prototyped the FBR and system. V.N.G., Y.H., J.J.S., L.E.R., J.Z., V.T., K.A.K., Y.Z., and A.M.F. performed experiments and data analysis. Y.S., A.R., D.P., and Y.F. performed modeling. D.M., J.L.W., Z.M., and T.M.U. guided the system development. Y.F. wrote the main manuscript, analyzed all data, and prepared all figures.

**References**

1. A. Aijaz, M. Li, D. Smith, D. Khong, C. LeBlon, O.S. Fenton, R.M. Olabisi, S. Libutti, J. Tischfield, M.V. Maus, R. Deans, R.N. Barcia, D.G. Anderson, J. Ritz, R. Preti, B. Parekkadan, Biomanufacturing for clinically advanced cell therapies. *Nat. Biomed. Eng.* **2018**, *2*, 362.
2. C.J. Bashor, I.B. Hilton, H. Bandukwala, D.M. Smith, O. Veiseh, Engineering the next generation of cell-based therapeutics. *Nat. Rev. Drug. Discov.* **2022**, *21*, 655-675.
3. H.P. Lesch, P. Valonen, M. Karhinen, Evaluation of the single-use fixed-bed bioreactors in scalable virus production. *Biotechnol. J.* **2021***, 16,* 2000020*.*
4. P. Young, Treatment to cure: Advancing AAV gene therapy manufacture. *Drug Discov. Today* **2023**, *28*, 103610.
5. J. Shupe, A. Zhang, D.C. Odenwelder, T. Dobrowsky, Gene therapy: challenges in cell culture scale-up. *Curr. Opin. Biotechnol.* **2022**, *75*, 102721.
6. T. Dobrowsky, D. Gianni, J. Pieracci, J. Suh, AAV manufacturing for clinical use: Insights on current challenges from the upstream process perspective. *Curr. Opin. Biomed. Eng.,* **2021**, *20*, 100353.
7. X. Wang, M. Olszewska, J. Qu, T. Wasielewska, S. Bartido, G. Hermetet, M. Sadelain, I. Riviere, Large-scale clinical-grade retroviral vector production in a fixed-bed bioreactor. *J. Immunother.* **2015**, *38*, 127.
8. H.P. Lesch, K. M. Heikkilä, E. M. Lipponen, P. Valonen, A. Müller, E. Räsänen, T. Tuunanen, M.M. Hassinen, N. Parker, M. Karhinen, R. Shaw, S. Ylä-Herttuala, Process development of adenoviral vector production in fixed bed bioreactor: from bench to commercial scale. *Hum. Gene Ther,* **2015**, *25*, 560-571
9. V. V. Emmerling, A. Pegel, E. G. Milian, A. Venereo-Sanchez, M. Kunz, J. Wegele, A. A. Kamen, S. Kochanek, M. Hoerer, Rational plasmid design and bioprocess optimization to enhance recombinant adeno‐associated virus (AAV) productivity in mammalian cells. *Biotechnol. J.* **2016**, *11*, 290.
10. Alicia D. Powers, Bryan A. Piras, Robert K. Clark, Timothy D. Lockey, and Michael M. Meagher. Development and optimization of AAV hFIX particles by transient transfection in an iCELLis® fixed-bed bioreactor. *Human Gene Ther. Methods* **2016**, 27,112-121.
11. A. J. Valkama, H. M. Leinonen, E. M. Lipponen, V. Turkki, J. Malinen, T. Heikura, H. P. Lesch, Optimization of lentiviral vector production for scale-up in fixed-bed bioreactor. *Gene Ther.* **2018**, *25*, 39.
12. H. Leinonen, S. Lepola, E. M. Lipponen, T. Heikura, T. Koponen, N. Parker, H. P. Lesch, Benchmarking of scale-X bioreactor system in lentiviral and adenoviral vector production. *Hum. Gene Ther.* **2020**, *31*, 376.
13. S. Kiesslich, J. P. Vila-Chã Losa, J.-F. Gélinas, A. A. Kamen, Serum-free production of rVSV-ZEBOV in Vero cells: Microcarrier bioreactor versus Scale-X™ hydro fixed-bed. *J. Biotechnol.* **2020**, *310*, 32.
14. A. McCarron, M. Donnelley, C. McIntyre, D. Parsons, Transient lentiviral vector production using a packed-bed bioreactor system. *Hum. Gene Ther: Methods* **2019**, *30*, 93.
15. FDA (2004) Guidance for industry: PAT—a framework for innovative pharmaceutical manufacturing and quality assurance, <http://www.fda.gov/cvm/guidance/published.html>
16. Jenzsch, M.; Bell, C.; Buziol, S.; Kepert, F.; Wegele, H.; Hakemeyer, C. Trends in process analytical technology: present state in bioprocessing. *Adv. Biochem. Eng. Biotechnol.* **2018**, *165,* 211-252.
17. Reyes, S.J.; Durocher, Y.; Pham, P.L.; Henry, O. Modern sensor tools and techniques for monitoring, controlling, and improving cell culture processes. *Processes* **2022**, *10*, 189.
18. Ducommun, P.; Bolzonella, I.; Rhiel, M.; Pugeaud, P.; Stockar, Uv.; Marison, I.W. On-line determination of animal cell concentration. *Biotechnol. Bioeng.* **2001**, *72*, 515–522.
19. Aehle, M.; Simutis, R.; Lübbert, A. Comparison of viable cell concentration estimation methods for a mammalian cell cultivation process. *Cytotechnology* **2010**, *62*, 413–422.
20. W.C. Heerens, Application of capacitance techniques in sensor design. *J. Phys. E: Sci. Instrum.* **1986**,*19*, 897
21. Hutmacher DW, Singh H. Computational fluid dynamics for improved bioreactor design and 3D culture. *Trends Biotechnol*. **2008**, *26,* 166-172.
22. B.J. Ballermann, A. Dardik, E. Eng, A. Liu, Shear stress and the endothelium. *Kidney Intl.* **1998**, *54*, S100-S108.
23. Reneman RS, Hoeks AP. Wall shear stress as measured in vivo: consequences for the design of the arterial system. *Med. Biol. Eng. Comput.* **2008**, *46*, 499-507.
24. Brindley D, Moorthy K, Lee JH, Mason C, Kim HW, Wall I. Bioprocess forces and their impact on cell behavior: implications for bone regeneration therapy. *J. Tissue Eng.* **2011**, *2011*, 620247.
25. H.S. Fogler, Elements of Chemical Reaction Engineering. **2017**, 5th Edition, Prentice-Hall Inc., New Jersey.
26. D. Wang, P.W.L. Tai, G. Gao, Adeno-associated virus vector as a platform for gene therapy delivery. *Nat. Rev. Drug Discov.* **2019**, *18*, 358–378.
27. J. Gálvez, M. Lecina, C. Solà, J.J. Cairó, F. Gòdia, Optimization of HEK-293S cell cultures for the production of adenoviral vectors in bioreactors using on-line OUR measurements. *J. Biotechnol.* **2012**, *157*, 214-222.
28. F. García-Ochoa, E. Gómez, Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview. *Biotenol. Adv.* **2009**, *27*, 153-176
29. M. Lecina, A. Soley, J. Gracia, E. Espunya, B. Lazaro, J.J. Cairo, F. Godia, Application of on-line OUR measurements to detect actions points to improve baculovirus-insect cell cultures in bioreactors. *J. Biotechnol.* **2006**, *125*, 385-394.
30. W. Zhou, J. Rehm, W.-S. Hu, High viable cell concentration fedbatch cultures of hybridoma cells through on-line nutrient feeding. *Biotechnol. Bioeng.* **1995**, *46*, 579–587.
31. A. E. Higareda, L. D. Possani, O. T. Ramírez, The use of culture redox potential and oxygen uptake rate for assessing glucose and glutamine depletion in hybridoma cultures. *Biotechnol. Bioeng.* **1997**, *56*, 555–563.
32. B.A. Wagner, S. Venkataraman, G.R. Buettner, The rate of oxygen utilization by cells. *Free Radic. Biol. Med.* **2011**, *51*, 700-712.
33. Z. Zaytseva, J.G. Lynn, Q. Wu, D.J. Mudaliar, P.Q. Kuang, Y. Fang, Resonant waveguide grating biosensor-enabled label-free and fluorescence detection of cell adhesion. *Sens. Actuators B Chem.* **2013**, *188*, 1064–1072.
34. H.S. Hou, K.L. Lee, C.H. Wang, T.H. Hsieh, J.J. Sun, P.K. Wei, J.Y. Cheng, Simultaneous assessment of cell morphology and adhesion using aluminum nanoslit-based plasmonic biosensing chips. *Sci. Rep.* **2019**, *9*, 7204.
35. M. Pappenreiter, B. Sissolak, W. Sommeregger, G. Striedner, Oxygen uptake rate soft-sensing via dynamic KLa computation: cell volume and metabolic transition prediction in mammalian bioprocesses. *Front. Bioeng. Biotechnol.* **2019**, *7*, 195.
36. P. Calik, P. Yilgör, P. Ayhan, A. Demir, Oxygen transfer effects on recombinant benzaldehyde lyase production. *Chem. Eng. Sci.* **2004**, *59*, 5075–5083.
37. F. Garcia-Ochoa, E. Gomez, V.E. Santos, J.C. Merchuk, Oxygen uptake rate in microbial processes: An overview. *Biochem. Eng. J.* **2010**, *49*, 289–307.
38. M.H. Zwietering, I. Jongenburger, F.M. Rombouts, K. van’t Riet. Modeling of the bacterial growth curve. *Appl. Environ. Microbiol.* **1990**, *56*, 1875-1881.
39. K. Konstantinov, S. Chuppa, E. Sajan, Y. Tsai, S. Yoon, F. Golini, Real-time biomass-concentration monitoring in animal-cell cultures. *Trends Biotechnol.* **1994**, *12*, 324–333.
40. B.A. Wagner, S. Venkataraman, G.R. Buettner, The rate of oxygen utilization by cells. *Free Radic. Biol. Med.* **2011**, *51*, 700-712.
41. I. Martínez-Monge, R. Roman, P. Comas, A. Fontova, M. Lecina, A. Casablancas, J.J. Cairó, New developments in online our monitoring and its application to animal cell cultures. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 6903–6917.

**Figure legends**

**Figure 1**. **Bioreactor configurations, flow characteristics, and metabolite profiles.**

(**a**) Benchtop bioreactors with 1, 2.5, or 5m2 effective surface areas. The photo shows the structure of the woven PET mesh. (**b**) A representative flow pattern in a 2.5m2 bioreactor, as revealed using macro-scale CFD modeling. (**c**) Shear stress distribution of interstitial flow through the packed bed; (**d**) Wall shear stress distribution on PET mesh surfaces, both of which were obtained using micro-scale CFD modeling. (**e**) The dimensionless residence time distribution function E(θ) *versus* the dimensionless θ, wherein both E(θ) and θ are normalized to compare bioreactors with different volume or tests using different flow rates. (**f**) The glucose concentrations or (**g**) the dissolved oxygen concentrations before (*inlet*) and after (*outlet*) the media passed through a 1m2 bioreactor, wherein HEK293T cells were seeded with a density of 22,000 cells/cm2 and cultured for 4 days. At morning of each day there was a medium exchange.

**Figure 2**. **Cell attachment, distribution, harvesting, growth pattern, and glucose consumption in five distinct types of bioreactors, 1, 2.5, 5, 50, and 100m2**.

(**a**) Cell attachment kinetics. (**b**) Cell density as a function of location within 5m2 bioreactors. (**c**) The relation between the manual and automated harvested cell density. Here, nine culture experiments in 5m2 bioreactors were analyzed (3 independent reactors for 3, 4, or 5 days, all started with 35,000 cells/cm2). (**d**) Doubling time distribution as a function of bioreactor size. (**e**) Cells harvested from 5m2 bioreactors as a function of culture duration. (**f**) Total glucose consumed per cell as a function of bioreactor size. All experiments were performed using a cell seeding density of ~22k/cm2, except for the 100m2 bioreactors and the 5-day culture experiments in 5m2 bioreactors whose seeding density was ~15k/cm2.

**Figure 3**. **Cell transfection efficiency and viral vector production**. (**a**) Flow cytometer graphs showing the number of GFP+ cells and mean fluorescence intensity for cells obtained from PET discs at three different locations (Top, center, and bottom). (**b**) The total genome copies obtained as a function of effective surface area (SA) of a vessel. (**c**) The yield of AAV-GFP per cell obtained as a function of different vessels.

**Figure 4**. **Oxygen consumption for cell growth modeling**.

(**a**) DO profiles of medium in the MCV (MCV DO%) and after the bioreactor (FBR DO%) as well as flow rate. Cells were seeded at a density of 22K/cm2 in a 2.5m2 bioreactor. (**b**) The cell attachment kinetics obtained by fitting the oxygen consumption rate or the percentage of cells found in the bioreactor as a function of time using an offline cell counting protocol with a one-phase association model. The was calculated by dividing the OUR by the initial seeding number. (**c**) The OUR profile (black dots) and the data fitted with a logistic cell growth model (red line). (**d**) The oxygen consumption rate profile. (**e**) Real-time biomass prediction using three different approaches: the logistic model (black line), the cell line specific historical normal -cell line value (green dot line), the culture experiment specific steady value (-steady) obtained seven hours after cell seeding started. (**f**) Comparison of cell numbers harvested with those predicted based on the OUR modeling.

**Figure 5**. **An in-house developed seed train for cell amplification**. Cells were first expanded in CellBind T-75 flasks, followed by 2-layer CellBind CellSTACK®, 12-layer CellBind HYPER*Stack*®-12, 5m2 FBR, and 50m2 bioreactor. Depending on the cell seeding numbers desired for the bioreactors, 2-3 CellBind HYPER*Stack®*-12 vessels were used for the N-1 step.

Figure 1

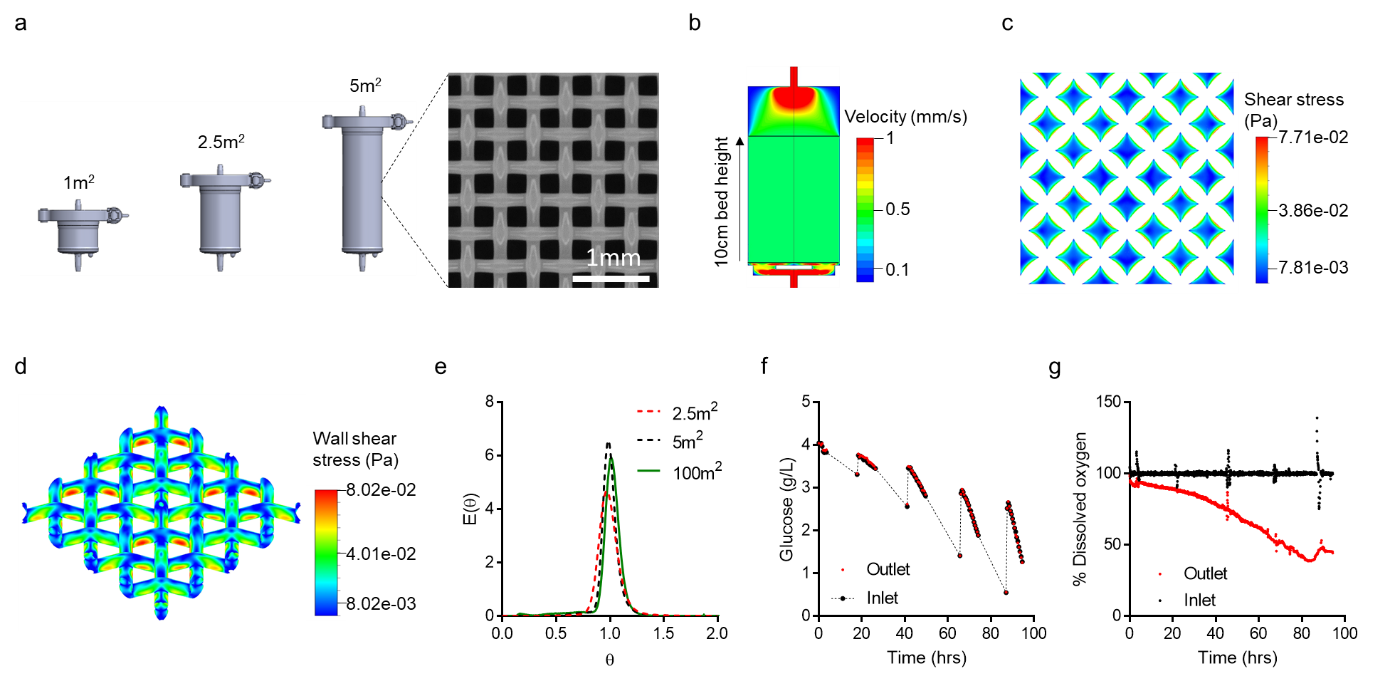


Figure 2

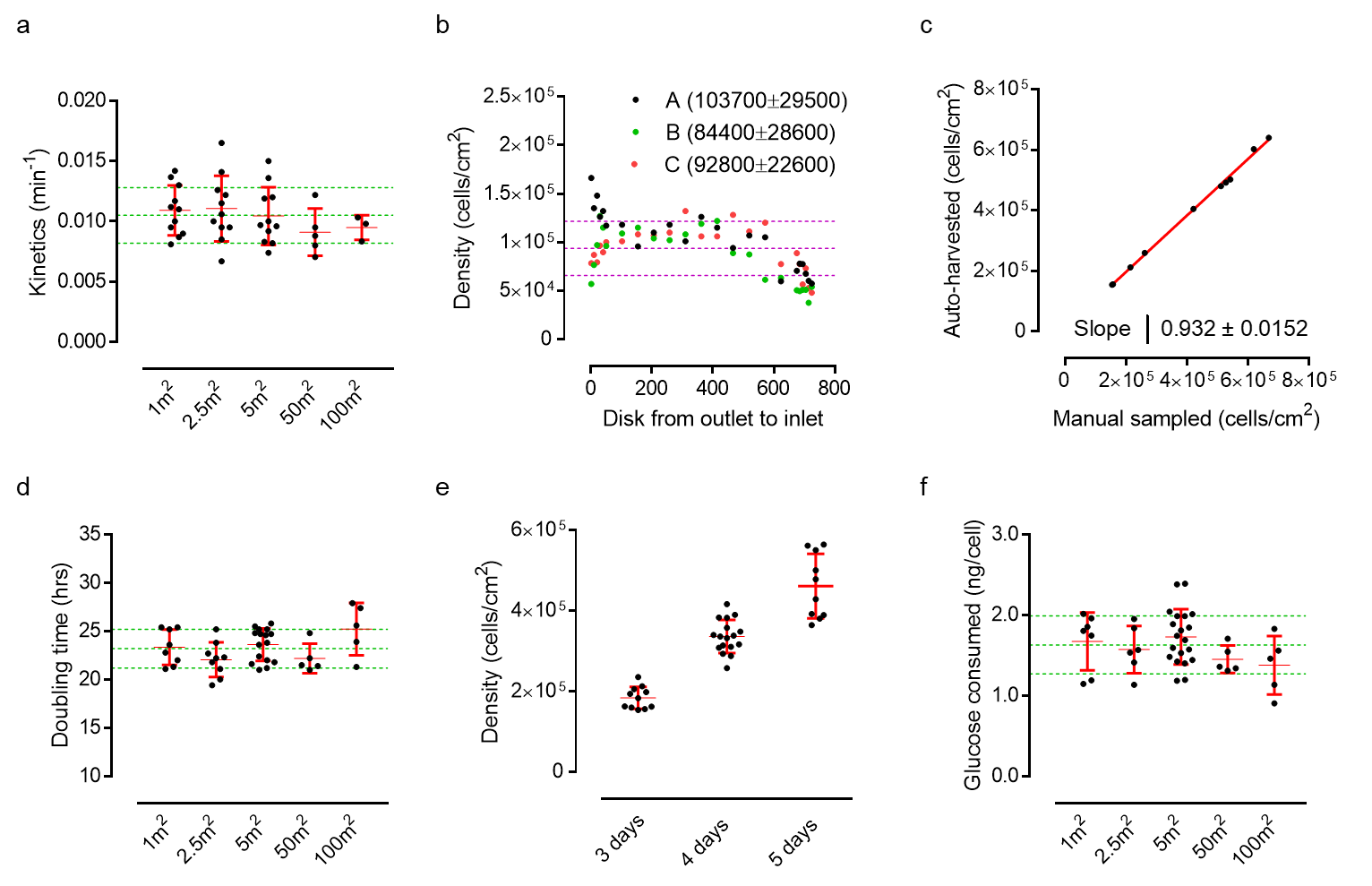


Figure 3

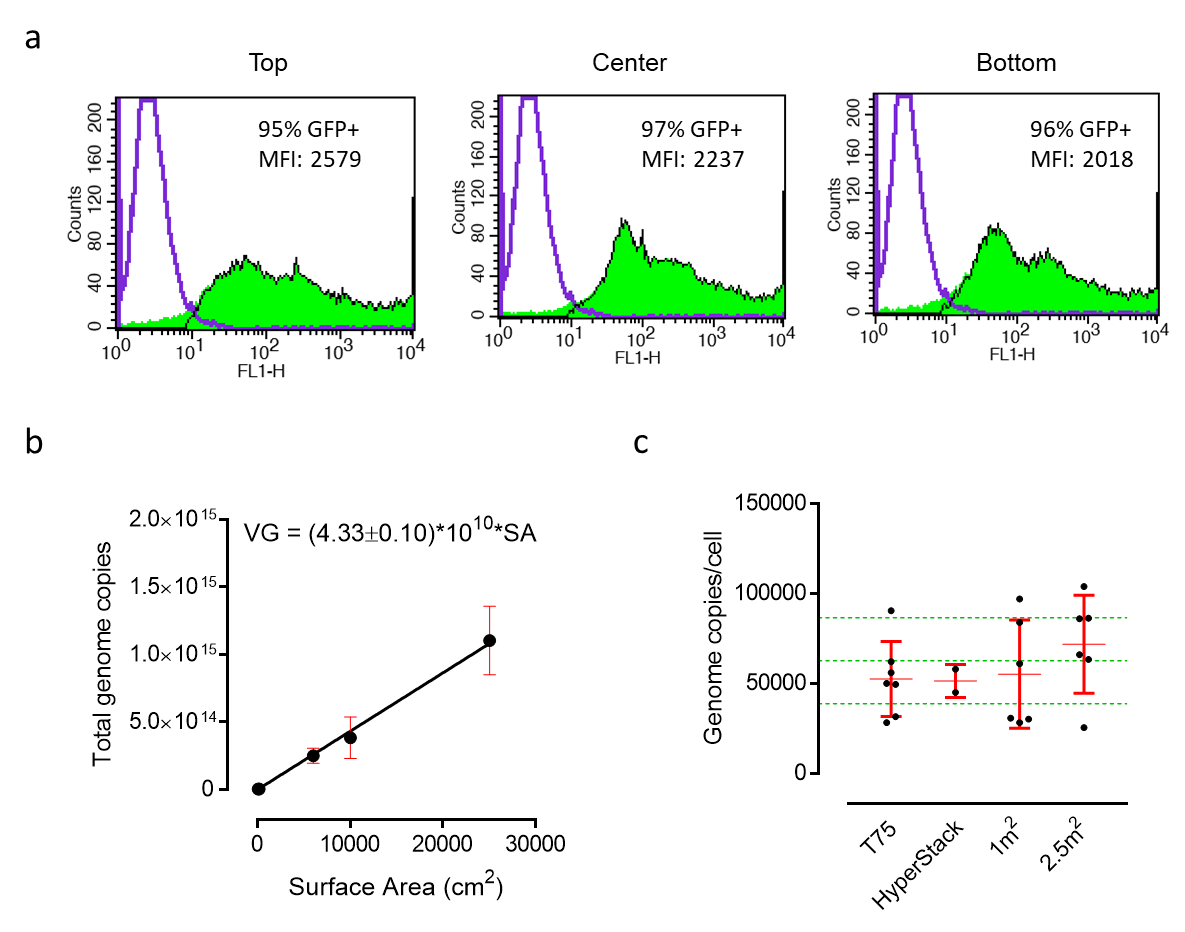


Figure 4

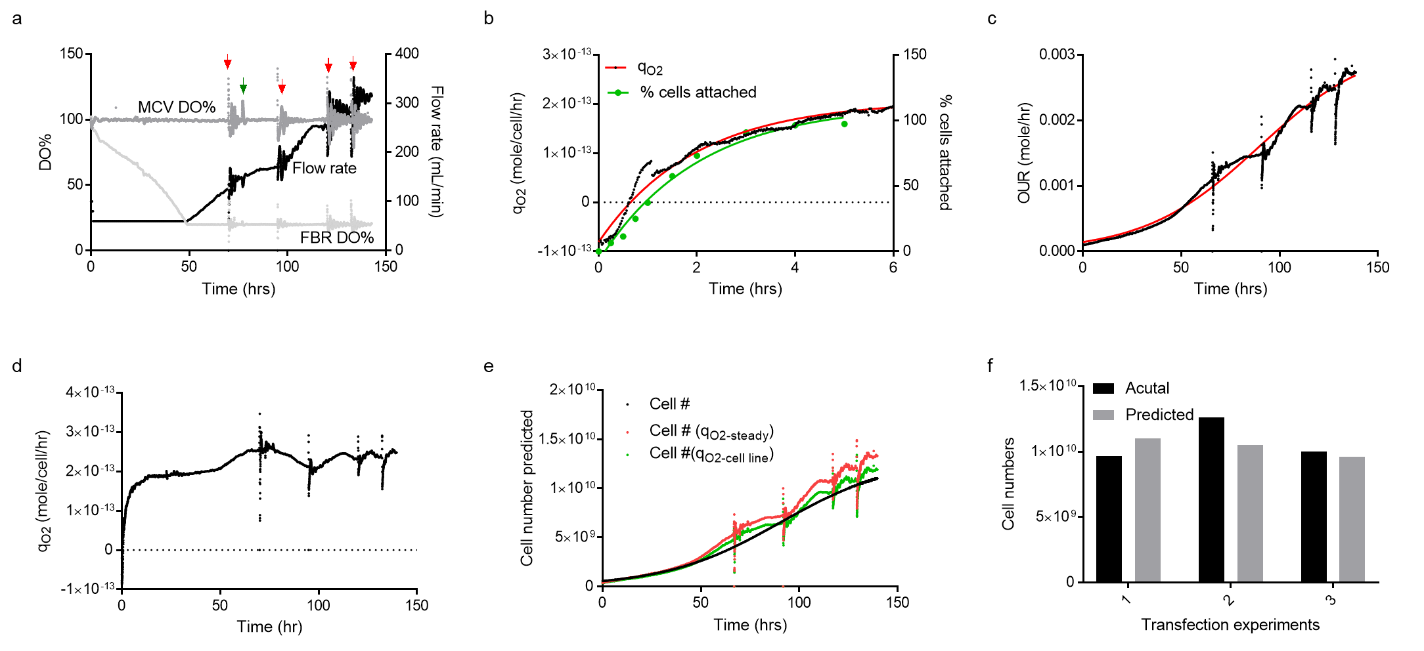


Figure 5

