RESEARCH ARTICLE

A novel LED-based 2D-fluorescence spectroscopy system for in-line bioprocess monitoring of Chinese hamster ovary cell cultivations—Part II

Jens Claßen¹ | Alexander Graf² | Florian Aupert¹ | Dörte Solle¹ | Marek Höhse² | Thomas Scheper¹

¹Institute of Technical Chemistry, Gottfried Wilhelm Leibniz University of Hannover, Hannover, Germany

²Sartorius Stedim Biotech GmbH, Göttingen, Germany

Correspondence

Dörte Solle, Institute of Technical Chemistry, Gottfried Wilhelm Leibniz University of Hannover, Callinstraße 5, 30167 Hannover, Germany.

Email: solle@iftc.uni-hannover.de

This study was performed in order to evaluate a new LED-based 2D-fluorescence spectrometer for in-line bioprocess monitoring of Chinese hamster ovary (CHO) cell culture processes. The new spectrometer used selected excitation wavelengths of 280, 365, and 455 nm to collect spectral data from six 10-L fed-batch processes. The technique provides data on various fluorescent compounds from the cultivation medium as well as from cell metabolism. In addition, scattered light offers information about the cultivation status. Multivariate data analysis tools were applied to analyze the large data sets of the collected fluorescence spectra. First, principal component analysis was used to accomplish an overview of all spectral data from all six CHO cultivations. Partial least square regression models were developed to correlate 2D-fluorescence spectral data with selected critical process variables as offline reference values. A separate independent fed-batch process was used for model validation and prediction. An almost continuous in-line bioprocess monitoring was realized because 2D-fluorescence spectra were collected every 10 min during the whole cultivation. The new 2D-fluorescence device demonstrates the significant potential for accurate prediction of the total cell count, viable cell count, and the cell viability. The results strongly indicated that the technique is particularly capable to distinguish between different cell statuses inside the bioreactor. In addition, spectral data provided information about the lactate metabolism shift and cellular respiration during the cultivation process. Overall, the 2D-fluorescence device is a highly sensitive tool for process analytical technology applications in mammalian cell cultures.

KEYWORDS

2D-fluorescence spectroscopy, bioprocess monitoring, CHO cell cultivation, multivariate data analysis, PAT

Abbreviations: CHO, Chinese hamster ovary; CQA, critical quality attribute; FAD, flavin adenine dinucleotide; FDA, Food and Drug Administration; MVDA, multivariate data analysis; NAD(P)H, nicotinamide adenine dinucleotide; PAT, process analytical technology; PC, principal component; PCA, principal component analysis; PLS, partial least square; RFI, relative fluorescence intensity; RMSEC, root mean square error of calibration; RMSEcv, root mean square error of cross-validation;

1 | INTRODUCTION

Chinese hamster ovary (CHO) cells are the most commonly used mammalian cell system in industrial processes for the production of therapeutic proteins [1]. This

RMSEP, root mean square error of prediction; TCC, total cell concentration; VCC, viable cell concentration.

well-characterized cell type has been used for the production of more than 100 biopharmaceuticals such as monoclonal antibodies. Seven of the top 10 selling biopharmaceuticals are manufactured in CHO cells and the biopharmaceutical market has an average yearly growth of 35% since 2001 [2].

In upstream processing, stirred tank bioreactors over 10 000 L scale are utilized for suspension cultures of recombinant CHO cells. In comparison to bacteria and yeasts, CHO cells are very sensitive to many variables in the cultivation process. Therefore, bioprocess monitoring of mammalian cell cultivation has a particular important role in the commercial production of biopharmaceuticals. However, several relevant process variables are not measured in standard upstream processes.

The process analytical technology (PAT) initiative of the United States Food and Drug Administration has motivated biopharmaceutical companies to intensify process understanding and process control during development and manufacturing with the goal of ensuring final product quality [3]. The PAT guidance entails the challenge to monitor the critical process variables (e.g., biomass, metabolites) and to control all critical process parameters during a bioprocess that influences the critical quality attributes (CQAs) of the final product (e.g., therapeutic proteins) [4]. Spectroscopic sensors are one of the most powerful tools within the initiative for in-line bioprocess monitoring of several relevant variables [5]. In-line measurements are particularly important to facilitate early process fault detection, minimize the risk of contamination, and observe real-time product release. By these sensors, an efficient process control of several process variables is accessible simultaneously with no delay between sampling and analysis [6].

Several optical sensors with different wavelength ranges, such as ultraviolet–visible (UV–Vis), near-infrared (NIR), mid-infrared (MIR), Raman, and fluorescence spectroscopy are in research for industrial application for the past decades [5]. These sensors enable simultaneous noninvasive, in-line, and online bioprocess monitoring of various process variables including biological and chemical variables during cell cultivation processes [7]. Each technique or rather each wavelength range has their own advantages and disadvantages.

Fluorescence spectroscopy is a highly sensitive technique and has been used to monitor upstream processes for more than 30 years [8]. It uses the special characteristic of fluorescent compounds called fluorophores, which emit light of a larger wavelength upon light excitation. Each fluorophore has maximum fluorescence intensity at a specific excitation/emission (λ_{exc} / λ_{em}) wavelength pairs. In bioprocess monitoring, several important biological components involved in cellular growth and metabolic activity operate as fluorophores such as proteins with certain amino acids (tryptophan, tyrosine, and phenylalanine), NAD(P)H,

PRACTICAL APPLICATION

The use of spectroscopic sensors for bioprocess monitoring is a powerful tool within the process analytical technology (PAT) initiative of the Food and Drug Administration. In-line measurements are particularly important during cost intensive manufacturing of biopharmaceuticals in order to facilitate early process fault detection, real-time product release, and minimize the risk of contaminations. As shown in this research article, the newly developed LED-based 2D-fluorescence spectrometer is a highly sensitive PAT tool for monitoring mammalian cell culture processes. The system enables a deep insight into the bioreactor and provides information about the cell concentration, cell viability, cell metabolism, and cellular respiration within the cultivation. In summary, this research article demonstrates the great potential of the new device for bioprocess monitoring and for the implementation of the PAT concept.

pyruvate, pyridoxines, co-enzymes, vitamins, and flavins [8,9]. Thus, fluorescence spectra provide significant information about critical process variables such as the cell count, cell viability, cell metabolism, and products during the cultivation process [10]. However, the interpretation of fluorescence data is not always easy because fluorescence interference with medium components or the incidence of inner filter effects, cascade effects, and quenching processes [11]. Multivariate data analysis (MVDA) has to be used to analyze large data sets of fluorescence spectra [5,12,13]. Principal component analysis (PCA) is commonly used for a qualitative approach to interpret data, detect outliers, and to extract relevant information [12]. Partial least square (PLS) regression is a quantitative method that needs reference data for model calibration and determines correlations between spectral data with relevant bioprocess variables.

A broad overview about many applications of fluorescence spectroscopy in bioprocess monitoring is given in the literature [8,10,14–16]. 2D-Fluorescence spectroscopy allows the measurement of several fluorophores simultaneously by using multiple combinations of excitation and emission wavelengths. In most applications, the BioView® instrument (Delta, Denmark) has been used to obtain spectral data [13]. This 2D-fluorescence spectrometer uses filter wheels for the multiple combinations of excitation and emission wavelengths. The disadvantages of this 2D-fluorescence spectrometer are the poor reproducibility of the spectral data resulting in batch to batch differences and reduced prediction accuracy. The filter wheels limit the sensitivity and dynamic range of the system [10,16]. Moreover, due to the

wear of moving parts and filter aging the operating life of the BioView® device is reduced.

The focus of the present work was the evaluation of a new prototype 2D-fluorescence spectrometer and probe for in-line monitoring of CHO cell cultivation processes. During development, relevant excitation wavelengths were selected for monitoring of CHO cell cultivations. For each excitation wavelength, the whole emission spectra are detected by a CCD detector. Therefore, the instrument is free from any moving part components.

PCA of the fluorescence data demonstrate the reproducibility of measurements, the interprocess variances from different cultivations, and the changes in the fluorescence during the cultivations. PLS regression generates quantitative predictions of relevant process variables representing the total cell count, viable cell count, viability, dead cell count, and cell metabolism in CHO cell cultivations. The new approach of 2D-fluorescence spectroscopy is discussed and advantages of this spectrometer are presented in this work.

2 | MATERIALS AND METHODS

2.1 | Cell line and culture conditions

A CHO-K1 cell line (Cell Culture Technology, University of Bielefeld, Germany) was cultivated in chemically-defined, serum-free, and protein-free CHOMACS CD® medium (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). A 200 mM stock solution of L-glutamine was diluted in the medium to maintain a start concentration of 8 mM.

The inoculum was prepared in three passages incubated in 5% CO₂/air mixture, 95% relative humidity at 37°C on a shaking platform (150 rpm). The cultures were expanded every 3 days in larger shake flasks from 25 mL up to 150 mL with an initial seeding concentration of 4×10^5 cells/mL.

Cultivations were performed in a 10-L bioreactor (Biostat® Cplus, Sartorius Stedim Biotech GmbH, Göttingen, Germany) with a maximum working volume of 7.5 L. Exponentially growing cells with a cell density of $6\text{--}9 \times 10^6$ cells/mL in shake flasks were used as an inoculum to get an initial cell concentration of 4×10^5 cells/mL in the bioreactor. Bioreactor cultures were maintained at 37°C, pH 7.1, 40% dissolved oxygen via ring sparger with an air-oxygen mixture, and 100–200 rpm agitation speed (rushton turbine). The pH was maintained by automatic addition of 1 M Na₂CO₃ or by aeration with CO₂. The feeding was implemented by CHOMACS feed medium (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) enriched with 20 g/L glucose and a total glutamine concentration of 37 mM. A volume of 315 mL was added to the culture once a day from day 3 onward. In the last cultivation (cultivation 6), an automated glucose control based on at-line measurements with

TABLE 1 Overview of the CHO cell cultivation processes

Cultivation	Process condition	Batch duration	Notes
1	Fed-batch	238 h	None
2	Fed-batch	189 h	None
3	Fed-batch	210 h	None
4	Fed-batch	189 h	None
5	Fed-batch	264 h	Introduced oxygen limitation of 15 h
6	Fed-batch	232 h	Automated glucose control, glucose is kept at 1 g/L; 6 h without stirring

a BioPAT® Trace analyzer system (Sartorius Stedim Biotech GmbH, Göttingen, Germany) was additionally implemented to realize a glucose concentration nearly at 1 g/L by feeding a volume of 50 mL, if the glucose measurement was below 1 g/L. In one cultivation process (cultivation 5), an oxygen limitation of 15 h was introduced to study changes in cell metabolisms. In another cultivation (cultivation 6), a technical problem represented a lack of stirring. All cultivations were terminated when the cell viability was less than 60%. Samples (10 mL) were collected every 4 h with an autosampler system. The samples were stored in a rack at 4°C and analyzed once a day. Table 1 gives an overview of the six CHO cell cultivation processes.

2.2 | Offline reference measurements

The viability and total and viable cell concentrations were counted using a Cedex (Roche Innovatis AG, Penzberg, Germany) based on a trypan blue staining (0.5% (w/v) trypan blue in phosphate buffer). For further analysis all samples were centrifuged (0.3 rcf, 5 min) to remove cells and particles. Glucose, lactate, glutamine, and glutamate concentrations in the supernatant were measured using an YSI 2950 biochemistry analyzer (YSI Inc., Yellow Spring, OH, USA). All determinations were conducted in triplicate. The offline data and spectral data are stored in <https://doi.org/10.25835/0005774>.

2.3 | 2D-Fluorescence spectroscopy

The 2D-fluorescence spectral data were collected with a spectrometer (J&M Analytik AG, Essingen, Germany) consisted of three high power LED with excitation wavelength of 280, 365, and 455 nm (full widths at half maximum between 10 and 16 nm) and a CCD sensor (Tidas OEM 800, J&M Analytik AG, Essingen, Germany) as detector. Each LED could be individually controlled without the use of a multiplexer and is connected to the probe (Art Photonics GmbH, Berlin, Germany) via an optical fiber. In addition, further fibers lead back from each LED to a photodiode for feedback

FIGURE 1 2D-Fluorescence spectrometer consists of three LEDs with $\lambda_{\text{exc}} = 280, 365$, and 455 nm and a CCD sensor. The probe is directly connected to the bioreactor

control of the LEDs light intensity. In the middle of the fluorescence probe, a central fiber is installed. The central fiber leads the emitted light to the spectrometer. Moreover, the probe was manufactured in such way that the in-situ sterilization was possible directly within a 25 mm Ingold® port of the bioreactor. Figure 1 shows a schematic view of the 2D-fluorescence device.

The software TIDASDAQ (J&M Analytik AG, Essingen, Germany) was used for operating the 2D-fluorescence spectrometer. With every measurement, a spectrum from each LED for the emission wavelength ranges from 180.5 to 997.5 nm in 4 nm steps was collected. 2D-Fluorescence spectra were collected continuously every 10 min during CHO cultivation and contained 615 wavelength combinations, 205 emission measurements for 280 nm excitation, 205 for 365 nm, and 205 for 455 nm.

2.4 | Multivariate data analysis

Spectral data were analyzed with SIMCA 15 (Sartorius Stedim Data Analytics, Umeå, Sweden). Data preprocessing was not necessary, because of the high reproducibility of the fluorescence and clearly defined signals. Only some wavelengths ($\lambda_{\text{exc}}: 365 / \lambda_{\text{em}} = 360.5 - 385$; $\lambda_{\text{exc}}: 455 / \lambda_{\text{em}} = 433 - 477$) of the Rayleigh scattering were excluded due to saturation. The unsaturated flanks of the Rayleigh scattering were not excluded as they include information on cell concentration. PCA was applied as a qualitative approach to identify spectral outliers and extract relevant information to determine the measurement variance of different cultivations. PLS regression was applied as quantitative method to correlate reference

data with spectral data. Each offline sample (4 h interval) was aligned to the spectra (10-min interval) right before the sample taking time point. Calibration models were built based on the data of five cultivations for several process variables (total cell concentration, viable cell concentration, and cell viability) and a sixth external cultivation data set was used to predict these variables as validation to examine the robustness of the PLS models. Reference data of the total cell count were log-transformed for the PLS model. The performances of each model were valued by R^2 and Q^2 respectively as well as the root mean square error of calibration (RMSEC), from cross-validation (RMSEcv) and prediction (RMSEP).

$$\text{RMSEC} = \sqrt{\sum \frac{(y_i - y_{\text{cal}})^2}{N}}$$

$$\text{RMSEcv} = \sqrt{\sum \frac{(y_i - y_{\text{cvpred}})^2}{N}}$$

$$\text{RMSEP} = \sqrt{\sum \frac{(y_i - y_{\text{pred}})^2}{N}}$$

where y_i is the observed value, y_{cal} is the value calculated by the calibration model, y_{cvpred} is the value by five-fold cross validation, y_{pred} is the value predicted by the model for an external data set, and N declares the number of samples used for the model.

3 | RESULTS AND DISCUSSION

3.1 | CHO cultivations and analysis of 2D-fluorescence spectra

The data derived from six fed-batch cultivations with two different feeding strategies were used in these investigations (Table 1). Figure 2A–D shows the batch to batch variances for the TCC, VCC, viability, and lactate concentration. Apart from batch 5, all the trajectories are very similar. Only in the course of the lactate, there are greater differences within the individual cultivations. TCC increases during the first 140 h up to 32×10^6 cells/mL. After that, the VCC decreased to a viable cell count of approximately 15×10^6 cells/mL at nearly 200 h. The viability is constant over 90% for the initial 136 h and drops subsequently down to less than 60% at the end of the cultivation. In fed-batch 5, a drastically decrease of the viability between 30 and 68 h is recognized, coming along with a reduction of the cell growth. This depends on an introduced oxygen limitation at a batch age of 26 h. The initial oxygen concentration in the bioreactor was consumed after 26 h and the oxygen control was started after 41 h. In all cultivations, the lactate concentration increases strongly during the exponential growth phase up to a concentration of

FIGURE 2 Offline measurements of all six CHO cultivations (Fed-batch 1–6): (A) TCC; (B) VCC; (C) viability; (D) lactate concentrations

4–5 g/L and afterward, there is a metabolic shift to metabolization of lactate as an alternative carbon source to glucose. Lactate is a product of an incomplete oxidative metabolism by rapid glucose consumption in highly proliferating CHO cells [17]. Thus, there are two significant changes during the cultivation process: At first the metabolic shift within the cells and later starts the decrease of the viability, cell lysis begins, and intracellular compounds are released into the culture media [18].

An important factor to evaluate the new 2D-fluorescence spectrometer for in-line monitoring of CHO cultivations is the comparability of a good data set from several cultivations. Exemplarily for all cultivations the emission spectra for each excitation wavelength of cultivation 3 are shown in Figure 3. Mammalian cells are incapable of synthesizing these fluorescent essential amino acids. Thus, CHO cells metabolize these amino acids for biomass synthesis and for protein production. Both effects reduce the fluorescence intensity of the amino acids during cultivation. The fluorescence intensity of the tyrosine ($\lambda_{\text{exc}}/\lambda_{\text{em}} = 280/303$ nm), tryptophan ($\lambda_{\text{exc}}/\lambda_{\text{em}} = 280/340$ nm), and the vitamin pyridoxine ($\lambda_{\text{exc}}/\lambda_{\text{em}} = 280/389$ nm) decrease during exponential growth phase, because the amino acid integration into proteins changes the fluorescence intensity depending on its placement within the protein structure [19]. A hydrophilic environment or the presence of quenchers within proteins

inhibits the fluorescence [19,20]. For example, tryptophan fluorescence is quenched by adjoining protonated acidic groups like aspartate and glutamate [21]. The fluorescent amino acid phenylalanine absorbs light by 260 nm and therefore cannot be detected by the device.

The emitted fluorescence of the reduced cofactor NADH ($\lambda_{\text{exc}}/\lambda_{\text{em}} = 365/437$) increases during the cultivation until the culture achieves the stationary growth phase. An increase of NADH is correlated to the cellular respiration and cell count. This coenzyme acts as a shuttle for electrons and is essential for the adenosine triphosphate (ATP) regeneration [22]. Therefore, monitoring of changes in the cytosolic NADH concentration can indicate energy respiration as well as the redox state of all cells under different metabolic and physiological states. A closer look at Figure 4, where the relative fluorescence intensity (RFI) signal of the NADH wavelength is normalized to the maximum, indicates an oxygen limitation of 15 h (batch age 26–41 h) in fed-batch 5. The oxygen concentration (pO_2) within the media drops below the set point of 40% after 25 h. At this point, nitrogen was added instead of oxygen to control the pO_2 -value to 0%. After 15 h, the oxygen control was switched on again at the batch age of 41 h. A rise of the NADH fluorescence intensity at the same time with the decrease of the viability is recognized. During an oxygen limitation, the equilibrium of the oxidized and the reduced form is shifted toward the reduced form [19]. NADH

FIGURE 3 Typical 2D-fluorescence emission spectra (fed-batch 3) for the excitation wavelengths of 280 nm (A), 365 nm (B), and 455 nm (C). The number before the low line is the excitation wavelength and the number behind is the emission wavelength. The color gradient from blue to red indicates the batch age

FIGURE 4 Normalized relative 2D-fluorescence intensity (RFI) of the NADH (solid line) wavelength region, cell viability (dotted line with open marks), and the oxygen concentration (pO_2) for cultivation 5

accumulates, because it cannot be oxidized via the oxidative phosphorylation without oxygen. The increase is shown in the zoom of Figure 4, where the normalized NADH fluorescence is plotted. However, when NADH cannot be oxidized to yield non-fluorescent NAD^+ , the glycolysis interrupts. Consequence is the cell death and a subsequent shift down of the NADH pool. After the oxygen supply a rapid drop in the NADH pool is to be seen at first. NADH is directly oxidized to the non-fluorescent NAD^+ . Afterward, the glycolysis/NADH oxidation restarts and an increase in the wavelength of the NADH fluorescence and cell growth is visible. Thus, the molecule is useful to monitor the metabolic activities in different physiological states of cells.

The vitamin riboflavin is consumed by growing CHO cells and therefore the fluorescence intensity declines in the spectra regions $\lambda_{exc}/\lambda_{em} = 280/525$ nm, $\lambda_{exc}/\lambda_{em} = 365/525$ nm, and $\lambda_{exc}/\lambda_{em} = 455/525$ nm during the exponential growth phase [23]. When the maximum cell concentration is achieved, it plateaus for a time and increase slowly at the end of the cultivation process. Riboflavin is essential for the biosynthesis of FAD and FMN, which are electron carrier in cellular oxidative metabolism in the TCA cycle and oxidative phosphorylation. These flavins are mostly cofactors

for enzymes involved in oxidation–reduction reactions. Thus, FAD is also associated with respiratory activities in mammalian cells [24]. The FAD fluorescence quantum yield is lower than the quantum yield of riboflavin and therefore decrease the fluorescence intensity in this wavelength region [25]. The small increase of the fluorescence signal in the death phase indicates that the added riboflavin concentration in the media is higher than the cells are able to consume and convert in FAD.

An intensive signal in the spectra is the Rayleigh scattering at $\lambda_{exc} = \lambda_{em}$ (Figure 3). The intensity increases during the cultivation process because more growing cells act as scattering centers and the excitation light penetration is reduced. Moreover, the accumulation of cell debris and released organelles effects scattered light [18]. In addition, higher cell concentrations result in more backward scattering.

These findings demonstrate that important process variables such as the TCC, VCC, viability, and nutrients as well as the cell metabolism have an influence on the detected fluorescence intensity. Due to the complexity of 2D-fluorescence spectra, the use of MVDA methods is indispensable for prediction of relevant process variables.

3.2 | Qualitative analysis of 2D-fluorescence spectra by PCA

The first step of multivariate data analysis is a qualitative analysis by PCA to reduce multivariate data to a smaller number of uncorrelated variables, the scores of the principal components (PCs). PCA is used to accomplish an overview of the spectral data from all six cultivations and is visualized in a score plot. No data preprocessing of the 2D-fluorescence spectra was used. In Figure 5A, the score plot of PC2 over PC1 for all six CHO cultivations is presented. The first two PCs together explain 96.3% of the spectral variance. The trend of the score values is similar for all six cultivations. In particular, the starting points on the upper left site are very close together, this describes the high reproducibility of the spectral measurement for similar initial conditions such as media and less cell

FIGURE 5 PCA of 2D-fluorescence spectra of all batches at the time of the reference values, showing the first and second PC (A). Score values of the second PC and the lactate concentration over the cultivation time (B). Trajectories of the normalized relative fluorescence intensity (RFI) of the wavelength regions for NADH, Rayleigh scattering (solid lines), and the offline values for lactate, TCC, and VCC (dotted line with marks) (C). Trajectories of riboflavin, tyrosine, tryptophan, pyridoxine (solid lines,) and the offline values for lactate and cell viability (dotted line with marks) (D). The vertical dotted line marks the second PC

counts. PC1 describes the changes in the Rayleigh scattering, amino acids and riboflavin metabolism, and NADH production (data are shown in <https://doi.org/10.25835/0005774>). Thus, the cell growth is responsible for the increasing trajectory in the horizontal direction along the first PC. The trajectory of the scores in batch 5 and 6 differentiate to the other batches in some score values. In batch 6, there is a distinction before the minimum in the score value of the second PC compared to the other batches and it is triggered at 6 h without stirring within the process. As already mentioned, in fed-batch 5, there was an oxygen limitation of 15 h, which influence the cellular respiration and final the cell growth. The distribution of the observations along PC2 with 16.7% of the spectral variance (the vertical direction) is recorded during the course of the cultivation and differences are observed within the cultivations. During any cultivation, the scores of PC2 in the vertical direction decreases with the batch age, achieving a minimum value and increasing afterward until the end of the cultivation. The corresponding loading plot demonstrates that particularly the amino acids and riboflavin wavelengths regions have a strong impact on PC2 (data are shown in <https://doi.org/10.25835/0005774>).

The minimum score value in the second PC is nearly to that point when the lactate metabolism shift from production to consumption (Figure 5B). Lactate production is monitored as a notable metabolite for cultured CHO cells. It is usually produced during the exponential growth phase and afterward, there is a metabolic shift to lactate consumption. Monitoring the lactate concentration is crucial because the accumulation of lactate has a toxic effect on cell growth and productivity [26]. Lactate does not influence fluorescence directly, therefore a quantitative correlation by PLS is not possible. Authors of other studies described a lactate correlation without causality [27]. A closer study of the RFI shows that the wavelength region of the Rayleigh scattering stagnates during the lactate metabolic shift (Figure 5C). This effect is visible in the other cultivations as well. It indicates that the proliferation of the CHO cells interrupts for a while during that culture phase. One reason for this might be that the cells use the energy instead for the growth but rather for the changes in metabolism. In addition, the signal in the wavelength region of NADH increases during the lactate metabolic shift (Figure 5C). One possible explanation for the signal rise is that the enzyme lactate dehydrogenase

generates NADH by the conversion of lactate to pyruvate [28]. Pan et al. showed that the TCA cycle activity increases at the same point [29]. The converted pyruvate and the lower NADH/NAD⁺ ratio support the higher TCA cycle activity and thus a higher proliferation. Furthermore, the PC2 trend represents the fluorescence of the amino acids. The minimum in the score value is also the minimum signal in the wavelength region of the fluorescence amino acid intensities. The RFI signal of the fluorescence amino acids drops only down to that point, stagnated for a short period, and increased afterward. The consumption of all the essential amino acids decreased after lactate metabolism shift [29]. Additionally, more amino acids were added with the feed media. Moreover, the cell viability begins to decrease after 120 h and by cell lysis, cell components are released in to the media.

3.3 | Quantitative analysis by PLS regression

PLS models were developed for quantitative analysis by correlating 2D-fluorescence spectra with critical process variables as offline reference values. Five fed-batch cultivations (1, 2, 4–6) were used as dataset for calibration (Table 1). The calibration data set include the most varying cultivations, so the model is calibrated to the observed process variance. A separate independent fed-batch process (3) was used for validation. The prediction of the validation set was based on the PLS model and spectral data, which were observed by collecting 2D-fluorescence spectra every 10 min during the whole cultivations. By this, a continuous in-line bioprocess monitoring was realized. All determined PLS models were evaluated in consideration of the goodness of fit and goodness of prediction, which are given by R^2 and Q^2 , respectively, as well as the root mean square error of calibration (RMSEC), from cross-validation (RMSEcv) and prediction (RMSEP).

3.3.1 | Monitoring of total cell concentration

The CHO cells contain several fluorophores, in particular, the cell metabolism includes a dynamic change of fluorescent compounds; therefore, fluorescence spectroscopy is predestined for monitoring the cell concentration. The description of the PLS calibration model for TCC is based on two PCs (Figure 6). Most of the variance (79.3%) is captured by the first component, which contains in particular the information of Rayleigh scattering, and amino acid and riboflavin decrease during the process as shown in the regression coefficients plot (Figure 6C). The coefficients point out how relevant the corresponding fluorescence regions are for the prediction of the variables [12]. This supports the theory that growing cells act as scattering centers, and therefore, the scattering light increases by higher cell count. Furthermore, the consumption of essential amino acids and riboflavin can be correlated to the TCC. The observed versus predicted plot shows an excellent

FIGURE 6 Calibration model for TCC (A) and the prediction for TCC (C). The regression line indicates an ideal correlation ($R^2 = 1$) between measured and predicted values. Regression coefficients reflecting the relevance of each excitation/emission wavelength pair for the TCC model (B)

correlation with R^2 of 0.95. As shown in Table 2, the RMSEC is 2.6×10^6 cells/mL and the RMSEcv is 3.2×10^6 cells/mL. A calibration error of nearly 9% of the maximum cell concentration together with an acceptable cross validation error stand for a high robustness of the model.

The prediction of TCC for a further cultivation (fed-batch 3), based on the calibration model was well realized with a Q^2 of 0.99 and an RMSEP of 1.82×10^6 cells/mL (Figure 6B). Especially, the lag phase and exponential growth phase till the batch age of 125 h was accurately predicted. One reason for the larger fluctuations at the end of the process is the measurement error of the reference measurement by Cedex of 5–10 % for high cell counts.

TABLE 2 PLS results for calibration models and validation /prediction by external data sets

Analyte	Range	Principal components	R ²	Q ²	RMSEC	RMSEcv	RMSEP	Number of calibrated batches (samples)	Number of validated batches (samples)
TCC (10 ⁶ cells/mL)	0.3–38	2	0.95	0.99	2.61	3.18	1.82	5 (322)	1 (56)
VCC (10 ⁶ cells/mL)	0.3–32	4	0.97	0.98	1.52	2.3	2.15	5 (322)	1 (56)
Viability (%)	53–100	5	0.93	0.96	2.93	3.79	3.82	5 (322)	1 (56)

FIGURE 7 Calibration models for VCC (A) and viability (D), Prediction of VCC (B), and viability (E). Scaled regression coefficients reflecting the relevance of each excitation/emission wavelength pair for the relevance of the VCC (C) and the viability model (F)

3.3.2 | Monitoring of viable cell concentration and viability

In addition to the total cell count, the viable cell density (VCC), and viability are important process variables to detect

when cells begin to die. Cell deaths are accompanied by cell lysis and result in accumulation of intracellular components and debris in the supernatant. Monitoring these variables provide information on the changes in cell physiology and indicates control strategies for next steps (e.g., for downstream

processing) to maximize product yield or reduce process time in view of the biopharmaceutical production. The PLS model developed for VCC demands four PCs and indicates a good model fit with R^2 of 0.97. Furthermore, a more precise VCC prediction (Q^2 of 0.98, RMSEP of 2.15×10^6 cells/mL) than for TCC was observed (Figure 7B). The accurate prediction of the cell concentration in the death phase is remarkable. Thus, the in-line 2D-fluorescence spectroscopy is a benefit for the monitoring of the VCC. PLS model for cell viability consists of five components and can precisely predict the viability of a further cultivation (fed-batch 3) with a RMSEP of 3.8% and Q^2 of 0.96. As shown in Figure 7, the most data points are by viability values above 90% from the first 120 h of the cultivation.

As illustrated in the regression coefficient plot (Figure 7C), the VCC model is dominated by the Rayleigh scattering, NADH, amino acids, and riboflavin wavelength region within the 2D-fluorescence spectra. The fluorescence region of tyrosine and tryptophan correlated well with the viable cell concentration as already described by Marose et al. [19]. The fluorescence at $\lambda_{\text{exc}} / \lambda_{\text{em}} = 365/437$ nm (NADH region) has a special relevance for the VCC and viability models (Figure 7C). As mentioned earlier, NADH plays an important role in a wide range of oxidative–reduction reactions in the cell metabolism. In dying cells, the intracellular $\text{NAD}^+:\text{NADH}$ ratio, the ATP production, and therefore, the metabolic activity changes during the cultivation. Thus, the change of the $\text{NAD}^+:\text{NADH}$ ratio between the non-fluorescence NAD^+ and fluorescent NADH molecule is visible in the fluorescence spectra and recognizable in the PLS model of the VCC and cell viability. These results support the observations in fed-batch 5, where the oxygen limitation has a significant influence on the NADH fluorescence region and VCC.

In the regression coefficients for cell viability, the riboflavin/FAD wavelength regions and the Rayleigh scattering have a great influence (Figure 7F). The property is the same as mentioned for NADH while FAD is involved in cellular oxidative metabolism in the TCA cycle. The TCA cycle activity decreases rapidly in dying cells and thereby the FAD-pool decreases. Cell deaths are accompanied by cell lysis and result in accumulation of intracellular components and debris in the supernatant. Therefore, the amount of cellular debris, suspended particles, and intracellular components in the culture media increases during the death phase. This also has the consequence of increases in the Rayleigh scattering. These changes in the culture media are feasible within the spectral data. The scattered light increases in the death phase and captured information about the cell morphology, size, and lysis. However, the new 2D-fluorescence device is capable to distinguish between total and viable cells. In further studies, it would be interesting if the device also has the ability to

distinguish between different forms of cell death such as apoptosis and necrosis.

4 | CONCLUDING REMARKS

This research clearly presents the potential of the new LED-based 2D-fluorescence device as a PAT tool for simultaneous in-line bioprocess monitoring of several important process variables in CHO cultivations. The results demonstrate that the new device combined with MVDA methods realize robust models and precise predictions of process variables within complex mammalian cell culture processes. Particularly it is to underline that the models are also robust for different process conditions such as various feeding strategies. The new approach of the device and the reduction of the relevant excitation wavelengths generate fluorescence spectral data, which are understandable to interpret without significant data preprocessing. However, this reduction does not lead to a loss of essential information for process understanding. This is an advantage for the implementation in industrial GMP manufacturing facilities. In addition, process variables like the viability and lactate metabolism shift, which are difficult to access with common in-line analytical analyzers, are feasible by 2D-fluorescence spectroscopy. This spectroscopic method executes a view into CHO cells and is best suited to get an understanding of the physiological state during the process in real-time. The biomass is frequently monitored only homogeneous as a total cell concentration. In CHO cell culture processes, the biomass consists of subpopulations of viable cells, dead cells, and lysed cells. The 2D-fluorescence spectroscopy represents a technology to distinguish between these subpopulations. In particular, the information about the cell lysis during the process would be interesting for further purification steps in downstream processing. In summary, the 2D-fluorescence device is a useful PAT tool to get information about the TCC, VCC, viability, lactate shift, and oxygen limitations during cultivation processes. The presented 2D-fluorescence technique can be seen as a step forward in the implementation of real-time bioprocess monitoring within the PAT goals. It offers the opportunity to be a sensible monitoring tool for highly complex biopharmaceutical upstream processes.

ACKNOWLEDGMENTS

The authors would like to thank the federal ministry of education and research for financial support.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

REFERENCES

1. Noh, S. M., Sathyamurthy, M., Lee, G. M., Development of recombinant Chinese hamster ovary cell lines for therapeutic protein production. *Curr. Opin. Chem. Eng.* 2013, 2, 391–397.
2. Jagschies, G., Lindskog, E., Lacki, K., Galliher, P., *Biopharmaceutical Processing: Development, Design, and Implementation of Manufacturing Processes*, Elsevier Science, Amsterdam, 2018.
3. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine, Office of Regulatory Affairs. *Guidance for Industry PAT—A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance*. 2004, USFDA, Silver Springs, MD 16.
4. Glassey, J., Gernaey, K. V., Clemens, C., Schulz, T. W., et al., Process analytical technology (PAT) for biopharmaceuticals. *Biotechnol. J.* 2011, 6, 369–377.
5. Claßen, J., Aupert, F., Reardon, K. F., Solle, D., et al., Spectroscopic sensors for in-line bioprocess monitoring in research and pharmaceutical industrial application. *Anal. Bioanal. Chem.* 2017, 409, 651–666.
6. Biechele, P., Busse, C., Solle, D., Scheper, T., et al., Sensor systems for bioprocess monitoring. *Eng. Life Sci.* 2015, 15, 469–488.
7. Hoehse, M., Alves-Rausch, J., Prediger, A., Roch, P., et al., Near-infrared spectroscopy in upstream bioprocesses. *Pharm. Bioprocess.* 2015, 3, 153–172.
8. Faassen, S. M., Hitzmann, B., Fluorescence spectroscopy and chemometric modeling for bioprocess monitoring. *Sensors* 2015, 15, 10271–10291.
9. Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*, Springer, Berlin, Germany 2006.
10. Hantelmann, K., Kolleck, M., Hüll, D., Hitzmann, B., et al., Two-dimensional fluorescence spectroscopy: A novel approach for controlling fed-batch cultivations. *J. Biotechnol.* 2006, 121, 410–417.
11. Konstantinov, K. B., Dhurjati, P., Van Dyk, T., Majarian, W., et al., Real-Time compensation of the inner filter effect in high-density bioluminescent cultures. *Biotechnol. Bioeng.* 1993, 42, 1190–1198.
12. Kessler, W., *Multivariate Datenanalyse: für die Pharma-, Bio- und Prozessanalytik*, Wiley, Hoboken, NJ 2013.
13. Faassen, S. M., Hitzmann, B., Fluorescence spectroscopy and chemometric modeling for bioprocess monitoring. *Sensor.* 2015, 15, 10271–10291.
14. Stärk, E., Hitzmann, B., Schügerl, K., Scheper, T., et al., In-situ fluorescence-probes: a useful tool for non-invasive bioprocess monitoring. *Adv. Biochem. Eng. Biotechnol.* 2002, 74, 21–38.
15. Schwab, K., Amann, T., Schmid, J., Handrick, R., et al., Exploring the capabilities of fluorometric online monitoring on chinese hamster ovary cell cultivations producing a monoclonal antibody. *Biotechnol. Prog.* 2016, 32, 1592–1600.
16. Li, B., Shanahan, M., Calvet, A., Leister, K. J., et al., Comprehensive, quantitative bioprocess productivity monitoring using fluorescence EEM spectroscopy and chemometrics. *Analyst* 2014, 139, 1661–1671.
17. Zagari, F., Jordan, M., Stettler, M., Broly, H., et al., Lactate metabolism shift in CHO cell culture: The role of mitochondrial oxidative activity. *N. Biotechnol.* 2013, 30, 238–245.
18. Farhat, G., Mariampillai, A., Yang, V. X. D., Czarnota, G. J., et al., Detecting apoptosis using dynamic light scattering with optical coherence tomography. *J. Biomed. Opt.* 2011, 16, 070505.
19. Marose, S., Lindemann, C., Scheper, T., Two-Dimensional fluorescence spectroscopy: A new tool for on-line bioprocess monitoring. 1998, 14, 63–74.
20. Callis, P. R., Liu, T., Quantitative prediction of fluorescence quantum yields for tryptophan in proteins. *J. Phys. Chem. B* 2004, 108, 4248–4259.
21. Teixeira, A. P., Oliveira, R., Alves, P. M., Carrondo, M. J. T., Advances in on-line monitoring and control of mammalian cell cultures: Supporting the PAT initiative. *Biotechnol. Adv.* 2009, 27, 726–732.
22. Heikal, A. A., Intracellular coenzymes as natural biomarkers for metabolic activities and mitochondrial anomalies. *Biomark. Med.* 2010, 4, 241–263.
23. Märkl, I. H., Animal cell culture technology. *J. Biotechnol.* 1994, 34, vii.
24. Huang, S., Heikal, A. A., Webb, W. W., Two-photon fluorescence spectroscopy and microscopy of NAD(P)H and flavoprotein. 2002, 82, 2811–2815.
25. Bessey, O. A., Lowry, O. H., Love, R. H., The fluorometric measurement of the nucleotides of riboflavin and their concentration in tissues. *J. Biol. Chem.* 1949, 180, 755–769.
26. Lao, M., Toth, D., Effects of ammonium and lactate on growth and metabolism of a recombinant Chinese hamster ovary cell culture. *Biotechnol. Prog.* 1997, 13, 688–691.
27. Schwab, K., Amann, T., Schmid, J., Handrick, R., et al., Exploring the capabilities of fluorometric online monitoring on chinese hamster ovary cell cultivations producing a monoclonal antibody. *Biotechnol. Prog.* 2016, 32, 1592–1600.
28. de Marcos, S., Galbán, J., Alonso, C., Castillo, J. R., Intrinsic molecular fluorescence of lactate dehydrogenase: An analytical alternative for enzymic determination of pyruvate. *Analyst* 1997, 122, 355–359.
29. Pan, X., Dalm, C., Wijffels, R. H., Martens, D. E., Metabolic characterization of a CHO cell size increase phase in fed-batch cultures. *Appl. Microbiol. Biotechnol.* 2017, 101, 8101–8113.

How to cite this article: Claßen J, Graf A, Aupert F, Solle D, Höhse M, Scheper T. A novel LED-based 2D-fluorescence spectroscopy system for in-line bioprocess monitoring of Chinese hamster ovary cell cultivations—Part II. *Eng Life Sci* 2019;19:341–351. <https://doi.org/10.1002/elsc.201800146>