Scaling-up cells in Falcon® Cell Culture Multi-Flasks

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Application Note

Contents

Introduction

A growing number of cell-based applications require a large number of cells. These applications typically commence in a small scale during development phase and are subsequently scaled-up once an assay or application is validated. Use of single-layer T-flasks may be adequate during development, but they can become cumbersome, laborious and inefficient when a large number of cells is required. For efficient scale-up of adherent mammalian cell cultures, it is important to use vessels designed to generate homogeneous cells on a consistent basis while retaining original cellular characteristics such as morphology, growth and function. Vessel design is equally important for optimal cell or product recovery.

To address these needs, we have engineered two multi-layered cell culture vessels with an intuitive design to facilitate easy scaling-up of cells from single-layered T-flasks.

Falcon Multi-Flasks–Features and Benefits

Falcon Cell Culture Multi-Flasks are stackable 3- or 5-layer vessels with the same footprint as a T-175 flask but increased growth surface area (525 cm^2 or 875 cm^2, respectively). The flasks have the same proven tissue culture-treated growth surface as all other Falcon flasks. The Falcon Multi-Flask design is compatible with reagent volumes and cell seeding densities that are typically utilized with T-175 flasks on a per unit area basis, which results in a familiar and simple process for cell expansion.

Falcon Cell Culture Multi-Flask Vessels

Figure 1. Falcon Cell Culture Multi-Flask vessels are available in 3- and 5-layer stackable formats for easy scale-up from T-flasks.

Falcon Multi-Flasks provide convenient pipette access for aspiration and addition of cells and reagents directly into the flasks, as well as efficient recovery of materials with reduced risk of contamination. For applications that require pouring, Falcon Multi-Flasks have minimal residual liquid retention, which reduces waste of valuable cells and reagents.

Another key feature of the Falcon Multi-Flask is a mixing/equilibration port that allows rapid in-vessel mixing, as well as uniform distribution of cells and reagents to all layers of the vessel. This design yields homogeneous cell growth on all layers of the vessel. The mixing port also eliminates the need for making large volumes of cell suspensions outside of the vessel, thereby streamlining workflow. The vessel design allows for a wide range liquid volumes (5 to 50 mL per layer). Typically, a volume range of 25 to 35 mL of medium per layer is recommended for adherent mammalian cell culture.

FALCON®

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Materials and Methods

We tested a broad range of cell lines in order to evaluate the design and performance of the Falcon® Multi-Flask. The various parameters evaluated included cell yield, cell patterning and spent media analysis. Some cell based assays were also performed on cells expanded using this vessel. In each case a T-175 flask was used as a control. In some instances competitive multi-layered devices were evaluated as well.

Cell Lines

LnCAP, HepG2, CHO-M1 WT3 cell lines were purchased from ATCC. BHK-21 cells were obtained from Sigma Aldrich; EcoPack™2-293 cells from Clontech; hMSCs from Lonza; and Corning® HUVEC-2 cells. Vendor's protocols were followed for culturing each of these cell lines.

Cell Yield

Cells harvested from culture vessels were counted using a Vi-CELL™ XR (Beckman Coulter, Inc.) automated cell counter. Cell yield was expressed as total number of cells recovered from a given vessel. Cells per unit area (cells/cm^2) was calculated by dividing the total cell yield of a vessel by the total growth surface area of that vessel. Cell yield per layer was determined by normalizing cells harvested from individual layers relative to the bottom layer of a vessel (set as 100%). For Falcon Multi-Flasks, holes were drilled in each layer to isolate cells from individual layers. For competitor vessels, cells were first isolated from the bottom layer by cutting the neck area. Next, the top two layers of the competitor vessels were cut and cells were isolated. Cell viability was not compromised (i.e., >95%) for any of the cell harvesting procedures used in this study.

Cell Patterning

Cultures that reached ~80% or higher confluence were fixed with 4% paraformaldehyde. Mean cell confluence was then determined using IncuCyte® (ESSEN) by imaging 134 distinct regions in the bottom layer of 3- and 5-layer Falcon Multi-Flasks. Intra-layer coefficient-of-variation (% CV) values were calculated for the mean cell confluence in the bottom layer of each flask. For qualitative assessment of cell patterning, fixed cells were stained with crystal violet and scanned using a laser scanner. Typically, the bottom layer of vessels was analyzed. In some experiments, the flasks were cut open and all individual layers of the flask were imaged.

Spent Media Analysis

A small volume (1-2 mL) of medium was removed from culture vessels (either from individual layers of Multi-Flask or from pre-mixed media of an entire flask) and analyzed for nutrient and metabolite concentration, pH, partial oxygen (% air saturation) and partial carbon dioxide pressures in the spent media using a BioProfile® FLEX analyzer (Nova Biomedical).

GPCR Calcium Mobilization Assay

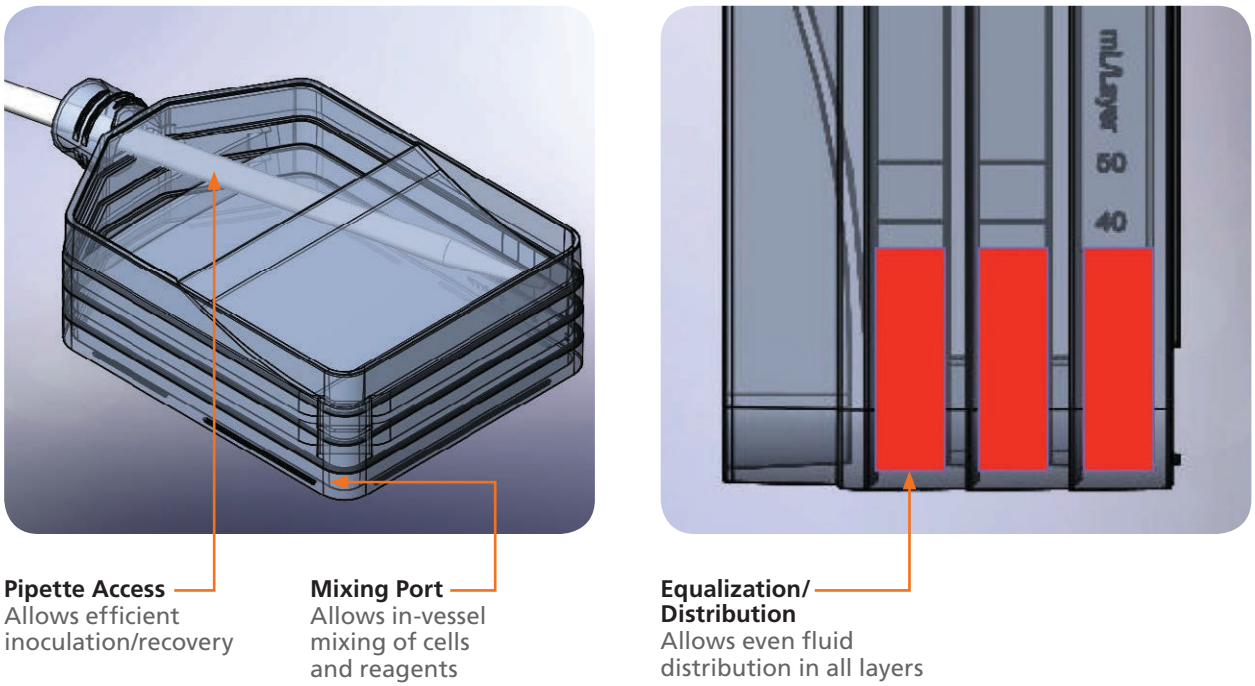
Calcium mobilization assays were performed using CHO-M1 WT3 cells according to guidelines provided for the FLIPR® Calcium-4 assay kit (Molecular Devices). CHO-M1 WT3 cells were seeded at a density of 5,000 –6,000 cells/cm^2 in growth medium (Ham's F12K medium supplemented with 10% FBS, 4 mM L-Glutamine and 0.1 mg/mL G418) in either T-175 or Falcon Multi-Flasks and cultured for 96 hours.

Cells were then harvested, spun, re-suspended and seeded in Corning® BioCoat™ Poly-D-Lysine 96-well Black/Clear Microtest™ Plates at a density of 40,000 cells per well in 100 µL growth medium. After an overnight incubation, cells were labeled with 50 µL of fluorescent calcium loading dye containing 2.5 mM probenecid for 1 hour followed by addition of 50 µL of buffer (HBSS with 20 mM HEPES, pH 7.4) or 4X drug (carbachol diluted in the same buffer). Drug-induced changes in fluorescent signal corresponding to calcium flux were measured using a fluorescent plate reader (EnVision®, Perkin Elmer) at a wavelength of 485/535 nm (Ex/Em) one minute after compound addition.

Cell Impedance Assay

Cell attachment, spreading, growth and GPCR-mediated modulation of cell morphology were measured using an impedance-based assay (xCELLigence™ RTCA MP System, Roche). Interactions between cells and electrodes were measured as changes in impedance and were reported as an arbitrary Cell Index (CI) value. CI values were continuously monitored for the above mentioned cell parameters after cell seeding and immediately after compound additions.

Figure 2. Design features of Falcon® Multi-Flasks



Results and Discussion

Even Media Distribution and Homogeneous Cell Growth across All Layers

A key objective in developing cell-based assays is to reduce variability (% CV) and maximize signal-to-noise ratio for a given cellular response (i.e., increase Z' values). Cell-based assay outcomes can be highly influenced by the cell culture environment, cell health and uniformity of cell populations. Using an optimized ratio of culture medium volume to cell density per unit area of the culture vessel is critical for controlling nutrient to metabolite ratio, gas exchange rate between air and media interface, and overall cell growth. Lower than optimal volume of medium results in rapid depletion of nutrients and build up of deleterious metabolites, which, in turn, can cause early cell death and detachment. Conversely, higher than optimal volume of medium can result in rapid cell growth and over-confluence. It can also lead to contact inhibition with some cell types. For instance, mesenchymal stem cells are particularly susceptible to contact-dependent replicative senescence that can limit the expansion potential of these costly cells (Ho, et al., 2010). Others, such as human leukemic HL-60 cells, are susceptible to cell density-induced apoptosis (Saeki, et al., 1997). Immortalized cells such as HEK293- or CHO-K1-derived lines appear to continue proliferation post-confluence with progressively reduced cell size. Falcon® Multi-Flasks have been designed with a mixing/equilibration port to evenly distribute cell suspensions and maintain a consistent volume of medium per layer to provide the same environment for all cells and thereby enable growth of homogenous cells on a consistent basis. Moreover, culture medium to cell ratio per unit area on all layers of the Falcon Multi-Flasks is identical to those used in T-175 flasks, thus enabling efficient expansion of cells without the need for protocol reoptimization.

Figure 3A (upper panel) demonstrates uniform cell attachment and growth patterns on all layers of the Falcon Multi-Flasks. Uniform cell growth is correlated with even medium distribution on each layer of Falcon Multi-Flask (Figure 3B). In this experiment, the highest recommended medium volume (50 mL per layer) was used for Falcon Multi-Flasks. Even medium distribution and cell growth has also been consistently observed using lower volumes per layer. Several diverse cell types were successfully cultured using 35 mL of medium per layer in the Falcon Multi-Flasks (Table 1). An experiment conducted to carefully remove cells from individual layers revealed equal cell yield from each layer of the Falcon Multi-Flask (Figure 3C). In other vessels (competitor), where medium distribution was uneven per layer (Figure 3B), cell patterning (Figure 3A, lower panel) and cell numbers (Figure 3C) per layer were affected.

Figure 3. The figures below illustrate cell patterning, media distribution and cell yield on individual layers of Falcon® Multi-Flasks and compares it to a competitive 3-layer flask.

Figure 3A. EcoPack™2-293 cells grown to >80% confluence in 3-layer Falcon Multi-Flasks and a competitive 3-layer flask. The cells were fixed and stained with crystal violet for observing cell attachment and growth patterns. The vessel was cut and each stained layer was scanned. The images clearly show how consistent and even the cell population is within each layer and between layers for the Falcon Multi-Flask. The competitive product demonstrated inferior cell distribution.

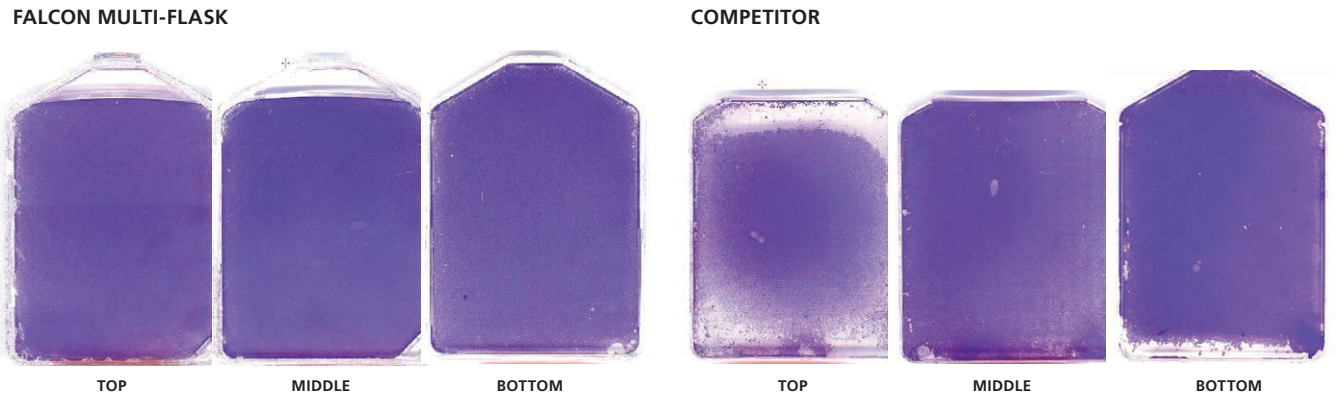


Figure 3B. Each bar shows medium distribution per layer as a percentage of total volume added per flask. Cell culture medium (150 mL, maximum recommended volume per flask) was added to 3-layer Falcon Multi-Flasks and equilibrated. Medium was pumped out of individual layers and fluid weights were recorded from each layer. The data shown is mean ± SD of seven flasks. For competitor vessels, a parallel experiment was conducted using vendor recommended media volume (100 mL) and equilibration steps.

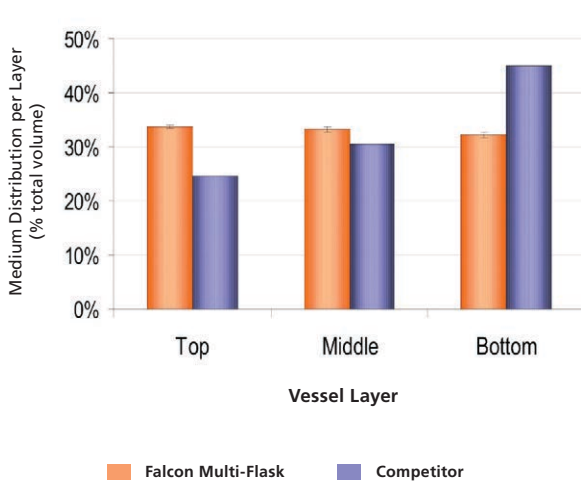


Figure 3C. CHO-M1 WT3 cells were seeded at a density of $6x10^6$ cells in 105 mL of total medium per vessel in 3-layer Falcon Multi-Flasks and competitor vessels. Following 72 hours of culture, cells were harvested from each individual layer of the vessel and cell yield per layer was determined using a Vi-CELL™ XR automated cell counter. Each bar on the graph illustrates the normalized mean cell yield per layer (% mean ± SD) relative to the bottom layer for three flasks.

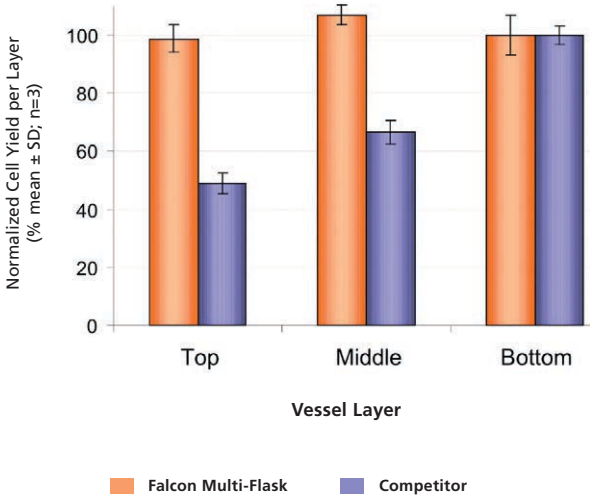
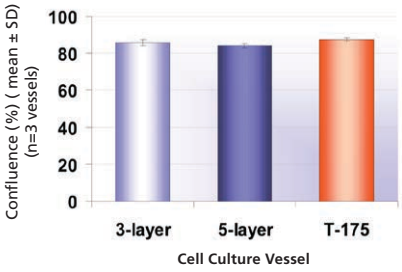
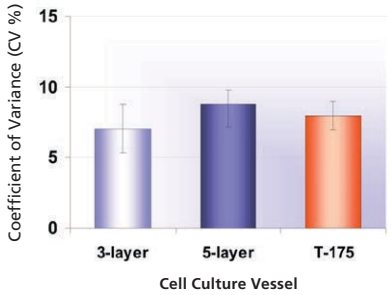


Figure 4. Cell confluence in Falcon Multi-Flasks. Shown below is mean intra-layer confluence (top panel) calculated by imaging 134 regions across the bottom layer of 3- and 5-layer Falcon Multi-Flasks and control T-175 flasks using IncuCyte® (ESSEN). BHK-21 cells grown to >80% confluence in 3-layer Falcon Multi-Flasks and T-175 control vessels were fixed and stained with crystal violet prior to cell confluence measurements. Intra-layer CV (%) values were calculated for mean confluence levels across each growth layer (bottom panel).





To further evaluate homogeneity of cultures, cell confluence was quantified in the Falcon® Multi-Flasks and control T-175 flasks. Measurements at various regions of interest across the bottom layer of 3- and 5-layer Falcon Multi-Flasks revealed uniform cell confluence with low intra-layer CV (<10%) comparable to T-175 flasks (Figure 4).

Nutrient (glutamine and glucose) and metabolite (lactate and ammonia) levels were measured in spent culture medium isolated from individual layers of Falcon Multi-Flasks after culturing EcoPack™2-293 cells for 96 hours (Figure 5A). No difference was observed in concentrations of nutrients or metabolites between individual layers of the Falcon Multi-Flask or control T-175 flasks. Similar results have been observed with pooled medium from Falcon Multi-Flasks (data not shown). Percentage of oxygen (O_2) and carbon dioxide (CO_2) saturation and pH levels were not different between spent medium isolated from 5-layer Falcon Multi-Flasks and control T-175 flasks (Figure 5B and 5C).

O_2 saturation in spent medium was the same compared to the levels detected in control T-175 flasks. These results further confirm the culture environment for cells in Falcon Multi-Flasks is consistent and equivalent to that observed in T-175 flasks.

Figure 5. Analysis of spent medium from Falcon Multi-Flasks. Spent medium analysis revealed no difference in concentration of nutrients (glutamine and glucose) and metabolites (lactate and ammonia) in media isolated from individual layers of 3-layer Falcon Multi-Flasks vs. T-175 flasks **(5A)**. Air saturation (% of ambient oxygen) **(5B)**, carbon dioxide saturation (CO_2%) and pH (–) **(5C)** of spent medium were determined using pre-mixed medium from 5-layer Falcon Multi-Flasks and control T-175 flasks. EcoPack2-293 cells were seeded at a density of 35,000 cells/cm² and cultured for 96 hours prior to analysis (n=3 flasks).

Figure 5A.

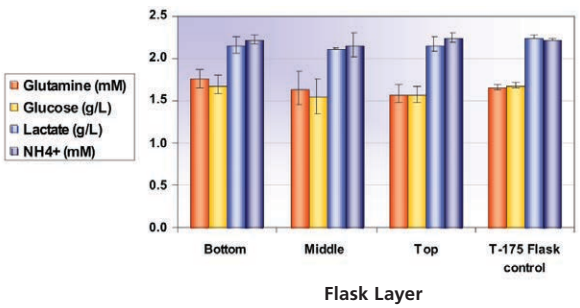


Figure 5B.

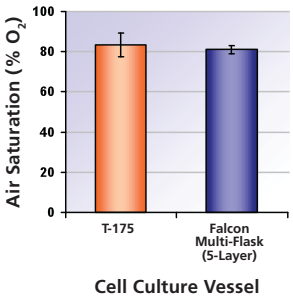
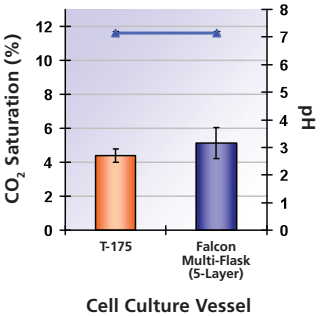


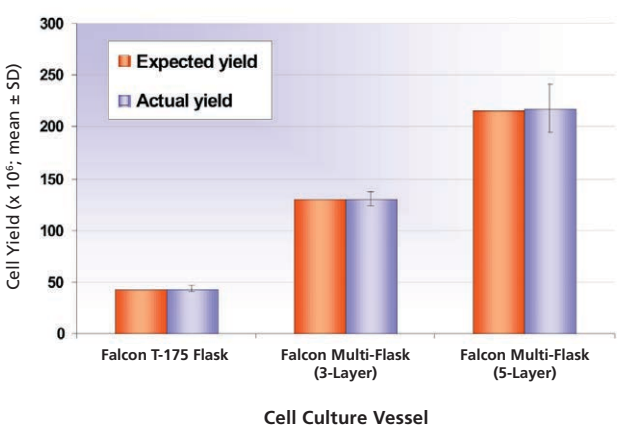
Figure 5C.

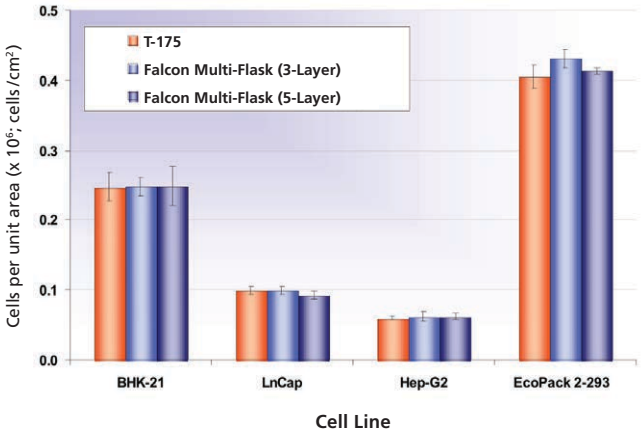


Easy Scale-Up from T-175 Flasks

To efficiently scale-up cells, it is not enough to simply increase the growth surface area of a vessel. It is also important to design a vessel that will enable optimal cell growth and efficient recovery of cells. Falcon® Cell Culture Multi-Flasks have three and five times the growth surface area (525 cm^2 and 875 cm^2, respectively, for the 3- and 5-layer vessels) compared to T-175 flasks. Both formats have a familiar T-175 footprint and are compatible with the same reagent volume and cell density requirements as T-flasks. Even media and cell distribution in all layers of the vessels, as well as convenient pipette access to recover cells and reagents, allows easy 3X and 5X scale-up from T-175 flasks. This is illustrated in Figure 6 (left panel) where three and five times the number of BHK-21 cells were grown and recovered from 3- and 5-layer Falcon Multi-Flasks as compared to T-175 flasks. Equivalent cell yield per unit area compared to control T-175 flasks was observed with various cell types for both 3- and 5-layer Falcon Multi-Flasks (Figure 6, right panel; and Table 1). Furthermore, scale-up of primary cultures, such as MSCs in serum-containing and serum-free media, yielded equivalent cell number per unit area in the Falcon Multi- Flasks compared to control T-175 flasks (Table 1).

Figure 6. Cell yield from Falcon Multi-Flasks compared to T-175 flasks. Three and five times the number of BHK-21 cells were grown and recovered from 3- and 5-layer Falcon Multi-Flasks compared to T-175 flasks (left pane; n=4 flasks). Expected yield was calculated by multiplying the mean cell yield of T-175 flasks by three and five times for the 3- and 5-layer Falcon Multi-Flasks, respectively. Cell yield per unit area (cm^2) was equivalent in 3- and 5-layer Falcon Multi-Flasks and T-175 flasks for BHK-21, LnCap, Hep-G2 and EcoPack™2-293 cells (right panel). Each bar represents a mean of 3 to 6 flasks.





CELL TYPE	T-175 (CELLS/CM²)	FALCON® MULTI-FLASK (3-LAYER)	
		(CELLS/CM²)	% OF T-175
BHK-21	2.47 x 10^5	2.48 x 10^5	100%
HUVEC-2	2.49 x 10^4	3.18 x 10^4	128%
LnCap	9.95 x 10^4	9.92 x 10^4	106%
Hep-G2	6.04 x 10^4	6.29 x 10^4	104%
EcoPack™2-293 (HEK 293 derived)	4.04 x 10^5	4.31 x 10^5	107%
CHO-M1	2.40 x 10^5	2.31 x 10^5	97%
MSC (WITH SERUM)	1.32 x 10^4	1.37 x 10^4	104%
MSC (SERUM FREE)	2.96 x 10^4	3.23 x 10^4	109%

A comparison of cell yield per unit area is shown in Figure 7. Cell recovery from both 3- and 5-layer Falcon Multi-Flasks was equivalent to control T-175 flasks.

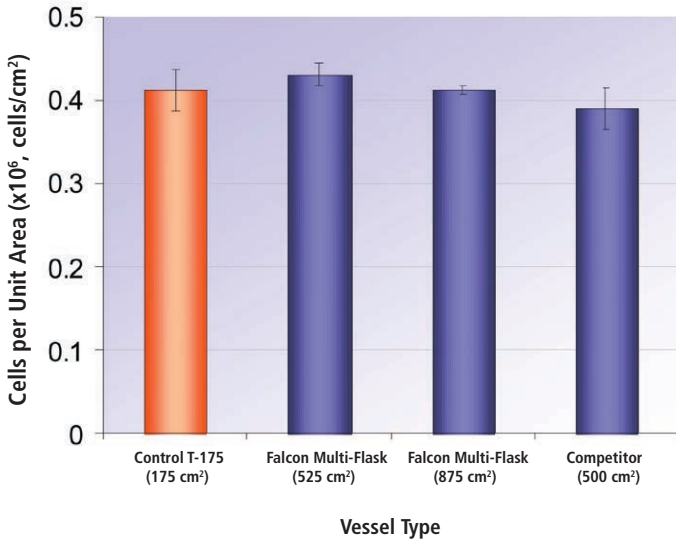
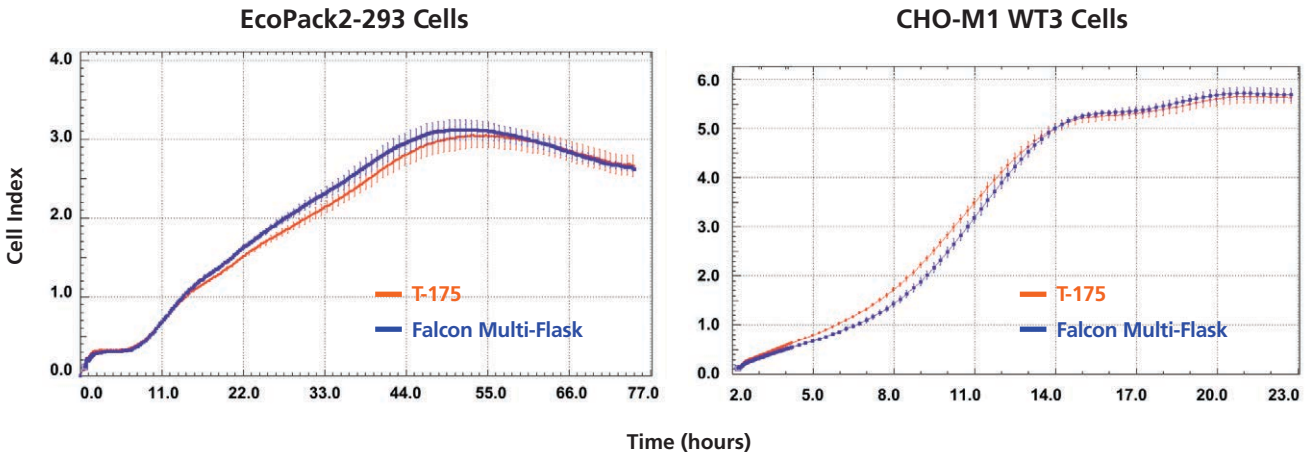


Figure 7. Efficiency of cell recovery from Falcon Multi-Flasks. EcoPack2-293 cells were seeded at a density of 34,500 cells/cm² in each of the vessels. At the end of 96 hours, cells were harvested and cell yield per cm² was calculated using the total growth surface area of each respective vessel (as indicated in parenthesis on the X-axis). Each bar on the graph depicts the mean ± SD of cell yield per unit area for three independent vessels.

Functional Performance of Cells Cultured in Falcon® Multi-Flasks

It is critical to ensure that cell quality or function is not altered by the culture vessel or cell environment while scaling-up cells. We used two methods to determine whether the performance of cells cultured in Falcon Multi-Flasks was equivalent to those grown in control T-175 flasks. First, we assessed the ability of two cells lines (EcoPack™2-293 and CHO-M1 WT3) to attach, spread and proliferate using an impedance assay. EcoPack2-293 (an HEK293–derived cell line) and CHO-M1 WT3 (a CHO-K1 derived transfected stable cell line) were selected as representative of commonly used cell types in drug discovery. Our results (Figure 8) confirmed that cell attachment, evident within a few hours after cell seeding, as well as cell spreading and proliferation, observed over one to three days, were nearly identical following culture in either control or Falcon Multi-Flasks.

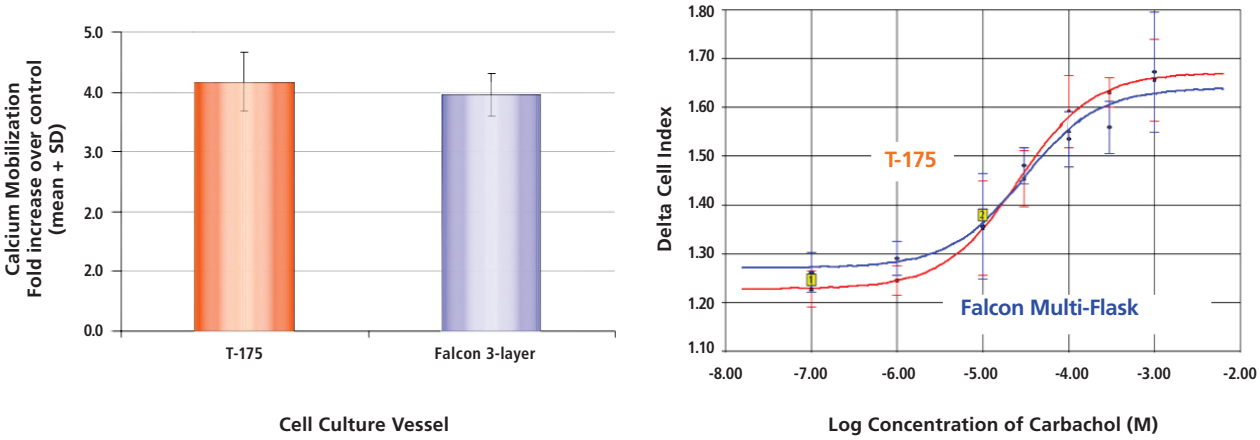
Figure 8. Detection of cell attachment, spreading and proliferation patterns in Falcon Multi-Flasks using an impedance assay. EcoPack2-293 cells (30,000 to 40,000 cells/cm^2) or CHO-M1 WT3 cells (10,000 to 20,000 cells/cm^2) were seeded in either control T-175 or Falcon Multi-Flasks and cultured for 96 hours. Cells were then harvested and seeded for impedance measurements in 96-well E-PLATES (Roche) and interactions between cells and electrodes, measured as changes in impedance, were reported as arbitrary cell index (CI) values. CI values are positively correlated with increased cell attachment, spreading and growth and in this experiment were recorded every 10 minutes for up to 3 days. The ability of either cell line to attach, spread, and proliferate following culture in T-175 and Falcon Multi-Flasks (3- and 5-layer) was remarkably similar.



Second, we evaluated the functional response of muscarinic G protein-coupled receptors (GPCR) in the same two cells lines using a non-selective muscarinic receptor agonist, carbachol (Figure 9). HEK293 cells, as well as derived lines such as EcoPack™2-293 cells, express endogenous muscarinic receptors and are responsive to agonists in a concentration-dependent manner (Atwood, et al., 2011, Hakak, et al., 2003, Luo, et al., 2008 and Abraham, et al., 2009). CHO-M1 WT3 is a transfected line with stable over-expression of M1 muscarinic receptors. GPCR response was tested in two ways: 1) by challenging the cells with a single concentration of agonist and monitoring a drug-induced calcium mobilization response (Figure 9, left panel); and 2) by performing a concentration-response study using a range of agonist concentrations and monitoring GPCR-induced changes in cell morphology (Figure 9, right panel). In both GPCR functional studies, performance of cells grown in Falcon® Multi-Flask was found to be equivalent to those from control T-175 flasks (Figure 9).

EcoPack2-293 cells (30,000 to 40,000 cells/cm^2) or CHO-M1 WT3 cells (10,000 to 20,000 cells/cm^2) were seeded in either control T-175 or Falcon Multi-Flasks and cultured for 96 hours. Cells were then harvested and seeded for impedance measurements in 96-well E-PLATES (Roche) and interactions between cells and electrodes, measured as changes in impedance, were reported as arbitrary cell index (CI) values. CI values are positively correlated with increased cell attachment, spreading and growth and in this experiment were recorded every 10 minutes for up to 3 days. The ability of either cell line to attach, spread, and proliferate following culture in T-175 and Falcon Multi-Flasks (3- and 5-layer) was remarkably similar.

Figure 9. Agonist-induced GPCR response of cells cultured in Falcon Multi-Flasks. CHO-M1 WT3 cells (left panel) were grown in T-175 or 3-layer Multi-Flask for 72 hours. Cells were harvested and re-seeded on Corning® BioCoat™ Poly-D-Lysine 96-well Black/Clear Microtest™ Plates for GPCR calcium mobilization assay. Cells were challenged with an agonist, Carbachol (300 uM), and responses were recorded one minute following compound addition. Data is expressed as fold-increase in calcium flux compared to control cells challenged with assay buffer (no drug). No significant difference in agonist response was observed between cells harvested from either vessel type. Data shown is representative of three independent experiments. Carbachol-induced changes in cell morphology were measured for EcoPack2-293 cells using an impedance assay (right panel). Cells harvested from T-175 flasks and 5-layer Falcon Multi-Flasks were seeded on 96 well plates and challenged with increasing concentrations of carbachol (0.1 to 300 uM) after 24 hours. Cells grown in T-175 flasks and Falcon Multi-Flasks both demonstrated concentration-dependent sigmoidal drug response following stimulation with carbachol. Moreover, carbachol was equipotent in stimulating morphological response in cells from both culture vessels. Data shown is representative of two independent experiments.



Conclusions

- Falcon® Multi-Flasks offer scientists the option of increasing productivity and overall cell yield during cell expansion without compromising cell behavior.
- Homogeneous cell growth is observed within and between layers of Falcon Multi-Flasks.
- Metabolic activity of cells, as well as pH, O_2 and CO_2 saturation in spent media (pooled or from individual layers) are equivalent for Falcon Multi-Flasks and T-175 flasks.
- Three and five times the number of cells can be grown and recovered from 3- and 5-layer Falcon Multi-Flasks, respectively, compared to T-175 flasks.
- Cell growth (cells/cm^2) for diverse cell lines and primary cells (cultured with and without serum) is equivalent for Falcon Multi-Flasks compared to T-175 flasks.
- Attachment and growth profile of CHO-M1 and EcoPack™2-293 cells cultured in T-175 and Falcon Multi-Flasks are indistinguishable.
- No difference was observed in functional performance (agonist-induced GPCR response) of CHO-M1 and EcoPack2-293 cells harvested from T-175 and Falcon Multi-Flasks.

The current study demonstrates how the design features of the Falcon Multi-Flask translates into homogeneous cell culture. The cell culture environment within, and between, layers of each Falcon Multi-Flask vessel is uniform, which enables the expansion of homogenous cells. Growth, yield (per unit area), attachment, proliferation and functional performance of cells cultured in the Falcon Multi-Flasks were equivalent to T-175 flasks. Diverse cell lines and primary cultures can be scaled-up efficiently using the Falcon Multi- Flasks without the need for re-optimizing existing culture conditions or compromising the quality, homogeneity or the performance of cells.

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Corning acquired the BioCoat™ and Falcon® brands.
For information, visit www.corning.com/discoverylabware.

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