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RESEARCH ARTICLE

BIOTECHNOLOGY PROGRESS

Single cell analysis of Chinese hamster ovary cells during a bioprocess using a novel dynamic imaging system

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Abstract

Reliable monitoring of mammalian cells in bioreactors is essential to biopharmaceutical production. Trypan blue exclusion is a method of determining cell density and viability that has been used for over one hundred years to monitor cells in culture and is the current standard method in biomanufacturing. This method has many disadvantages however and there is a growing demand for more detailed and in-line measurements of cell growth in bioreactors. This article assesses a novel dynamic imaging system for single cell analysis. This data shows that comparable total cell density, viable cell density and percentage viability data shown here, generated by the imaging system, aligned well with conventional trypan blue counting methods for an industrially relevant Chinese Hamster Ovary (CHO) cell line. Furthermore, detailed statistical analysis shows that the classification system used by the PharmaFlow system can reveal trends of interest in monitoring the health of mammalian cells over a 6-day bioreactor culture. The system is also capable of sampling at-line, removing the necessity for taking samples off-line and enabling real time monitoring of cells in a bioreactor culture.

KEYWORDS
bioprocess, cell viability, CHO cells, PharmaFlow

1 | INTRODUCTION

Effective monitoring and control of a bioprocess involving mammalian cells is an important feature of the consistent and robust biomanufacturing of biotherapeutics.[1,2] Sensors that allow continuous monitoring of pH, dissolved oxygen and temperature have been available for some time.[3] However, cell concentrations are typically measured by manual sampling, staining and counting with a stand-alone counter.[4]

The ability to accurately assess cell viability is vitally important in bioprocessing. The gold standard method of assessing viability and counting mammalian cells is the trypan blue method which has been successfully used for decades.[5,6] This method is based on exclusion of the trypan blue dye by the cell membrane allowing simple, color-based identification of live and dead cells. Many alternative viability methods exist using other dyes and fluorescent stains such as acridine orange and propidium iodide[7] and 7-AAD.[8] Despite some of these alternative methods proving more accurate, trypan blue staining remains the standard in most academic and many industrial settings. Automated cell counting using trypan blue has become commonplace and multiple platforms are available.[9,10] In more recent years, there has been a move towards less invasive methods of cell health analysis in bioprocessing. There is also an aspiration within the biopharmaceutical industry to move away from offline measurements in favor of automated bioprocessing encompassing in-line or at-line monitoring[11,12]

using various sensor and imaging technologies that have become available.[13]

The distinctions between at-line and in-line monitoring are well described in a recent review.[14] In-line monitoring is typically performed by the use of sterilizable probes that are inserted into the bioreactor and will allow continuous monitoring. This is routinely used for the determination of pH, dissolved oxygen and temperature. However, cell counting is typically performed by off-line measurements where discrete samples are taken manually from the culture, stained and then analyzed by a cell counter, a process that can take tens of minutes. At-line involves the analysis of discrete samples from a culture that can be taken automatically and analyzed within minutes. The development of liquid autosamplers has helped the advance of at-line monitoring tools. In-line or at-line measurements of viable cell numbers provide real-time or near real-time analysis of the growth of cells during a bioprocess and usually without the need for staining. This has advantages that enable operators to monitor the progress of a bioprocess and allow early detection and correction of any unwanted deviations.

Changes in the optical density of cultures could be exploited as a means of continuous on-line monitoring of cells. Turbidity sensors are available to measure back scatter of light and have been used extensively in microbial bioprocesses.[15,16] There are examples of these being applied to mammalian cell lines.[17] However, they are generally not well suited because of the presence of cell debris that can arise at high densities leading to inaccurate readings.[18]

Significant advances have been made in the development of in-line capacitance probes that use dielectric methods for monitoring cells in a bioprocess. The dielectric methods are based on the interactions between the cells and an electromagnetic field.[19] This is the basis of the biocapacitance probes that can be sterilized and placed in situ within the culture. Live cells with intact membranes act as electrical capacitors resulting in an increase in signal, that is proportional to the number of viable cells. This technology has been rapidly accepted by the brewing industry and its value in biopharmaceutical manufacture has been realized for many years.[20] A great deal of data can be acquired with such a system as the frequency of the applied alternating electric field is altered to obtain a spectrum of response. However, the data has to be interpreted carefully as the capacitance signal obtained is dependent upon multiple factors including total cell volume, membrane characteristics and cytoplasmic conductivity.

Image analysis of a cell population provides complementary data based on morphological characteristics as cells progress through a bioprocess. The analysis of digital images of individual cells by machine learning enables the identification not only of viable cells but the changing state and metabolism as cells become non-viable and enter a death phase.[21] Digital holographic microscopy provides detailed 3-dimensional images of cells that can be used to examine various morphological changes of cells.[22] An application of this approach to cell bioprocessing was developed through the Ovizio Imaging System (Brussels, Belgium). This system loops cells from a bioreactor into a flow chamber from which are constructed digital holographic images. The 3-D holograms are monitored through software that allows the identification of cells by their morphological characteristics.[23]

In the current study we investigated the applicability of an alternative optical system to monitor CHO cells during culture. This microscopic imaging system provides a moderately high resolution of a population of cells that enabled rapid identification of morphological features that could distinguish viable from non-viable cells in samples taken from a bioreactor. The measurements obtained from the system were compared with traditional counting measurements. The advantages of using this imaging system compared to trypan blue are: (1) thousands of cells are analyzed in each sample; (2) more information is gathered about each cell with over 35 individual parameters measured and (3) no dye or stain is needed. Process analytical technology such as this would help greatly in the move away from off-line measurements. Furthermore, we show how statistical methods could be applied to the imaging data to provide information about the health of the CHO cells over the duration of the bioreactor culture.

2 | METHODS

2.1 | Cell culture

CHO-EG2 cells which express a human-llama chimeric antibody (EG2) against EGFR were kindly provided by Yves Durocher of the NRC, Canada.[24] The cells were cultured in BIOGRO-CHO serum free medium (BioGro Technologies Inc., Winnipeg, Canada) supplemented with 4 mM L-Glutamine, under standard cell culture conditions (5% CO_2, 95% humidity, 37°C and 120 rpm). Cells at low passage number were scaled up to inoculate 1 L stirred bench-top bioreactors (Applikon Biotechnology, Delft, Netherlands) at a density of 3×10^5 cells/mL (7.2 pH, 30% dO_2, 37°C and 400 rpm). Each culture was grown as a batch process over 7 days. Samples (5 mL) were taken under aseptic conditions using a sampling valve and sterile syringes, at defined time points.

2.2 | Trypan blue cell measurements

Cell numbers and viability were determined using the Trypan blue exclusion assay on a LUNA automated cell counter (Logos Biosystems, South Korea). Briefly, 10 µL of sample was incubated with 10 µL trypan blue and duplicate samples were added to the chambers of a LUNA cell counting chip before being analyzed on the automated counter.

2.3 | Flow imaging microscopy

Flow imaging measurements were performed using the PharmaFlow imaging system (JM Canty, Inc., Buffalo, New York). as shown in Figure 1a. A 1 mL sample containing cells was presented via pipette to the analyzer. A peristaltic pump was connected to this system to

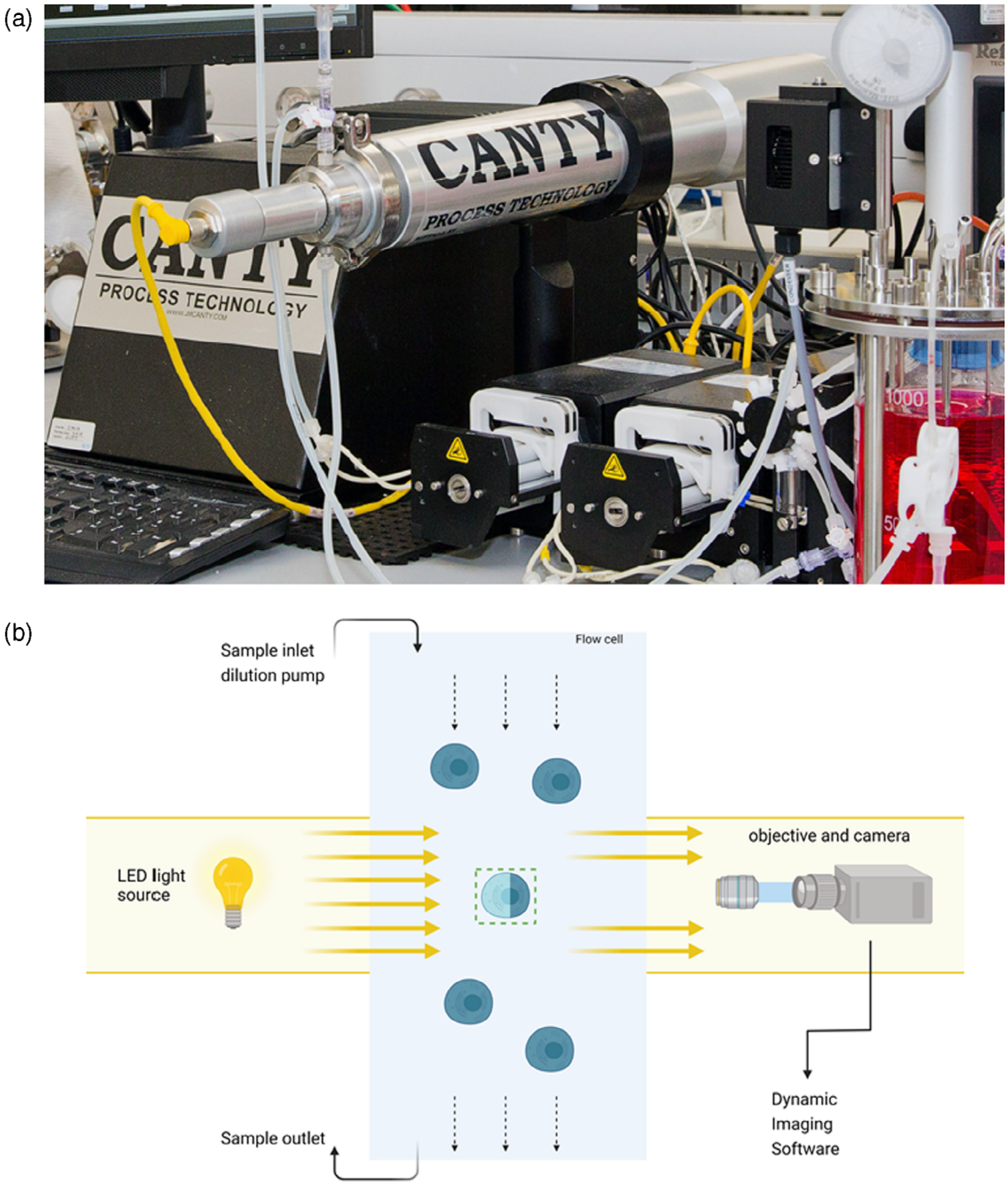


FIGURE 1 PharmaFlow imaging system. (a) Photograph of the PharmaFlow attached to 2 peristaltic pumps allowing sampling of a mammalian cell culture from a bench-top bioreactor.[25] (b) Schematic diagram showing the flow of cells between an LED light source and an objective lens. The magnified images of the cells are captured and sent digitally to the dynamic imaging software (created with BioRender.com).

provide phosphate buffered saline (PBS) solution for automated dilution. The system utilizes an AI based platform coupled with PID controller to auto adjust the pump dilution ratio to ensure cells are dispersed and not overlapping or touching during measurement analysis. The automatic dilution feature is not fixed a dilution, thus ensuring the system is capable of measuring a wide range of cell densities up to 10^8/ml. The sample containing cells is diluted to within a range of the optical imaging density required by the software for detecting the number of cells within a frame of the flow cell. The liquid flow allows a frame rate of 30–60 frames per second. Image capture is constant at that frame rate which continues until more than 1000 cells are captured, to allow a statistically significant sample per analysis. The glass on the flow cell was siliconized to prevent cell attachment. Greyscale intensity of each individual cell was measured from microscopic images taken by an 8.9 Megapixel digital CCD camera at 12× optical zoom delivering a field of view of 1315 µm × 867 µm, illuminated by an LED light source. As the sample flows through the flow cell the individual cells pass an LED light source and camera fitted

with an objective where images of individual cells are detected. Figure 1b shows a schematic of the dynamic imaging system. Cell densities were determined by the software from the number of cell images within a calibrated frame of known volume within the flow cell. Data was analyzed using the CantyVision imaging analysis (CVIA) software, which captures and displays single-cell images and dynamically converts the raw grayscale form to binary (black/white) formats, while extracting values of a set of 39 morphological descriptors. The morphological characteristics were used to assign cells as viable, necrotic or apoptotic based upon a previously developed pretrained model using a machine-learning approach in which the images of Chinese Hamster Ovary (CHO) were related to measurements of stained cell populations.[26,27]

2.4 | Apoptosis assay

Apoptosis was measured in offline bioreactor samples using the FITC Annexin V Apoptosis Detection Kit (BD) following the manufacturer's instructions. Briefly, samples at a concentration of 1×10^5 cells/mL were washed and resuspended in a binding buffer containing Annexin V and propidium iodide (PI) and incubated at room temperature in the dark for 15 min. Samples were analyzed using an Accuri C6 flow cytometer (Becton Dickinson, BD, New Jersey).

2.5 | Statistical analysis

Data analysis, calculation of mean and standard error and principal component analysis (PCA) were performed using GraphPad Prism software. Data was standardized (centred and scaled) and the component selection method was parallel analysis.

3 | RESULTS

3.1 | PharmaFlow analysis

As described in the methods section, cell samples taken from the bioreactor were analyzed on the PharmaFlow imaging system. The digital images were sent to the accompanying CantyVision software for analysis. A thresholding algorithm distinguished cells from the background providing high contrast cell images. Thresholding is the process of replacing pixels on the image of a specific intensity with either a zero or one thereby converting the image to binary.

The PharmaFlow system provides the opportunity to examine these morphological differences in more detail as an image is generated for every cell analyzed. Representative images are shown in Figure 2. Figures 2a and 2b show examples of cells classified as viable or necrotic by the system at two different timepoints (24 h and 120 h). Both methods used for measuring viability in this study classify the cells shown in Figure 2a as viable, despite the clear differences in morphology and health of the two cell populations at the two time points can be observed. This observation is expected due to the overall declining health of the cell population in the bioreactor at 120 hrs. The images of cells classified as necrotic (or non-viable) in Figure 2b are less clear, probably due to cell lysis as the membranes become damaged or broken. Figure 2c shows the breakdown of classified cells on each day of the bioreactor run by the CantyVision software. As expected, the proportion of cells considered necrotic increases over time as the proportion of viable cells decreases. The inset table in Figure 2c lists the number of cells analyzed at each timepoint from cell suspension samples (1 mL) taken from the bioreactor.

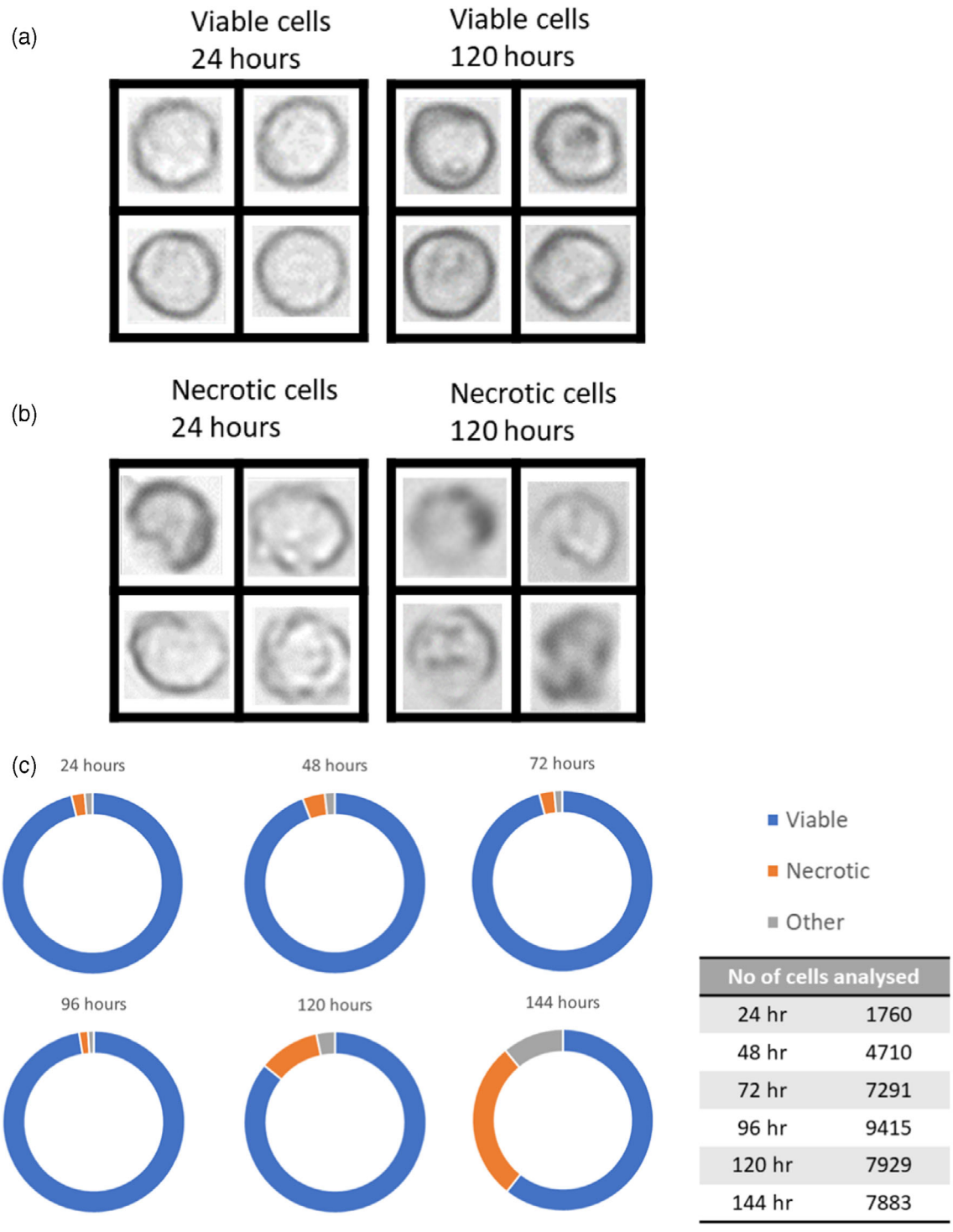
3.2 | Comparison with trypan blue measurements

In order to evaluate the capability of the PharmaFlow to accurately measure cell density and viability measurements of CHO-EG2 cells grown in a bench-top bioreactor (1 L) were taken and compared to traditional trypan blue assessment. Total cell density (TCD), viable cell density (VCD) and percentage viability were measured as described above using the trypan blue dye exclusion method and the automated cell counter (Luna), at regular intervals, over a 6-day bioreactor culture. The same information was calculated by the PharmaFlow imaging system software. Figure 3 shows the measurements from three independent bioreactor runs for total (A) and viable (B) cell density and percentage cell viability (C), comparing the measurements from the imaging software (black) to trypan blue measurements (pink). There is no notable difference between the viable cell density (VCD) measured with either method. For total cell density (TCD) the two curves are closely matched up to 96 h; after this timepoint there is a divergence in the measurements with the PharmaFlow detecting a lower total cell density. A similar divergence is apparent when measuring percentage viability.

Simple linear regression analysis was carried out to compare the measurements for PharmaFlow and trypan blue. Figure 4 shows the scatterplot of PharmaFlow measurements as a function of trypan blue measurements. For each of these analyses, 51 measurements were included. For TCD the cell densities measured ranged from 2.8×10^5 to 4.2×10^6 cells/mL and the R^2 was 0.94 (Figure 4a). For VCD the cell densities measured ranged from 2.7×10^5 to 3.9×10^6 cells/mL and the R^2 was 0.95 (Figure 4b). The percentage viability measurements ranged from 35% to 100% and the R^2 was 0.93 (Figure 4c). These analyses indicate that the PharmaFlow measurements correlated quantitatively with trypan blue for cell density and viability.

3.3 | Apoptosis estimation

The PharmaFlow classification system provides an estimation of the percentage of cell population that is apoptotic at the point of analysis. We compared this information with offline measurements of respective bioreactor samples using flow cytometry to identify Annexin-labeled cells to quantify apoptosis. These cells were positive to



No of cells analysed	
24 hr	1760
48 hr	4710
72 hr	7291
96 hr	9415
120 hr	7929
144 hr	7883

FIGURE 2 Classification of cells by imaging software. Images shown are examples of cells classified as (a) viable and (b) necrotic analyzed on Day 1 and Day 5 of bioreactor culture. (c) Proportional analysis of the number of cells classified as viable/necrotic over six days. Inset table shows the number of cells analyzed at each timepoint.

Annexin V that indicates an early stage of apoptosis but negative to propidium iodide (PI) that indicates an intact cell membrane. Figure 5a shows the percentage apoptotic cells alongside percentages of non-viable/ necrotic (PI positive) and viable as determined by the two methods. In Figure 5b it can be seen that estimations of the percentage of apoptotic cells by the PharmaFlow correlate with Annexin measurements despite higher values with the Annexin V label. However, over three bioreactor runs a higher standard error was observed with the Annexin measurements compared to those by the PharmaFlow.

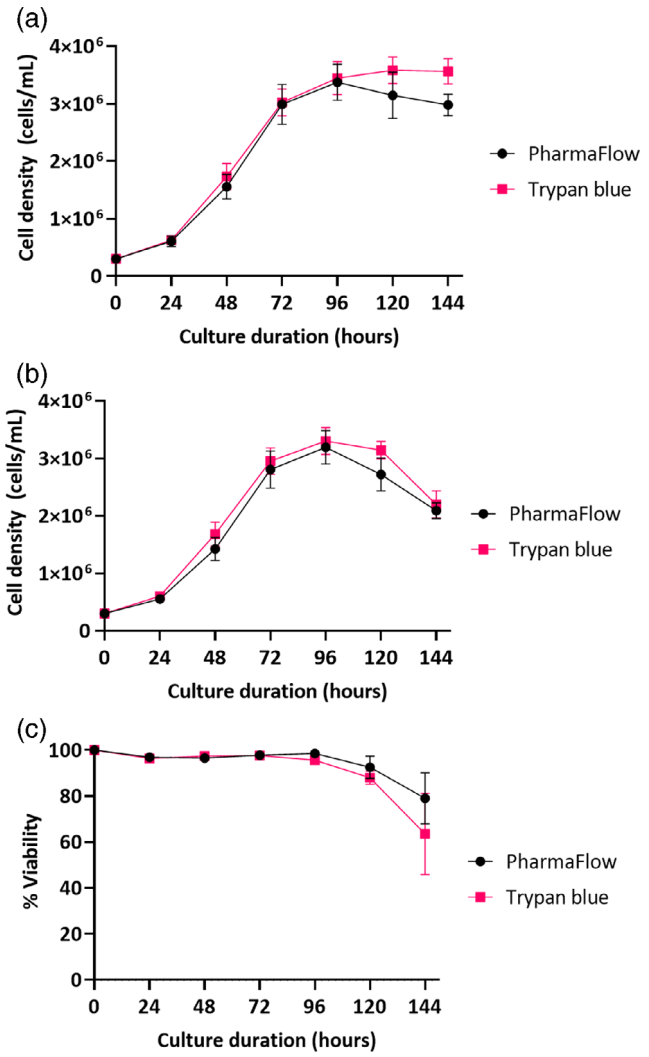


FIGURE 3 Comparison of cell density and viability between the PharmaFlow imaging system and traditional trypan blue exclusion method. (a) Total cell density, (b) Viable cell density and (c) Viability of CHO-EG2 cells over the course of a 6-day bioreactor culture (1 L) inoculated at 3×10^5 cells/ml. Results are presented as means of three independent bioreactor cultures ± standard error.

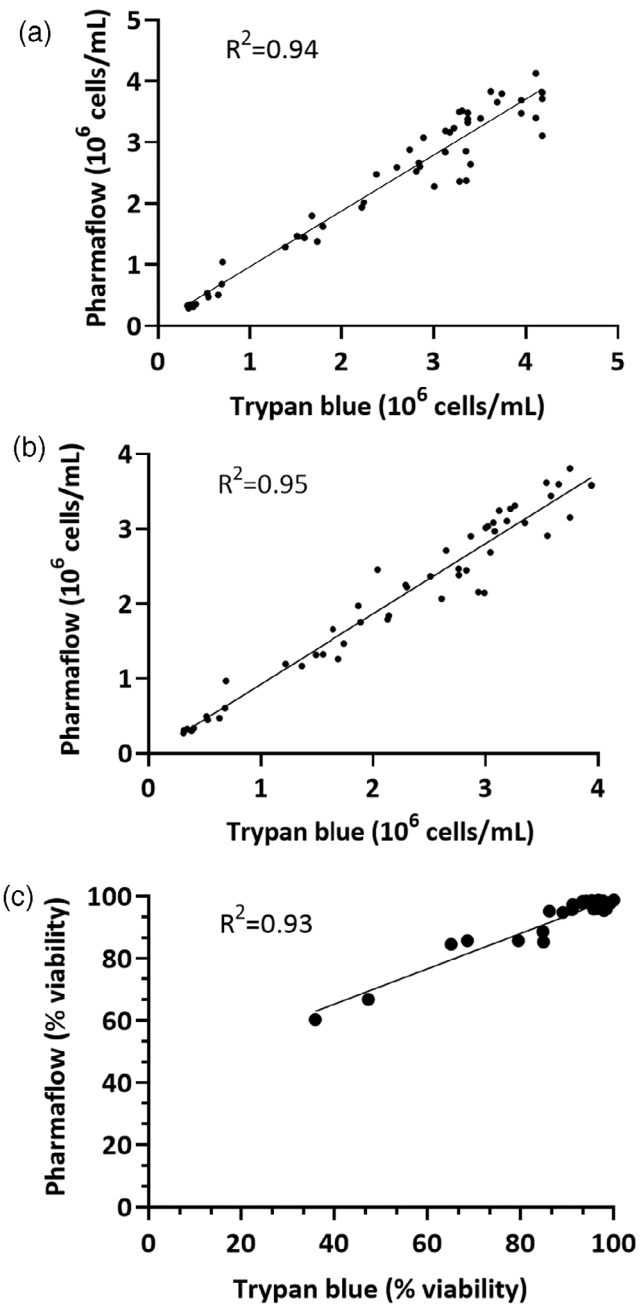


FIGURE 4 Scatterplots of PharmaFlow measurements as a function of Trypan blue measurements. (a) total cell density, (b) viable cell density and (c) viability; $n = 51$ for each graph and R^2 value is displayed.

3.4 | Principal component analysis

A large amount of data was generated by the imaging system with a range of 1760–9415 cells analyzed per sample and measurements of 39 variables gathered by the software for each cell analyzed. To better visualize this data, PCA was performed in GraphPad. For each timepoint in a bioreactor run, 36 of the variables were included for PCA. These variables were key morphological features obtained from the cell images that included cellular circularity, convexity, perimeter and hydraulic radius. The software calculated two principal components (PC1 and PC2) that contributed most highly to the variance from all the data analyzed at each time point and these were plotted against each other. The resulting scatter plots are shown in Figure 6 with viable and necrotic cells colored in green and red respectively. Over the 6-day bioreactor run the increasing number of necrotic cells can be seen. The data points from the cell population move towards the right of the plot as time progresses and may be indicative of the decreasing health of the cell population over time.

4 | DISCUSSION

There are clear drivers in the biopharmaceutical industry to establish intensive and continuous bioprocesses that require reliable monitoring

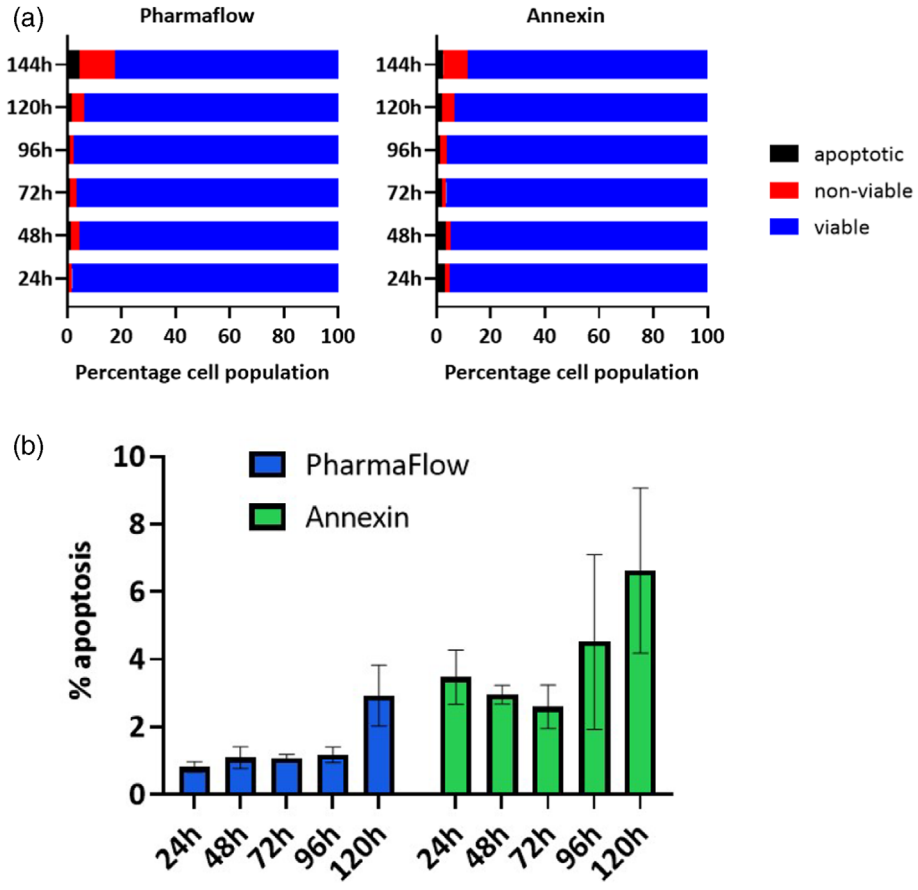


FIGURE 5 Comparison of apoptosis measurements. (a) PharmaFlow (left) and Flow cytometry with Annexin assay (right) determination of percentage of total cells that are apoptotic, non-viable or viable over each day of the bioreactor culture. The Pharmaflow classifies cells as viable, necrotic (labeled "non-viable" here and apoptotic. The annexin assay uses two stains to distinguish between groups of cells (here annexin +/PI- = "Apoptotic". Annexin −/PI- = "Viable" and Annexin +/PI+ = "Non-viable"; (b) comparison of percentage apoptotic cells at each time point for both methods.

and control of critical process parameters.[11] While some of these parameters such as temperature, agitation, pH and dissolved oxygen (DO) have been routinely controlled to defined ranges of set-points by PID controllers, others have relied upon manual sampling and off-line analysis. In the past nutrient concentrations and viable cell density have been critical measurements that have relied upon such off-line analysis. In situ sensors are ideal in that they remove the need for sampling which reduces the risk of contamination of cultures.[28] There are inherent problems associated with offline analysis caused by time delays that prevent immediate bioprocess intervention and remediation.

In this study we focused on the measurement of viable cell density. In the past this has been determined conventionally by manual cell sampling followed by counting using a hemocytometer or by an automated cell imager using trypan blue as a dye to identify cell viability. The trypan blue exclusion method is an off-line analysis to enumerate viable cells based on the membrane integrity of cells to exclude the high molecular weight dye. This method has been used for well over a century for the determination of the viability of a cell population.[6,29] Although this method is thought of as the "gold standard" for small scale mammalian cell cultures, it has several problems when applied to large-scale controlled bioreactor processes. Firstly, the process is inherently off-line, meaning that samples are taken manually, and the viability assessed by an optical counter or by microscopic counting. Secondly, membrane damage as detected by the trypan blue method is a late-stage event during the loss of cell viability. Metabolic changes associated with the gradual loss of cell viability occur much earlier than membrane damage. There are distinct advantages of being able to determine these early stages of the loss of viability in a bioprocess. Through early detection, it may be possible to replenish nutrients to extend the life-span of the culture or to choose an earlier termination of the culture to prevent the accumulation of host cell proteins that are extruded from cells once membranes are damaged.[30] Early loss of viability typically through the on-set of apoptosis may be detected by metabolic, electrochemical and morphological changes that are observed following the maximum cell density in a mammalian cell culture.[31,32]

In this article, we investigated a label-free imaging system for analyzing mammalian cells in a bioprocess. The CHO-EG2 cells were chosen as representative of antibody-producing CHO cells that are used extensively in large-scale production bioprocesses.[33,34] The cells were grown up to a maximum cell density in a controlled bench-top bioreactor. The identification of viable cells was based on detailed morphological information in the imaging system and used to determine values for viable cell density (VCD) and % viability. We compared the results obtained from the imaging system to the conventional method of trypan blue dye exclusion for cell analysis. Viable cell density was found to be directly comparable by the two methods of analysis. Total cell density and percentage viability measurements were well correlated for up to 96 h of culture by the two methods. After this timepoint there was a slight but notable divergence between the analysis

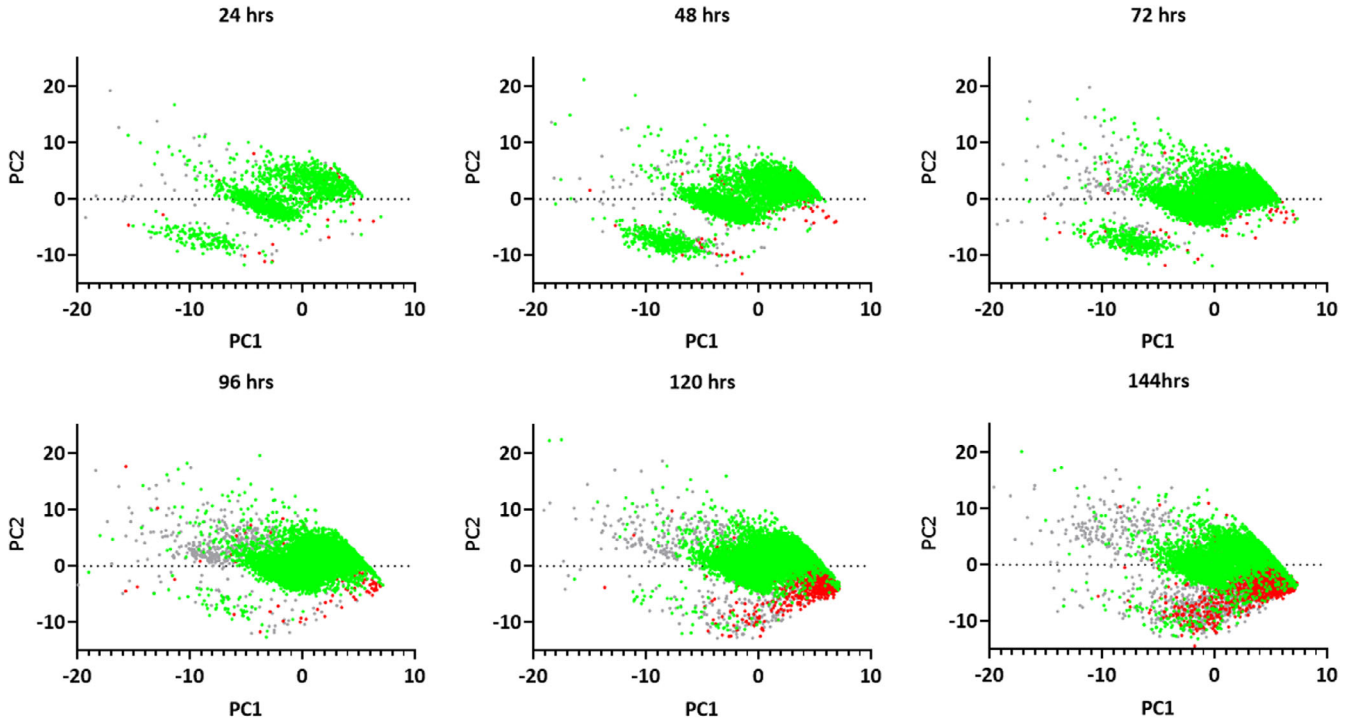


FIGURE 6 Scatter plots from principal component analysis (PCA) of a single bioreactor run over a 6-day period. Green represents viable cells; red represents necrotic cells; and all other cells are shown in gray.

methods. The divergence of viable cell density measurements after a growth phase in culture has been observed in previous studies when comparing trypan blue exclusion with other methods and attributed to an over-estimation when using membrane damage as a criterion for loss of cell viability.[35]

During the later stages of a culture, towards the end of exponential phase growth, the cell population is heterogeneous with subpopulations of cells that can be classified in various ways by staining such as viable/non-apoptotic, early apoptotic, mid-apoptotic or necrotic.[35] These stains are indicators of the various stages that cells go through from viable to non-viable. The changes during the loss of viability of cells are also associated with morphological changes that can be visualized microscopically. Most notably apoptosis is associated with blebbing of the surface and a shrinkage of cells. Autophagy can be recognized by the presence of large vacuoles, the autophagosomes. The digital imaging enabled by the PharmaFlow system records the morphological changes in multiples of individual cells by recording up to 39 measurable parameters for each cell. An estimation of the apoptotic cells by the PharmaFlow was found to be repeatable over bioreactor runs, however the actual percentage of apoptosis was slightly underestimated compared to offline measurements using the Annexin assay. Non-viable (or necrotic) cells are generally recognized as being trypan blue positive which means that the blue stain passes into the intracellular milieu through a damaged membrane. These cells are well recognized by the PharmaFlow system by the changed morphology. Thus a strong positive correlation was shown for viability measurements by the trypan blue and PharmaFlow methods.

Many optical methods developed for measuring cell growth are reliant on colorimetric or fluorometric staining. However many groups have focused recently on developing label-free optical methods. Online optical sensors have been used for a number of applications of bacterial and yeast cultures.[36,37] Optical methods have also been applied to mammalian cells in bioreactors[38] and in smaller scale flask cultures.[39,40] Optical methods that are developed for imaging cells have to overcome a number of obstacles such as the inherent heterogeneity of cell populations and the enhanced light penetration that might be necessary to obtain reasonable levels of image resolution.[41] Nevertheless some sophisticated optical methods for cell imaging have been reported in the literature, many of which describe cell analysis by 3D models such as in cancer research[42,43] or tissue engineering.[44,45] Other imaging techniques being used for analysis of cells include holographic methods,[46] microfluidics[47] and even smartphones.[48] A limitation of using some of the more sophisticated analytical techniques for bioprocessing is that real-time processing may not be possible.[49]

Real time analysis is a requirement for rapidly acquired data of the state of the cell population at-line or in-line of the bioreactor. This enables tight monitoring and control of a bioprocess as required in commercial biomanufacturing. In one recent article an imaging system applied to a mammalian cell CHO culture was described in which an imaging probe was installed in the bioreactor and immersed in the culture liquid.[50] In this system images of cells were acquired through a continuous fluid flow over the face of the probe. Although this is an attractive system for acquiring real time data it is dependent upon the

internal liquid flow based on the agitator as well as the geometry and structure of the bioreactor.

Using the imaging system (PharmaFlow) we describe for a mammalian cell culture, samples are taken directly from the bioreactor at time intervals either manually or automatically and added without modification into the flow chamber of the imaging system. Although we collected samples manually in our study the PharmaFlow can be connected directly to a bioreactor autosampler such as SegFlow™ for automatic sample delivery.[26] In our study real-time information on cell viability and density was collected alongside more detailed data that could be analyzed later. The cells were numerated and classified according to morphological characteristics based on digital imaging without staining. An advantage of using an imaging system of this type is the potential for its use as an online measurement system in which samples are removed automatically at pre-determined time intervals from the bioreactor and introduced into the flow chamber using a pump. As these samples are diluted and not returned to the bioreactor the monitoring can be termed at-line or more precisely an on-line ex-situ measurements.[49] An advantage of the dilution system is that it enables high densities of cells to be analyzed without the potential problems of overlapping or aggregated cells. The fluid flow associated with dilution as well as the siliconization of the internal surface of the flow cell minimizes cell aggregation or surface interaction. We have shown reliable analysis up to 15×10^6 cells/ml and technical specifications reporting maximum concentrations possible of up to 100×10^6 cells/ml.[25] The system has potential to be useful as an online process analytical technology tool in large-scale bioprocesses. Measurements of cell density and viability are consistent and correlate well with current conventional methods. Furthermore, the system provides much more detailed data that can be used to classify sub-populations particularly during the decline phase of a bioprocess.

Multivariate data analysis (MVDA) can be applied to large data sets to enable "deep learning". Such analysis of cell populations may provide a real-time understanding of the dynamics of sub-populations and the possibility of intervention when there are signs of decreasing cell health. MVDA techniques such as PCA have been applied previously to bioprocessing systems.[51,52] In the present study we used PCA to determine if more information about the overall health of the cells could be extracted. The analysis showed that the data points for the classification categories of viable and necrotic formed distinct groupings that shifted over the 6-day bioreactor culture. In our next article we will provide further analysis of these sub-population groups of cells.

AUTHOR CONTRIBUTIONS

Laura Breen: Conceptualization; methodology; validation; formal analysis; investigation; data curation; writing – original draft; visualization; writing – review and editing; project administration. **James Flynn:** Conceptualization; writing – original draft; investigation; visualization; writing – review and editing; methodology; validation; formal analysis; data curation. **Adam Bergin:** Investigation; conceptualization; methodology; visualization; formal analysis; data curation. **Evangelia Flampouri:** Conceptualization; investigation; methodology; visualization; formal analysis; data curation. **Michael Butler:** Conceptualization; project administration; funding acquisition; resources; writing – review and editing; methodology; supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All original data is available on request.

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REFERENCES

1. Ducommun P, Kadouri A, von Stockar U, Marison IW. On-line determination of animal cell concentration in two industrial high-density culture processes by dielectric spectroscopy. *Biotechnol Bioeng*. 2002; 77(3):316-323. doi:10.1002/bit.1197
2. Teixeira AP, Oliveira R, Alves PM, Carrondo MJT. Advances in on-line monitoring and control of mammalian cell cultures: supporting the PAT initiative. *Biotechnol Adv*. 2009;27(6):726-732. doi:10.1016/j.biotechadv.2009.05.003
3. Hanson MA, Ge X, Kostov Y, Brorson KA, Moreira AR, Rao G. Comparisons of optical pH and dissolved oxygen sensors with traditional electrochemical probes during mammalian cell culture. *Biotechnol Bioeng*. 2007;97(4):833-841. doi:10.1002/bit.21320
4. Butler M, Spearman M. Cell counting and viability measurements. In: Pörtner R, ed. *Animal Cell Biotechnology: Methods and Protocols*. Humana Press (Methods in Biotechnology); 2007:205-222. doi:10.1007/978-1-59745-399-8_8
5. Altman SA, Randers L, Rao G. Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. *Biotechnol Prog*. 1993;9(6):671-674. doi:10.1021/bp00024a017
6. Patterson MK. [11] Measurement of growth and viability of cells in culture. *Methods in Enzymology*. Academic Press (Cell Culture); 1979: 141-152. doi:10.1016/S0076-6879(79)58132-4
7. Mascotti K, McCullough J, Burger SR. HPC viability measurement: trypan blue versus acridine orange and propidium iodide. *Transfusion*. 2000;40(6):693-696. doi:10.1046/j.1537-2995.2000.40060693.x
8. Johnson S, Nguyen V, Coder D. *Assessment of Cell Viability'. Current Protocols in Cytometry*, Chapter 9; 2013:9.2.
9. Cadena-Herrera D, Esparza-de Lara JE, Ramírez-Ibañez ND, et al. Validation of three viable-cell counting methods: manual, semi-automated, and automated. *Biotechnol Rep*. 2015;7:9-16. doi:10.1016/j.btre.2015.04.004
10. Louis KS, Siegel AC. Cell viability analysis using trypan blue: manual and automated methods. *Method Molecul Biol*. 2011;740:7-12. doi:10.1007/978-1-61779-108-6_2
11. Rathore AS, Mishra S, Saxena N, Nain P. Bioprocess control: Current progress and future perspectives. *Life*. 2021;11(6):557. doi:10.3390/life11060557
12. Whitford B. Bioprocess intensification: aspirations and achievements. *BioTechniques*. 2020;69(2):84-87. doi:10.2144/btn-2020-0072

13. Kim KR, Yeo W-H. Advances in sensor developments for cell culture monitoring. *BMEMat*. 2023;1(4): e12063. doi:10.1002/bmm2.12047
14. Fung Shek C, Betenbaugh M. Taking the pulse of bioprocesses: at-line and in-line monitoring of mammalian cell cultures. *Curr Opin Biotechnol*. 2021;71:191-197. doi:10.1016/j.copbio.2021.08.007
15. Mira P, Yeh P, Hall BG. Estimating microbial population data from optical density. *PLoS One*. 2022;17(10):e0276040. doi:10.1371/journal.pone.0276040
16. Yamane T. Application of an on-line turbidimeter for the automation of fed-batch cultures. *Biotechnol Prog*. 1993;9(1):81-85. doi:10.1021/bp00019a012
17. Konstantinov KB, Pambayun R, Mataguihan R, Yoshida T, Perusicn CM, Hu WS. On-line monitoring of hybridoma cell growth using a laser turbidity sensor. *Biotechnol Bioeng*. 1992;40(11):1337-1342. doi:10.1002/bit.260401107
18. Kroll P, Stelzer IV, Herwig C. Soft sensor for monitoring biomass subpopulations in mammalian cell culture processes. *Biotechnol Lett*. 2017;39(11):1667-1673. doi:10.1007/s10529-017-2408-0
19. Bergin A, Carvell J, Butler M. Applications of bio-capacitance to cell culture manufacturing. *Biotechnol Adv*. 2022;61:108048. doi:10.1016/j.biotechadv.2022.108048
20. Austin GD, Watson RW, D'Amore T. Studies of on-line viable yeast biomass with a capacitance biomass monitor. *Biotechnol Bioeng*. 1994;43(4):337-341. doi:10.1002/bit.260430411
21. Verduijn J, van der Meeren L, Krysko DV, Skirtach AG. Deep learning with digital holographic microscopy discriminates apoptosis and necroptosis. *Cell Death Dis*. 2021;7:229. doi:10.1038/s41420-021-00616-8
22. Scholz I, Montoya C, Vela E. Examination of vesicular stomatitis virus-induced morphology changes in individual Vero cells by QMod microscopy. *BioTechniques*. 2020;68(6):305-310. doi:10.2144/btn-2019-0137
23. Sargent B. Continuous suspension cell culture monitoring in bioreactors using quantitative phase imaging. *Cell Culture Dish*. 2017; Available at: https://cellculturedish.com/continuous-suspension-cell-culture-monitoring-in-bioreactors-using-quantitative-phase-imaging/ (Accessed: 22 February 2024)
24. Zhang J, Liu X, Bell A, et al. Transient expression and purification of chimeric heavy chain antibodies. *Protein Expr Purif*. 2009;65(1):77-82. doi:10.1016/j.pep.2008.10.011
25. JM Canty. *PharmaFlow*. *JM* Canty; 2021 Available at: http://www.jmcanty.com/product/2604/ (Accessed: 2 December 2021)
26. Corredor C, Ketcham SA, McHugh K, Lane G, Vale N, Rehmann M, Both D. Process analytical technologies (PAT) applications for upstream biologics. *Am Pharm Rev*. 2022.
27. McHugh K. Correlation of Online and Offline Techniques to Measure CHO Cell Health. *Upstream Bio-Process Development*. *Bioprocessing Summit*. Cambridge HealthTech; 2021.
28. Beutel S, Henkel S. In situ sensor techniques in modern bioprocess monitoring. *Appl Microbiol Biotechnol*. 2011;91(6):1493-1505. doi:10.1007/s00253-011-3470-5
29. Pappenheimer AN. Experimental studies upon lymphocytes. *J Exp Med*. 1917;25(5):633-650.
30. Butler M, Bergin A, Braasch K, Salimi E. The case for re-evaluating cell viability in bioprocesses. *Bioproc Int*. 2019;17:11-12.
31. Afshar S, Salimi E, Fazelkhah A, Braasch K, Mishra N, Butler M. Progression of change in membrane capacitance and cytoplasm conductivity of cells during controlled starvation using dual-frequency DEP cytometry. *Anal Chim Acta*. 2019;1059:59-67. doi:10.1016/j.aca.2019.01.046
32. Fazelkhah A, Afshar S, Braasch K, et al. Cytoplasmic conductivity as a marker for bioprocess monitoring: study of Chinese hamster ovary cells under nutrient deprivation and reintroduction. *Biotechnol Bioeng*. 2019;116(11):2896-2905. doi:10.1002/bit.27115
33. O'Flaherty R, Bergin A, Flampouri E, Mota LM, Obaidi I, Quigley A, Xie Y, Butler M. Mammalian cell culture for production of recombinant proteins: a review of the critical steps in their biomanufacturing. *Biotechnol Adv*. 2020;43:107552. doi:10.1016/j.biotechadv.2020.107552
34. Villacrés C, Tayi VS, Butler M. Strategic feeding of NS0 and CHO cell cultures to control glycan profiles and immunogenic epitopes of monoclonal antibodies. *J Biotechnol*. 2021;333:49-62. doi:10.1016/j.jbiotec.2021.04.005
35. Braasch K, Nikolic-Jaric M, Cabel T, et al. The changing dielectric properties of CHO cells can be used to determine early apoptotic events in a bioprocess. *Biotechnol Bioeng*. 2013;110(11):2902-2914. doi:10.1002/bit.24976
36. Fan R, Ebrahimi M, Quitmann H, Aden M, Czermak P. An innovative optical sensor for the online monitoring and control of biomass concentration in a membrane bioreactor system for lactic acid production. *Sensors*. 2016;16(3):E411. doi:10.3390/s16030411
37. Geinitz B, Rehmann L, Büchs J, Regestein L. Noninvasive tool for optical online monitoring of individual biomass concentrations in a defined coculture. *Biotechnol Bioeng*. 2020;117(4):999-1011. doi:10.1002/bit.27256
38. Ude C, Schmidt-Hager J, Findeis M, John GT, Scheper T, Beutel S. Application of an online-biomass sensor in an optical multisensory platform prototype for growth monitoring of biotechnical relevant microorganism and cell lines in single-use shake flasks. *Sensors*. 2014;14(9):17390-17405. doi:10.3390/s140917390
39. Aijaz A, Trawinski D, McKirgan S, Parekkadan B. Non-invasive cell counting of adherent, suspended and encapsulated mammalian cells using optical density. *BioTechniques*. 2020;68(1):35-40. doi:10.2144/btn-2019-0052
40. Rue SM, Anderson PW, Miller JM, et al. Mammalian cell culture density determination using a laser through-beam sensor. *BioTechniques*. 2018;65(4):224-226. doi:10.2144/btn-2018-0059
41. Rosenberg JN, Cady NC. Surveilling cellular vital signs: toward label-free biosensors and real-time viability assays for bioprocessing. *Curr Opin Biotechnol*. 2021;71:123-129. doi:10.1016/j.copbio.2021.07.004
42. Booij TH, Price LS, Danen EHJ. 3D cell-based assays for drug screens: challenges in imaging, image analysis, and high-content analysis. *SLAS Discovery*. 2019;24(6):615-627. doi:10.1177/2472555219830087
43. Cacopardo L, Costa J, Giusti S, et al. Real-time cellular impedance monitoring and imaging of biological barriers in a dual-flow membrane bioreactor. *Biosens Bioelectron*. 2019;140:111340. doi:10.1016/j.bios.2019.111340
44. Bar A, Ruvinov E, Cohen S. Live imaging flow bioreactor for the simulation of articular cartilage regeneration after treatment with bioactive hydrogel. *Biotechnol Bioeng*. 2018;115(9):2205-2216. doi:10.1002/bit.26736
45. Schädli GN, Vetsch JR, Baumann RP, et al. Time-lapsed imaging of nanocomposite scaffolds reveals increased bone formation in dynamic compression bioreactors. *Commun Biol*. 2021;4(1):110. doi:10.1038/s42003-020-01635-4
46. Allier C, Bordy T, Cioni O, Herve L, Esteban G, Pisaneschi M, Morales S. Label-free cell viability assay using lens-free microscopy. *Imaging, Manipulation, and Analysis of Biomolecules, Cells, and Tissues XVI. Imaging, Manipulation, and Analysis of Biomolecules, Cells, and Tissues XVI*. SPIE; 2018:42-48. doi:10.1117/12.2289811
47. Lu H, Caen O, Vrignon J, et al. High throughput single cell counting in droplet-based microfluidics. *Sci Rep*. 2017;7(1):1366. doi:10.1038/s41598-017-01454-4
48. Imashiro C, Tokuoka Y, Kikuhara K, Yamada TG, Takemura K, Funahashi A. Direct cell counting using macro-scale smartphone images of cell aggregates. *IEEE Access*. 2020;8:170033-170043. doi:10.1109/ACCESS.2020.3024100

49. Lourenço ND, Lopes JA, Almeida CF, Sarraguça MC, Pinheiro HM. Bioreactor monitoring with spectroscopy and chemometrics: a review. *Anal Bioanal Chem*. 2012;404(4):1211-1237. doi:10.1007/s00216-012-6073-9
50. Wang X, Zhou G, Liang L, et al. Deep learning-based image analysis for in situ microscopic imaging of cell culture process. *Eng Appl Artif Intell*. 2024;129:107621. doi:10.1016/j.engappai.2023.107621
51. Mercier SM, Diepenbroek B, Dalm MCF, Wijffels RH, Streefland M. Multivariate data analysis as a PAT tool for early bioprocess development data. *J Biotechnol*. 2013;167(3):262-270. doi:10.1016/j.jbiotec.2013.07.006
52. Metze S, Blioch S, Matuszczyk J, et al. Multivariate data analysis of capacitance frequency scanning for online monitoring of viable cell concentrations in small-scale bioreactors. *Anal Bioanal Chem*. 2020;412(9):2089-2102. doi:10.1007/s00216-019-02096-3

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