Optimizing Cell Viability in Continuous and Batch Bioprocessing through Real-time Monitoring with Laser Force Cytology™

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LumaCyte®

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Introduction

Maintaining optimal cell health is crucial for the successful production of cell-based therapeutics and biologics. Particularly in the case of cell-based therapeutics, complex biomanufacturing methods are necessary that introduce a variety of challenges during upstream (cell culture and harvest) and downstream (purification) processing[1]. For example, in the use of continuous biomanufacturing processes, upstream process conditions, such as media components have been found to influence the heterogeneities in impurity and product profiles. As upstream conditions can be detrimental to downstream productivity and yield, inline monitoring of key cell-culture process parameters is essential to ensuring final product quality[2]. Cell stressors during upstream processing may include shear stresses and poor gas exchange in bioreactors, changes to media components, chemical treatments, and freeze thaw cycles. Changes to cells during this time can have major impacts on the final formulated product and reduce their efficacy or lead to out of trend (OOT), out of spec (OOS), or even batch failures[3]. One such critical factor influencing cell viability and productivity is oxidative stress, a condition that impacts large-scale bioprocesses due to the accumulation of reactive oxygen species (ROS)[4]. Vero cells, commonly used in vaccine production and other biomanufacturing processes, are generally considered to be a robust cell line, but even resilient cells like these can be altered by the introduction of ROS to the cell culture medium[5, 6]. In this tech note, we explore LumaCyte's label-free Laser Force Cytology™ instrument, Radiance®, as a robust tool for real-time monitoring of cell health during culture as a cell-based Process Analytical Technology (PAT). By inducing oxidative stress through the controlled addition of hydrogen peroxide, we stimulate the ROS buildup that can occur within bioreactors. LFC allows us to assess the impact of oxidative stress on cellular integrity, providing valuable insights into the adaptive responses of Vero cells beyond what can be captured using traditional cell viability assays such as trypan blue dye exclusion. The findings presented here underscore the potential of LFC to enhance process control and improve biomanufacturing outcomes by enabling more precise monitoring of cell health under stress conditions.

Experimental Results

Vero cells were seeded into 48-well tissue culture plates and subjected to oxidative stress conditions the following day using various concentrations of hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), for 24 hours. Cells were harvested after 24 hours and then measured with Radiance®. **Figure 1** shows single-cell scatter plots of velocity and eccentricity for cell populations impacted by varying levels of oxidative stress. As the H_2O_2 concentration increases, cells shift to lower velocity and increased eccentricity in the scatter plot. In order to

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compare population-wide trends across the different treatment levels, a threshold metric was applied to the scatter plots by calculating the percentage of cells within a population with a velocity greater than 2400 µm/s and eccentricity less than 0.6. As shown in **Figure 1**, 69% of the cells from this control sample are contained within the shaded region, compared to only 40% of the cells in the sample shown from the highest treatment group (450 µM H_2O_2). As the cells are interrogated by the laser in the Radiance® instrument, the cell's velocity in the fluid flow decreases proportionally to its optical force, which arises as a result of the transfer of momentum from the photons in the laser as they scatter and refract through the cell. Thus, the cell's velocity is a function of its biophysical and biochemical properties. Therefore, the changes in velocity and eccentricity seen as cells experience increasing oxidative stress indicate changes not only to the shape of the cell, but also to the optical force characteristics, likely due to changes in refractive index and other internal cellular changes.

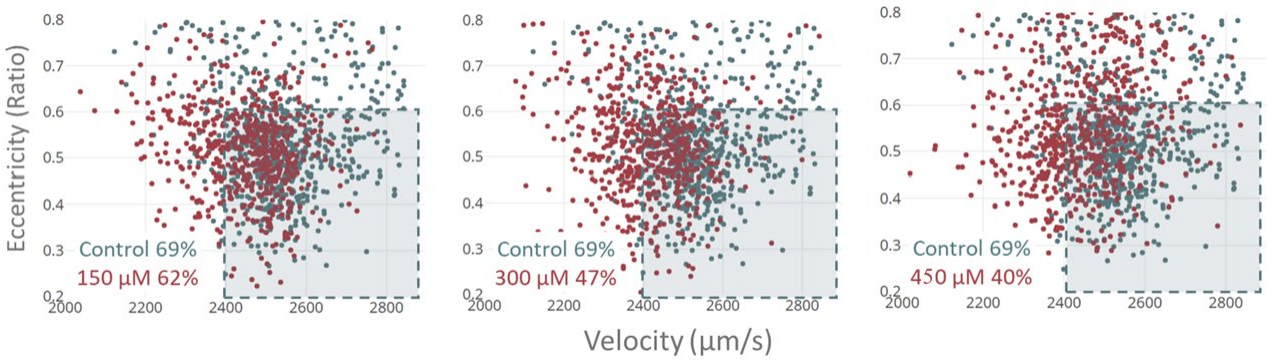

Figure 1: Scatter Plots of Vero Cells Subjected to Oxidative Stress Compared to Untreated Controls. Single cell velocity and eccentricity measurements of the individual cells measured with Radiance for each sample are used to create scatter plots. The green circles in each plot are the control Vero cells, which received no treatment. The red circles in each of the three plots are the treated cells at 150, 300, and 450 µM of H_2O_2, respectively. The graphs illustrate a decrease in velocity and increase in eccentricity as the level of treatment increases, reducing the percentage of cells within the shaded threshold region (Velocity >2400 µM/s, Eccentricity <0.6).

Changes in the velocity-eccentricity threshold metric are shown in **Figure 2** and illustrate the progression of the metric across treated groups. Higher levels of ROS treatment correspond to a continually decreased percentage of cells within the threshold metric region, indicating the capability of Radiance to not only detect insults to the cells, but also continually quantify cell conditions as they deteriorate due to oxidative stress. As mentioned above, the decrease in velocity observed is related to an increase in exerted optical force on the cell by the laser due most likely to the cellular components within the cell changing its refractive index. To associate changes seen in Radiance metrics, orthogonal assays were performed to determine whether there were shifts in the viability of the cells or changes in the state of apoptosis. Cells collected at 24 hours were also measured using a Caspase-3 activity assay to assess apoptotic activity as well as trypan blue exclusion for cell viability.

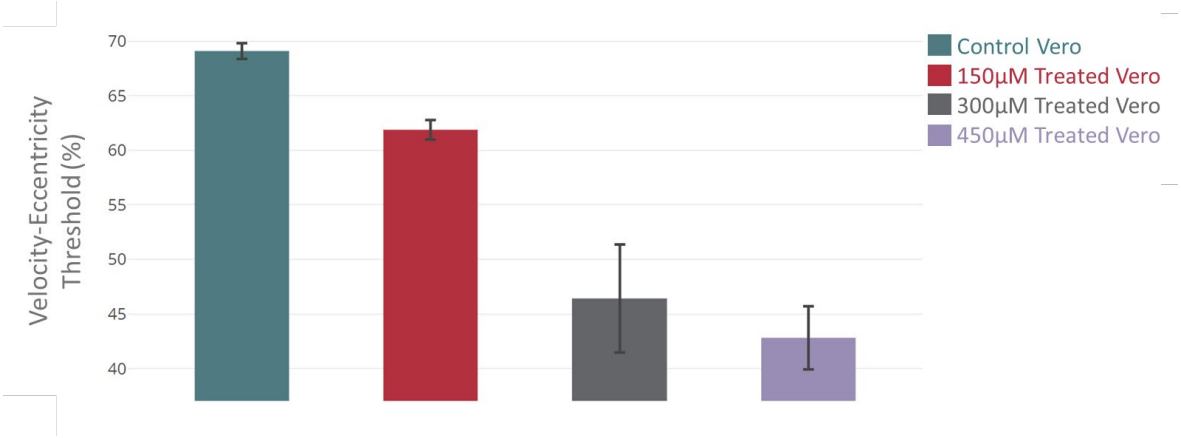

Figure 2: **Velocity and Eccentricity Threshold Metric Comparing Populations of Control Cells to Oxidative Stressed Cells.** The threshold metric was created to represent the percentage of cells within the population sample with a velocity greater than 2400 µm/s and an eccentricity greater than 0.6. As the level of H_2O_2 treatment increases from 150 µM up to 450 µM, the threshold metric continually decreases. The control population has 69% of cells falling within the threshold region, whereas the highest treatment group only has 43% of cells falling in this range. Error bars represent one standard deviation of the mean across replicates (N=3).

Cell viability is one common method for assessing cell health in biomanufacturing processes[7, 8]. However, cell viability alone is not always an accurate representation of cell health or potency in the biomanufacturing of cell therapeutic products[1]. In **Table 1**, trypan blue cell viability measurements for each of the conditions are compared to the corresponding Velocity-Eccentricity metric data measured by Radiance. There were no significant changes in cell viability seen in the 150 µM and 300 µM conditions when compared to the untreated control. However, the 450 µM condition saw a marked decrease in cell viability, measuring in at 55.82% versus 93.01% for the untreated control. This indicates that cell viability is impacted by oxidative stress, but at this timepoint it is only measurable in cells treated with the highest concentrations of H_2O_2. To further investigate the cell health effects of the 150 µM and 300 µM conditions, a fluorometric caspase-3 assay was also performed for each of the sample conditions. While the viability measurements did not change until the cells were exposed to 450 µM H_2O_2, both caspase-3 activity and the Radiance Velocity-Eccentricity metric showed significant changes from the untreated control in all conditions, even at the lowest concentration of H_2O_2. As shown in **Figure 3**, Radiance metrics demonstrated very similar changes to the caspase-3, with the added benefit of rapid time to result without the need for cell lysis, cell markers or labeling, or any additional reagents. This allows a more in-depth analysis of the cells as they would be in their cultured habitat and provides additional information beyond trypan blue viability measurements.

Sample	Trypan Blue Cell Viability (%)	Velocity-Eccentricity Threshold Metric (%)	Caspase-3 Activity (nmol AMC)
Control	93 ± 6	69 ± 0.7	0.10 ± 0.01
150 µM H_2O_2	89 ± 4	62 ± 0.9	0.12 ± 0.01
300 µM H_2O_2	90 ± 5	46 ± 5.0	0.15 ± 0.01
450 µM H_2O_2	56 ± 3	43 ± 2.9	0.16 ± 0.02

Table 1. Comparison of cell health measurements taken after 24 hours of H_2O_2 treatment. Cell viability (%), Radiance Velocity-Eccentricity metric (%), and caspase-3 activity (nmol of AMC) are compared across conditions. Cell viability shows minimal change in the 150 µM and 300 µM conditions, but drops significantly with the highest level of treatment, 450 µM. The Radiance metric and caspase-3 activity show significant changes from the untreated control at all conditions, with progressively increasing measurements as the level of treatment increases.

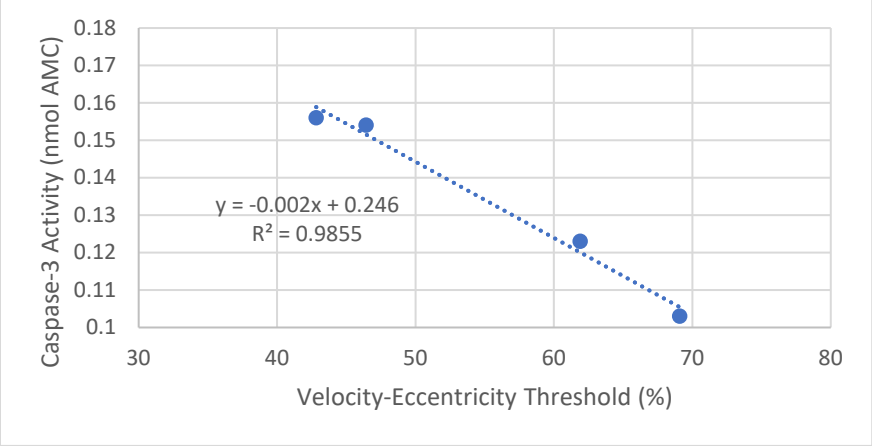

Figure 3. Correlation of cell health measurements taken after 24 hours of H_2O_2 treatment. Radiance Velocity-Eccentricity metric (%), and caspase-3 activity (nmol of AMC) are compared across conditions. The control samples are the farthest right point on the graph and the highest treatment condition (450 µM) is the farthest left point on the graph. Both the Caspase-3 assay and Radiance metrics show consistent changes between conditions, indicating the ability of Radiance to detect subtle changes to cells, such as those that occur during early stages of apoptosis. The coefficient of determination (R^2) comparing the metrics is 0.9855, showing a very strong correlation between the two parameters.

To further examine the capabilities of Radiance as a PAT in the biomanufacturing of cell-based therapeutic products, additional studies were performed to measure Vero cells subjected to ROS at timepoints shorter than 24 hours. Briefly, Vero cells were seeded in 48 well plates and H_2O_2 was added at concentrations of 750 and 1500 µM. At 2, 5, and 24 hours post treatment, cells were harvested and analyzed with the Radiance. Principal Component Analysis (PCA), a visualization tool for multivariate data, was performed using all the LFC metrics collected by Radiance. The PCA plot seen in **Figure 4** shows the capability of Radiance to distinguish healthy, control cells from cells subjected to oxidative stress even as quickly as 2 hours post H_2O_2 exposure. There is a clear separation between the untreated and hydrogen peroxide treated cells in PC1 highlighting the ability of Radiance to be used as a rapid PAT by identifying "In Spec" parameters for healthy cells, as illustrated by the ellipse drawn with a 3 standard deviation confidence interval. Furthermore, there is separation in PC2 between the treated samples, showing a further progression of the treated cells as they are exposed to stressed conditions for

longer periods of time. This illustrates the ability of LFC-based analysis to detect as well as track cellular changes throughout a process.

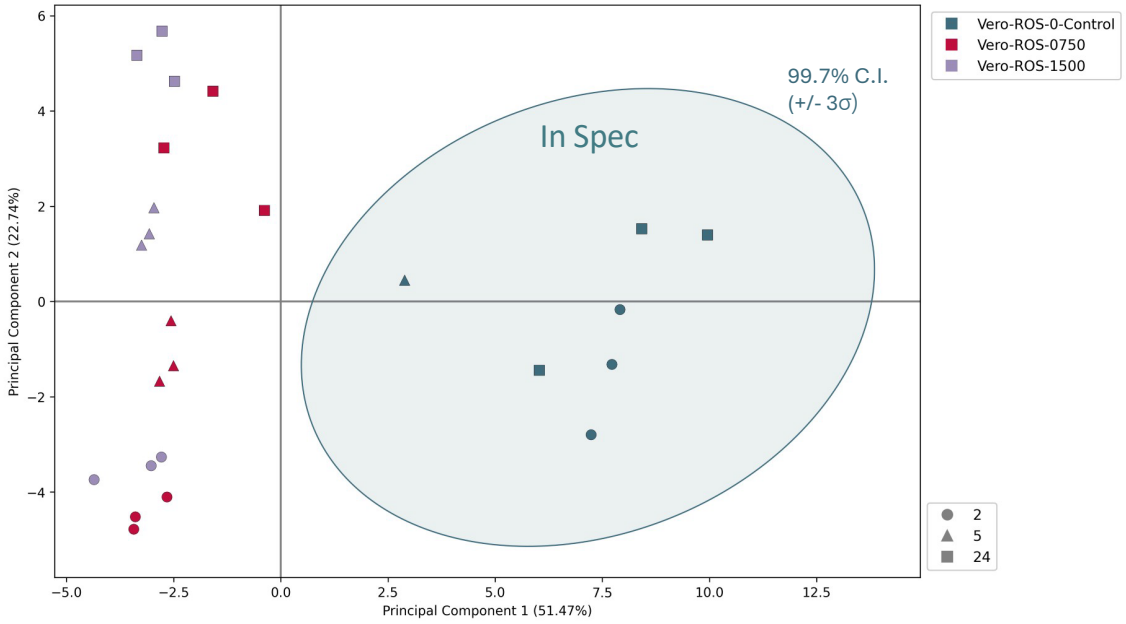

Figure 4: **Principal Component Analysis (PCA) Plot of Radiance Metrics for Vero Cells After Treatment with H_2O_2 from 2-24 hours.** The PCA uses all the LFC parameters collected from Radiance. The green shapes indicate the control samples, and the green ellipse represents theoretical boundaries to determine whether cells within a given bioprocess are in spec or out of spec. The 750 µM (grey shapes) and 1500 µM (red shapes) treated groups are clearly separated from the controls in PC1 and would be outside of a confidence interval of 3 standard deviations as indicated by the green ellipse even after only 2 hours of exposure to H_2O_2. There is a progression of treated cells with time of exposure seen in PC2 as the treated cells collected at 2, 5, and 24 hours cluster together in groups generally according to timepoint, highlighting the capability of Radiance as a PAT to capture early cellular changes.

Discussion

In this study, Laser Force Cytology was used to evaluate the effects of oxidative stress on Vero cells induced by H_2O_2. The application of LFC enabled real-time, non-invasive monitoring of cellular health and provided insights into cellular dynamics under oxidative stress. LFC metrics changed significantly in response to the H_2O_2 concentration and showed strong correlation with the caspase-3 activity assay. This appears to be a more sensitive measure of cell health than traditional viability assays, as after 24 hours of exposure, the lower concentrations of H_2O_2 has low to no impact on cell viability based on Typan blue exclusion assay. Compared to traditional methods, such as cell viability or protein assays, for cell health assessment, LFC offers distinct advantages by allowing live-cell analysis without the need for staining, lysis, or fixation. Most traditional assays are endpoint measurements, whereas LFC supports dynamic and continuous monitoring of cell health throughout bioprocess applications. This study highlights the potential of LFC as a robust tool for real-time monitoring of oxidative stress or similar stressors of cells throughout a biomanufacturing process. The insights gained through integration of LFC measurements as a PAT could inform strategies to optimize cell culture conditions, minimize cell stressors, and enhance process stability and predictability.

Cell Culture and Experimental Methods

For the first part of the experiment, Vero cells were seeded in a 48-well plate at a density of 8E4 cells/well in MEM-α media (Gibco-12571-048) supplemented with 10% fetal bovine serum (Corning-35-016-CV). Cells were allowed to grow overnight, incubated at 37°C in a humidified incubator with 5% CO2, and then treated with H_2O_2 (Thermo Scientific-426001000) for 24 hours at concentrations of 150 µM, 300 µM, and 450 µM plus a control receiving no treatment. After 24 hours, cells to be analyzed with Radiance were harvested by removing the cell culture medium, then adding 100 µL of TrypLE (Gibco-12604-021) and collecting the cells in centrifuge tubes. The cell samples were then centrifuged at 300g for 5 minutes, resuspended in 200 µl of LumaCyte Stabilization Buffer and transferred to a Radiance 96-well plate for analysis with the Radiance instrument. Cell viability was also measured from samples immediately after dissociation from the plate by collecting a 10 µl sample, mixing 1:1 with trypan blue (Corning-25-900-CI) and counting using a hemacytometer. Cells to be used for Caspase-3 activity assay remained adhered to the 48 well plates following 24 hours of treatment with H_2O_2. A Caspase-3 Fluorometric Assay Kit from Sigma Aldrich (CASP3F) was used to measure caspase activity per the manufacturer's protocol. In brief, cell culture medium was removed and 200 µl lysis buffer was added to the cells for 20 minutes on ice. 50 µl of assay buffer containing substrate was then added to each well and mixed thoroughly, then 200 µl was transferred to a 96-well fluorimeter plate. Substrate blanks and Caspase 3 positive controls were used, and plates were read on a Multiplate Reader (Spectramax iD3) every 10 minutes for 60 minutes. A standard curve was also made using the AMC standard provided with the kit and used to calculate nmol of AMC per 200 µl of buffer. For additional information, refer to the CASP3F Product Information Sheet provided on the Sigma Aldrich website. For the impact of oxidative stress over time experiment seen in Figure 3, Vero cells were seeded in a 24-well plate at a density of 1E5 cells/well in MEM-α media (Gibco-12571-048) supplemented with 10% fetal bovine serum (Corning-35-016-CV). Cells were allowed to grow overnight, incubated at 37°C in a humidified incubator with 5% CO2, and then treated with H_2O_2 for 2, 5, or 24 hours at concentrations of 750 µM and 1500 µM plus a control receiving no treatment. At each timepoint, cells to be analyzed with Radiance were harvested by removing cell media, then adding 250 µL of TrypLE (Gibco-12604-021) and collecting in centrifuge tubes. The cell samples were then centrifuged at 300g for 5 minutes, resuspended in 200 µl of LumaCyte Stabilization Buffer and transferred to a Radiance 96-well plate for analysis with the Radiance instrument.

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