Cell confluency analysis on microcarriers by micro-flow imaging

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Received: 14 December 2015 / Accepted: 17 March 2016 / Published online: 14 May 2016
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Abstract The productivity of cell culture-derived vaccines grown in anchorage-dependent animal cells is limited by bioreactor surface area. One way to increase the available surface area is by growing cells as monolayers on small spheres called microcarriers, which are approximately 100–250 μm in diameter. In order for microcarrier-based cell culture to be a success, it is important to understand the kinetics of cell growth on the microcarriers. Micro-flow imaging (MFI) is a simple and powerful technique that captures images and analyzes samples as they are drawn through a precision flow cell. In addition to providing size distribution and defect frequency data to compare microcarrier lots, MFI was used to generate hundreds of images to determine cell coverage and confluency on microcarriers. Same-day manual classification of

these images provided upstream cell culture teams with actionable data that informed in-process decision making (e.g. time of infection). Additionally, an automated cell coverage algorithm was developed to increase the speed and throughput of the analyses.

Keywords Microcarriers · Micro-flow Imaging · Confluency · Mammalian cell culture

Introduction

Vaccines have had a tremendous impact on global human health, particularly due to the reduction of disease burden caused by infectious diseases (Andre et al. 2008). Many life saving vaccines are grown in adherent cell lines. While adherent cells are traditionally viewed as having higher productivity than suspension cells (Nilsson 1989; Iyer et al. 1999), scale-up of adherent cells presents a formidable challenge due to surface area requirements. In addition, it is difficult, if not impossible, to adapt many adherent cells to suspension growth.

One way to increase the available surface area in culture is the use of microcarriers, small spherical particles used for the growth of adherent cells. Microcarriers have been used for the growth of mammalian cells, including MRC-5 cells (Forestell et al. 1992), and the production of both viruses and vaccines (van Hemert et al. 1969; Spier and Whiteside

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1976; Giard et al. 1977; Sinskey et al. 1981; Mered et al. 1981; Brands et al. 1997). The initial development of microcarriers for adherent cell growth used the anion-exchange resin DEAE-Sephadex A-50 (van Wezel 1967). Over the years the physical and chemical properties of microcarriers (density, size, porosity, substrate, rigidity, surface charge, optical properties) have been refined and currently there are numerous vendors and multiple varieties of microcarriers available commercially. In order to appropriately control the production of biotechnology products during upstream bioprocess unit operations, it is important to understand cell growth, viability and distribution of cells on microcarriers. In traditional suspension cultures, cell growth is measured using whole cell and nuclear counts, while viability is determined by dye exclusion (Patterson 1979). Microcarrier culture requires removal or lysing of cells from the microcarrier substrate to carry out this analysis. This removal process may be impacted by microcarrier aggregation and other factors that lead to incomplete detachment, non-uniform dispersal, or cellular changes which may impact the measurement. Importantly, any spatial information (e.g. cell morphology, cell confluency, distribution on microcarriers) is lost.

Micro-flow imaging (MFI, ProteinSimple, San Jose, CA, USA) is a flow microscopy-based particle counting technique. As the sample stream is drawn through a precision flow cell by a peristaltic pump, high resolution digital images of the particles are automatically captured. In addition to collecting approximately 700 images per 1 ml of sample, the instrument software generates a database of every particle in every image, allowing for the determination of particle size [equivalent circular diameter (ECD)], particle concentration, and other optical properties such as morphology (shape), aspect ratio, circularity, and intensity. MFI is accurate, reproducible, has a broad size range (2–1500 μm) and a reasonable level of throughput (approximately 5 min per sample). One important use of MFI has been the analysis of sub-visible particles in protein formulations (Huang et al. 2009; Sharma et al. 2010). MFI can also be used to differentiate sub-visible proteinaceous particles from silicone oil based on particle morphology (Majumdar et al. 2011; Strehl et al. 2012). In addition, the hundreds of images captured per sample provide a rich data set that can be probed for further insight into the nature of the tested sample.

In the current study, we demonstrated the ability of MFI to assess cell confluency of epithelial and fibroblast cell lines cultured on Cytodex 1 microcarriers (GE Healthcare, Uppsala, Sweden), a commonly used microcarrier composed of a cross-linked dextran matrix that is substituted with positively charged *N,N*-diethylaminoethyl (DEAE) groups. MFI was chosen for this analysis because it is a fast and straightforward method that provides same day information about the progression of cell growth and confluency that can help upstream bioprocess teams to make timely decisions. Additionally, we were able to collect information on the size and defect rate of Cytodex 1 microcarriers, indicating that MFI could be used to evaluate the quality of microcarrier lots.

Materials and methods

Cell lines

The experiments were carried out with adherent human retinal pigmented epithelial cell line ARPE-19 and adherent lung fibroblast cell line MRC-5 (ATCC, Manassas, VA, USA). Cells were cultivated on 1 g/l Cytodex 1 microcarriers (GE Healthcare Life Sciences, Uppsala, Sweden) in 3L bioreactors or 125 ml spinner flasks with either Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, for ARPE-19, Corning Life Sciences, Oneonta, NY, USA) or Williams' Modified Eagle Medium (WMEM, for MRC-5, GE Healthcare HyClone, Logan, UT, USA) and supplemented with 10 % fetal bovine serum (FBS, GE Healthcare HyClone, Logan, UT, USA). A detailed and optimized cell attachment procedure for MRC-5 cells on Cytodex 1 microcarriers has been previously published (Forestell et al. 1992).

Cells attached to microcarriers were washed twice by settle-decant with phosphate buffered saline (PBS) and fixed by incubating microcarriers with 4 % paraformaldehyde in PBS at room temperature for 20 min. Microcarriers were then washed twice with PBS and stained with 10 % (v/v) Giemsa stain, modified (Sigma-Aldrich, St. Louis, MO, USA) for 20 min and washed 4 times in PBS.

SLS

Static Light Scattering (SLS) measurements were performed with a Malvern Mastersizer 2000 (Malvern

Instruments, Malvern, UK) using a Micro Precision Hydro 2000 μ P sample dispersion unit. Microcarrier samples were rehydrated according to manufacturer's instructions to 20 g/l in phosphate buffered saline (PBS). Prior to measurement, samples were diluted to target an obscuration of 5 % (approximately 250 microliters (μ l) of 20 g/l microcarriers diluted in 20 milliliters (ml) of physiological saline). Data were acquired using a stir speed of 1500 rotations per minute (rpm) to prevent settling and maintain consistent obscuration values throughout the measurement. A refractive index of 1.347, absorption of 0.1, and a general purpose (irregular) analysis model were used for data analysis.

MFI

MFI measurements were performed with a ProteinSimple DPA 4100 particle analyzer (ProteinSimple, San Jose, CA, USA) with a 400 μ m SP1 silane coated flow cell under low magnification. For a typical analysis, approximately 600 microcarriers are imaged in 5 min (about 2 microcarriers per second) with a sample volume of 1 ml, total available volume of 0.9 ml, purge volume of 0.1 ml, and flow rate of 200 μ l per minute. Bins were 2–5, 5–10, 10–15, 15–20, 20–30, 30–40, 40–50, 50–75, 75–100, 100–125, 125–150, 150–175, 175–200, 200–225, 225–250, 250–275, 275–300 and 300–1500 μ m. Samples were run at 1–2 g/l and loaded with a 1 ml aerosol-barrier pipette tip.

Manual classification of cell-containing microcarrier MFI images was performed for the first 100 images of the approximately 700 collected for each sample in order to increase sample throughput and allow for same day analysis. For each sample, MFI images are acquired in 5 min while manual classification requires approximately 1 h per 100 images. Each microcarrier was visually inspected and classified according to the percent of the microcarrier that was covered with cells, into 0–25, 26–50, 51–75, and 76–100 % categories. The total number of microcarriers in each category was normalized to the total number of classified microcarriers.

Automated cell coverage algorithm

A customized algorithm for automatic detection of microcarriers and for quantification of confluency was developed using Matlab (R2015a Mathworks, Natick,

MA, USA). Prior to quantifying cell coverage, individual microcarriers were identified from background by evaluating the local gray value changes using a Canny edge detection algorithm (Canny 1986). Thresholding and morphological operations were later applied to remove the noise from background (Fig. 6b). Since the individual microcarriers are visualized as circles in 2-D due to their spherical nature in 3-D, a Hough transform (a method commonly used in image analysis literature to detect circles in images) was applied to find and isolate the individual microcarriers. Microcarrier bead aggregation was calculated by applying a connected component analysis on the individually segmented microcarriers (Fig. 6c). Whole microcarrier coverage was measured by estimating the area using the radius information as πr^2 . A histogram based thresholding algorithm was then applied to measure the cell coverage on microcarriers. After this thresholding step, morphological operations were applied to remove any noise that may have been included in the segmentation (Fig. 6e). Edge artifacts were excluded from the analysis. Confluency was calculated by taking the ratio of the segmented area to the whole microcarrier coverage. The % measurements were later grouped into 4 categories as 0–25, 26–50, 51–75 and 76–100 % confluent (Fig. 6f). Figure 6 shows the steps for the detection of the individual microcarriers, analysis of the bead aggregation and the measurement of the confluency within individual microcarriers with various confluency levels.

Results

Cytodex 1 microcarrier size was assessed by Static Light Scattering (SLS) and Micro-flow imaging (MFI). SLS (Fig. 1a) indicated an average mass median diameter (d_{50}) of 186 μ m (0.01 % Relative Standard Deviation, RSD). The average mean equivalent circular diameter (ECD) of three independently tested lots as measured by MFI (Fig. 1b) was 141 μ m (7 % RSD). When debris produced during the manufacturing process (<50 μ m) and large particles that are the result of overlapping individual microcarriers (>300 μ m) are excluded, the average equivalent circular diameter (ECD) as measured by MFI was 176 μ m (3 % RSD). The size range with the highest concentration of microcarrier particles was 150–175 μ m, while the range with the second highest concentration of

Fig. 1 Cytodex 1 microcarrier particle size as measured by SLS and MFI. **a** SLS particle size distribution overlay of triplicate measurements on the same sample, y axis indicates vol % of total measured particle volume; **b** MFI particle size distribution

histogram of three independent lots (black, white, gray fill) of Cytodex 1 microcarriers. Inset shows representative images of microcarriers

microcarrier particles was 175–200 μm . SLS is an ensemble particle sizing method. All of the particles in the sample are measured at the same time, and the volume-based particle size distribution is extracted from the raw light scattering data using Mie light scattering theory calculations. MFI, on the other hand, is a counting method that measures individual particles one at a time and the particle size distributions are number based. These differences (ensemble vs. counting, volume- versus number-based distributions) account for the ability of the MFI to detect the small particles that are missed by SLS. These measured sizes are consistent with the manufacturer's reported size of diameter at 50 % of the volume (d_{50}) of 190 μm , and range between the diameter at 5 and 95 % of the volume of a sample of microcarriers (d_{5-95}) of 147–248 μm . Independent testing of three lots by MFI demonstrated that while the distribution of particles among size ranges is similar, it is not identical (Fig. 1b). This suggests that MFI is capable of resolving differences in size distribution between different lots of microcarriers.

In addition to measuring particle size and concentration, MFI provides hundreds of images that can be mined for further information. Visual inspection of

approximately 600 microcarriers from 100 MFI images for each of three lots of microcarriers indicated the presence of Cytodex 1 microcarrier defects upon re-hydration (Fig. 2). These defects were classified according to the severity of the defect. Minor defects were defined as affecting less than half of the observable surface area, moderate defects affecting more than half, and major defects appearing as shriveled non-circular particles. Overall, the frequency of defects is less than 10 % of the total population, with minor defects being the most prevalent and major defects being the least prevalent. These defects cannot be seen by SLS, since the vast majority of them do not substantially affect the size of the microcarriers as detected by an ensemble method like SLS. Further, the size of the defects was generally within the size range of unblemished microcarriers. MFI allowed detection of the defects because it provides physical images that allow visualization of the morphology of the particles, in addition to sizing information.

The confluency of cells on microcarriers was also measured using MFI. ARPE-19 epithelial cells were cultured on Cytodex 1 microcarriers and approximately 700 images were captured for each sample. Microcarriers from the first 100 images were classified

Fig. 2 Cytodex 1 microcarrier defects **a** representative images of minor, moderate, and major defects as captured by MFI; **b** manual quantitation of total number of defects, minor, moderate, and major defects from 100 images. Three independent lots (black, white, gray fill) of Cytodex 1 microcarriers

into four categories according to microcarrier coverage: 0–25, 26–50, 51–75, 76–100 % (Fig. 3a, b). The percentage of microcarriers that were classified into each category was tabulated. This analysis was performed for four different 3L bioreactors across 4 time points, day 3, day 4, day 5 and day 6 post cell plant (Fig. 3c–f). The increase in the percentage of cells in the 76–100 % category is reflective of cell growth and indicated an increase in the confluency of cells on the microcarriers. A similar analysis was performed for MRC-5 fibroblasts (Fig. 4). Representative images demonstrate the increased incidence of bead-to-bead bridging and elongated cells typical of fibroblast cell growth (Fig. 4c, d). Manual classification of 100 images was performed in about 1 h. This allowed for the determination of confluency for 6 samples per day per analyst (inclusive of data

collection and analysis time). Due to the straightforward nature of the method and data analysis, MFI is being used routinely as a real time decision making tool to support upstream bioprocessing teams.

One way to improve the speed and accuracy of cell coverage determination on Cytodex 1 microcarriers is to enhance the contrast between cells and microcarriers. To this end, various cytological staining procedures were evaluated, including Coomassie Blue, hematoxylin and eosin, and Giemsa. The cytological stain that demonstrated the best contrast between cells and microcarriers was 10 % (v/v) Giemsa for 20 min (Fig. 5). This staining was effective for both epithelial (ARPE-19) and fibroblast cell lines (MRC-5). The other stains tested suffered from low contrast due to too little staining (0.1–3 % Coomassie Blue, 30 min-overnight) or staining both cells and microcarriers (1–5 % hematoxylin and eosin, 30 min-overnight) at the conditions tested. The other advantage of cell staining was that the enhanced contrast enabled automated classification.

Although manual classification of cell coverage on microcarriers was adequate for providing data for upstream process decision making, an automated cell coverage algorithm was developed to increase the throughput of the assay and remove any potential biases between individual analysts who performed the manual classifications. Images of Giemsa stained microcarriers were collected and subjected to an automated analysis workflow. The workflow for each image (Fig. 6) consisted of detection of the individual microcarriers using a Canny edge detection algorithm (Fig. 6b), analysis of bead aggregation with connected component analysis (Fig. 6c), and the measurement of the confluency of cells on individual microcarriers by taking the ratio of the segmented area to the area of the microcarrier (Fig. 6e, final results Fig. 6f). We validated the automated image analysis algorithm by applying it to 522 test images and comparing the confluency measurements with those obtained manually using Cohen's kappa statistic measure (McHugh 2012). The Cohen's kappa statistic is a measure of interrater reliability. It takes into account the agreement between two independent data collectors, in this case manual classification and automated classification. Table 1 shows the number of microcarriers that were classified into each bin by manual classification and automated classification to show concurrence. The Cohen's kappa coefficient between the two

Fig. 3 Cell coverage of ARPE-19 cells on Cytodex 1 microcarriers. **a, b** Representative images for each of the classification bins; **c–f** growth curves from four independent bioreactors,

indicating the percent of microcarriers that were manually classified into each bin from day 3, 4, 5, 6 post cell plant

classifications (manual and automated) was determined to be 0.4304, which demonstrated a moderate agreement. As expected, the concordance was highest when the cells on the microcarriers were closest to confluency. This is most likely because the automated algorithm is more accurate at measuring white space between cells on subconfluent beads compared with manual classification.

Discussion

Adherent cell growth is inherently limited by available bioreactor surface area. One scalable approach to improve the yield of biotechnology products derived from adherent cells is to increase the available surface area by growing cells on microcarriers. However, microcarrier cell culture presents unique technical challenges including the monitoring of cell growth.

Traditionally, cell growth and viability of adherent cells grown on microcarriers are monitored by removing cells from the substrate with trypsin, followed by trypan blue dye exclusion staining and counting on a hemocytometer (Forestell et al. 1992;

Frondoza et al. 1996; Boudreault et al. 2001; Mendonça et al. 2002; Bleckwenn and Shiloach 2004; Fernandes et al. 2007; Lock and Tzanakakis 2009; Sart et al. 2009). Alternatively, cell nuclei may be counted by crystal violet staining (Forestell et al. 1992; Serra et al. 2009). Nuclear counts should be used with caution because of the potentially confounding issue of a significant subpopulation of bi-nucleated cells (Berry et al. 1996). These widely used methods are direct and quantitative, but require the removal of cells from the microcarriers. This may lead to inaccurate cell counts due to incomplete cell detachment following trypsinization (Varani et al. 1985), poor dispersal, or altered cellular structure.

Another method to follow cell growth in a bioreactor is to measure nutrient consumption (e.g. glucose, glutamine) and waste accumulation (e.g. lactate, ammonia) as a proxy for metabolic activity (Bleckwenn and Shiloach 2004; Kino-Oka et al. 2005; Fernandes et al. 2007; Abranches et al. 2007; Serra et al. 2009). Metabolic activity can also be assessed using the tetrazolium dye reduction (MTT) assay. This colorimetric assay indirectly measures metabolic activity to estimate the number of viable cells in

Fig. 4 Cell coverage of MRC-5 cells on Cytodex 1 microcarriers. Representative images from **a** day 1; **b** day 2; **c** day 5; **d** day 6 post cell plant; **e** growth curve indicating the percent of microcarriers that were manually classified into each bin from day 1, 2, 4, 5, 6 post cell plant

culture (Lu et al. 2006; Abranches et al. 2007; Fernandes et al. 2007). Cytotoxicity can be assessed using the lactose dehydrogenase (LDH) assay which monitors the release of LDH into the media from damaged cells as a biomarker for cytolysis (Lock and Tzanakakis 2009; Serra et al. 2009). While these assays provide useful information about the culture, they are less sensitive than direct (i.e. cell count) methods and do not provide direct information about the uniformity of cell distribution across microcarriers. Importantly, these methods do not address the critical issue of bead to bead uniformity and reproducibility of cell growth, a parameter critical for a robust process.

Particle sizing methods can be used to follow the growth of adherent cells on microcarriers. These methods rely on the linear relationship between the cell density (number of cells per microcarrier) and the size distribution of the microcarriers. The particle size

Fig. 5 Cell staining with Giemsa. **a, b** Representative images of ARPE-19 cells; **c, d** MRC-5 cells on Cytodex 1 microcarriers **a, c** unstained or **b, d** stained with 10 % Giemsa to enhance the contrast between cells and microcarriers

distribution can be measured by either the Coulter principle as in Aperture Impedance Pulse Spectroscopy (Degouys et al. 1996) or by laser diffraction (Kenda-Ropson et al. 2002). These methods have the advantage that biomass can be estimated in situ (i.e. cells on the microcarriers) without the need for enzymatic removal. A challenge with these methods is that they require significant development work in order to establish the empirically determined and cell type specific relationship between cell density and particle size distribution, since each cell type has a different average particle size.

Direct microscopic observation is a simple, quick and easy way to qualitatively follow the growth of cells directly on microcarriers. In order to get more quantitative information, for example the percent of colonized beads (Lock and Tzanakakis 2009), time consuming and tedious manual counting is required. In situ microscopy (Rudolph et al. 2008; Bluma et al. 2010), an online alternative to traditional offline direct microscopy, consists of capturing images of microcarriers as they flow through the sampling zone of the in situ microscope that is attached to a bioreactor via a side port. Images are computationally segmented to generate gray value gradients, and these gradients are compared with a manually classified training set. A neural network based analysis is then used to extract and measure % cell coverage of the microcarriers.

Fig. 6 Workflow for automatic detection of microcarriers and quantification of confluency. **a** Original image; **b** application of Canny edge detection and morphological operations; **c** isolation of individual microcarriers and bead aggregation analysis. *Red circles* single microcarriers, *yellow circles* double microcarriers, *green circles* triple microcarriers; **d** after applying histogram based thresholding and morphological operations; **e** exclusion of edge and segmentation of cell coverage; **f** classification of microcarriers based on cell coverage. *Blue circles* 0–25 % confluent, *green circles* 26–50 % confluent, *yellow circles* 51–75 % confluent, *red circles* 76–100 % confluent

Table 1 Comparison of manual versus automated analysis of confluency in microcarriers

	Manual classification			
	0–25 %	26–50 %	51–75 %	76–100 %
Automated classification				
0–25 %	11	0	0	0
26–50 %	9	5	12	1
51–75 %	0	1	37	120
76–100 %	0	0	6	1419

This method has the advantage of direct online observation of the microcarriers in the bioreactor and high time resolution (a window of 7.5 h in one bioreactor). These advantages come at the expense of throughput, with data from more than one bioreactor necessitating multiple in situ microscopes. In addition, due to size limitations of the sampling zone, bead

agglomerates can not be seen, but only inferred from the decrease in sampling rate over time.

The low contrast of biological samples presents a formidable challenge when probing the fine features of cellular morphology with microscopy. There are several approaches to increasing the resolution of images. Scanning Electron Microscopy (SEM) has been used to provide detailed morphological information (e.g. cell shape and size) that is especially useful for investigating changes in cell phenotype over time with cells grown on microcarriers (Pons et al. 1992; Frondoza et al. 1996). Digital Holographic Microscopy (DHM) captures both intensity and phase information as a digital hologram that is computationally reconstructed as an image. This obviates the need for staining to increase contrast and enables live cellular dynamics studies (Rappaz et al. 2015). Multidimensional image-based analysis (Baradez and Marshall 2011) uses laser scanning confocal microscopy to image fluorescently labeled cells on microcarriers. Microcarriers are stained and imaged with a laser scanning confocal microscope. Confocal images are processed for microcarrier detection and quantification of confluency. This method has the advantage of multiparametric monitoring (e.g. confluency, cell morphology, live/dead stain) and the enticing possibility of incorporating direct labeling of subcellular components with fluorescently-labeled probes. SEM and DHM provide high resolution images at the expense of sample throughput, while the time scale of laser scanning confocal microscopy (inclusive of labeling, imaging and automated image analysis) is in the same order of magnitude as confluency analysis by MFI. All three of these high resolution techniques are limited by their low throughput, as well as the need for expensive and specialized equipment and highly trained operators.

In the current study, cell confluency on microcarriers was measured by MFI coupled with either manual classification or an automated cell coverage algorithm. This allowed for the direct imaging of cells on microcarriers and did not suffer from the liabilities of conventional methods (i.e. cell- or nuclear counting, indirect metabolic measurements). The kinetics of cell growth were determined by following the increase of the percent of beads in the 76–100 % confluent category. In addition to cell confluency measurements, this method provided hundreds of MFI images that allowed for detailed visual inspection of microcarriers

(e.g. defects) and cell morphology (e.g. fibril formation of fibroblasts).

One analyst can manually classify microcarriers from 100 images in about 1 h. This allows for the same day analysis of about 6 samples, inclusive of staining, data acquisition, and manual classification. One major advantage of the manual approach is the ease of implementation. The use of the automated cell coverage algorithm increased the throughput to 700 images in about 30 min. In addition to increased throughput, the classification of microcarriers from about 700 images also increased the statistical confidence in the cell coverage of the sample. The quick turnaround enables the upstream bioprocess team to make informed same day process decisions (e.g. day of infection).

Considerations for future improvement of the MFI confluency assay include easier data acquisition, faster data analysis, technology development to eliminate the need for sample staining, and extending the assay by investigating other aspects of the rich data set generated by MFI. The data acquisition could be improved through the use of a MFI system incorporating an autosampler (i.e. ProteinSimple MFI5100 with BOT1). While this would not necessarily improve the throughput, the autosampler bot would enable walk-away automation. Currently, the biggest bottleneck in the throughput of the assay is the data analysis, since the data acquisition step (5 min per sample) is much faster than the image analysis (approx. 1 h for manual counting, 30 min for automated cell coverage algorithm). Parallel processing could improve the throughput, enabling analysis of more samples (e.g. testing of sampling replicates). Another limitation to the speed of analysis is the need for cell staining. This limits the throughput somewhat, as the samples need to be fixed, stained and washed prior to collecting images. One way to remove the staining step would be to blend the label-free imaging of DHM with the quick image capture of MFI. This would require significant technology development. Besides ease of use and increased throughput, the rich data set generated by MFI could be mined for other types of experiments. Examples include microcarrier distribution across different bioreactors, or the presence of microcarrier agglomerates (Fig. 4c, d).

In summary, cell confluency analysis by MFI is a simple method that is straightforward to implement. Same day microcarrier cell confluency results provided

upstream process teams with timely, actionable data that can be used to inform bioprocess decisions.

Acknowledgments The authors thank our colleagues in Vaccine Bioprocess Research and Development and Global Vaccines and Biologics Commercialization for samples and technical assistance.

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