***In-situ* microscopy: Online process monitoring of mammalian cell cultures**

Klaus Joeris¹, Jan-Gerd Frerichs^{2*}, Konstantin Konstantinov¹ & Thomas Scheper²

¹ Bayer Corporation, Pharmaceutical Division, Department of Cell Culture and Bioprocess Engineering, 800 Dwight Way, Berkeley, CA, USA; ² Institut für Technische Chemie, University of Hannover, Callinstr. 3, 30167 Hannover, Germany

(* Author for correspondence; E-mail: frerichs@iftc.uni-hannover.de)

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Abstract

The *in-situ* microscope is a system developed to acquire images of mammalian cells directly inside a bioreactor (*in-situ*) during a fermentation process. It requires only minimal operator intervention and it is well suited for either batch or long-term perfusion fermentation runs. The system fits into a 25 mm standard port and has a retractable housing, similar to the industry standard InTrac. Therefore, it can be cleaned and serviced without interruption of the process or risking contamination. A sampling zone inside the bioreactor encloses a defined volume of culture and an image sequence is taken. The height of the sampling zone is set by the control program and can be adjusted during the cultivation to accommodate a wide range of change in cell density. The system has an infinity corrected optical train and uses a progressive scan CCD camera to acquire high quality images. Process relevant information like cell density is extracted from the images by digital image processing software, currently in development for mammalian cells (CHO, BHK). The first version of the software will be able to estimate the cell density, cell size distribution and to give information of the degree of aggregation (single and double cells, cell clusters).

Introduction

Two of the most important parameters for reliable fermentation control are cell density and cell viability. Ideally, these measurements are taken online. Currently available instruments are based on turbidity (e.g. Aquasant Messtechnik AG, Bubendorf, Switzerland), conductivity (e.g. Aber Instruments Ltd, Aberystwyth, UK), optical density (e.g. generic Photometer with dip probe or bypass) or fluorescence (Bioview, Delta Light & Optics, Lyngby, Denmark). The results of all these online measurements are verified using offline techniques, usually by counting the cells using a hemocytometer and a microscope.

This in-process re-calibration is necessary, as the described online systems depend on more parameters than just cell density like sensor drift over time or changes in the physical or chemical environment. A promising concept that eliminates the problems of existing systems is to develop microscopy from a pure

offline into an online technology. The result is called *in-situ* microscopy, a technique that delivers images of cells in a defined volume inside a bioreactor without sampling or bypass construction. This concept has been described earlier in the literature (Bittner et al., 1998; Suhr, 1995, 1997), but the complexity of the task prevented the development of a system with the potential of a routine tool so far. In this article we describe a new approach.

System development

In general, a cell counting procedure can be broken down into the four common steps described in Table I. A defined volume of a sample is enclosed between two parallel glass surfaces (slide and cover slip). After focussing multiple cell-counts are performed. The sample is discarded and a new sample can be analysed.

Figure 1. The retraction principle of the *in-situ* microscope.

Figure 2. Principle of the variable-volume flow-through sampling zone.

In case of the *in-situ* microscopy, the movement of two parallel sapphire windows inside the bioreactor replaces the offline hemocytometer assembly and disassembly. The offline method moves different squares of the hemocytometer into the field of view of the microscope to get multiple counts after mounting and focussing. Since the *in-situ* microscope has a fixed field of view, the sample itself is exchanged after every count and multiple counts are averaged as shown later.

The key requirements in designing an *in-situ* microscope for an industrial application are:

(1) *Long term stability:*

The sampling zone of the system stays inside the bioreactor during the entire cultivation process. Al-

though the right choice of materials can minimize cells or debris sticking to the surfaces, an option is needed to access and clean this sampling zone without interrupting the cultivation process or compromising sterility. For long-term high cell density processes this will be a routine maintenance procedure, so a high reliability is needed. The design satisfying this requirement is based on a retractable probe housing commonly used for pH and DO probes, the Intrac system by Mettler-Toledo (2000). It is an accepted industry standard proven to be highly reliable. The microscope, once installed in the bioreactor port, can be retracted for cleaning of the sampling zone. It is reinserted into the bioreactor after maintenance and steam sterilisation of the cleaning chamber. Figure 1 shows the principle of operation.

The retractable housing can either be an integral part of the system as shown above or an independent unit. While the latter can be removed completely from the bioreactor for cleaning or servicing, this version is technically far more complicated and requires further miniaturisation of all components. Therefore, the completely retractable system is a special development and will be applicable to very long cultivation runs of several months at high cell densities.

Table 1. Procedure of cell counting offline and *in-situ*

Operation	Offline microscope	<i>In-situ</i> microscope
Sampling/ mounting	<ul style="list-style-type: none"> – sample bioreactor aseptically – assemble hemocytometer (place coverslip on slide) – inject sample 	<ul style="list-style-type: none"> – closing of a sampling zone: moving the coverslip down towards the slide while enclosing a sample
Focussing	Manual focus (usually)	Autofocus
Counting	Fixed sample, changing position	Fixed position, changing sample
Discarding	Disassemble and clean hemocytometer	<ul style="list-style-type: none"> Opening of sampling zone: Moving the coverslip away from the slide

Figure 3. Schematics and operation principle of the retractable *in-situ* microscope.(2) *Standardisation:*

The system was designed to fit into a 25 mm standard port. Sterilisation is possible by either autoclaving or steam-in-place (SIP) cleaning. During sterilisation the optical system (CCD-camera, objective and linear stages, see Figure 3) is separated from the mechanical part.

(3) *Measuring range:*

To cover a complete cultivation from start to finish the system needs the ability to function in a wide range of cell densities. This is achieved by introducing the 'variable-volume flow-through sampling zone'.

The exact height of the sampling zone (and therefore the measuring volume) is defined by the moving piston-like part of the sampling zone (Figure 2) mounted on a linear positioning stage outside the bioreactor. This stage allows very precise and reproducible positioning of the piston as shown in Figure 3. Three spacers protrude from the moving part of the sampling zone. These spacers define the minimum sampling zone height and are a reference point for the position calibration. They also prevent clogging the sapphire windows with cells by ensuring a minimal distance between the two panes. A minimum height of 50 micrometers has

Figure 4. The retractable *in-situ* microscope.

Figure 5. Characteristic images taken by the *in-situ* microscope at different cell concentrations (cell density determined by hemocytometer hand count).

Figure 6. Cell density during the cell accumulation phase as determined by hand count, Cedex and *in-situ* microscope.

proven to be a good choice for mammalian cell culture.

This sampling zone does not enclose a sample completely, but allows a continuous flow of cells through it even in the measuring (closed) position and a sequence of different images taken during one closing cycle. This increases the measurement frequency by an order of magnitude and thus the number of images available to the image processing software, which enhances reliability and accuracy of the results.

(4) *Reproducibility and accuracy:*

Precise movements are necessary since the sampling zone height is not fixed but adjusted to the requirements of the process. The sampling zone positioning piston is mounted on a linear positioning stage with crossed roller bearings to obtain the necessary accuracy and repeatability needed for this application. Figure 3 shows the principle of operation. The camera with the optical train attached is mounted on a second stage parallel to the sampling zone stage. Both are staged on a U-shaped support and are driven by stepper motors coupled to micrometer heads. Figure 4 shows a photo of the system.

(5) *Image quality:*

To facilitate the development of the image processing routines an image quality comparable to conventional bench top microscopes is needed. An achromatic objective with a 5-fold magnification and a numerical aperture of 0.1 were used in all systems. The objective can be exchanged easily

to adjust the magnification without retracting the system from the bioreactor. The overall magnification is approx. 100-fold. A progressive scan CCD-camera with an exposure time of 0.5 ms is used to get focussed pictures of the moving cells inside the sampling zone.

The condenser consists of an LED and an aspheric condensing lens to achieve an homogenous background illumination. The very long lifetime of an LED makes the condenser virtually maintenance free. Illumination and positioning devices are controlled by a microcontroller and windows-based software that also handles the image acquisition. It has autofocus capability for automated operation where required.

The 5-fold objective is used for cell counting. An objective with 20-fold magnification can be used to obtain more detailed images and morphological information about the cells.

Results

Monitoring of a CHO-culture

The *in-situ* microscope was tested in a perfusion system with 5 L working volume and a cultivation of CHO cells in a serum and protein free medium. After autoclaving (30 min, 121 °C) and a sterility test the system was inoculated at a seeding cell density of 1×10^6 cells mL^{-1} . The culture was monitored during the cell accumulation phase of the perfusion process. The process was controlled using the following setpoints, see Table II:

The image processing routines are not yet fully automated and the images must to be loaded manually into the software (AdOculus 3.1, DBS, Germany) running the algorithms described in (Frerichs, 2000). Instead of continuously calculating the cell density and a moving average, image series at different stages of the process were recorded and 20 images of each stage were analysed. Figure 5 shows typical images for each of these stages.

Two prominent features are visible in the images: The cells and bubbles, created by microsparging. The first step of the image processing routine separates the cells from background and bubbles using a threshold operation followed by region labelling (Frerichs, 2000). The bubble area is measured because these regions do not contribute to the cell counting, are different for each image and thus must be subtracted from the total field of view for cell density

Figure 7. First and last image taken during a long-term test of focal stability.

Table 2. Process control setpoints

Parameter	pH	DO	Agitation	Temperature	Total gas flow
Setpoint	6.80	50%	40 rpm	36.5 °C	0.05 L min ⁻¹ , microsparged

calculation. The sampling zone height was adjusted from 100 micrometer at low cell density sequentially to the hardware-defined minimal height of 50 microns at high cell densities.

The results were compared to hand counts with a hemocytometer and an automated cell counting system, Cedex (Innovatis, Bielefeld, Germany), both using the trypan blue exclusion method (Figure 6). Since determination of viability with the *in-situ* microscope is not possible so far, only the total cell number was counted. The shown standard deviation was calculated for 5 hand counts, 20 Cedex- and 20 *in-situ* microscope images. The results for each point in time were obtained from the same sample, but plotted slightly shifted to allow space for error bars.

Stability of the system

Figure 7 shows the first and the last image of a long-term image series taken over a 12 h period without adjusting the focus during the image sequence recording. The identical image quality shows an excellent stability and reliability of the linear stage construction.

Monitoring of the process with the *in-situ* microscope was continued for several days after the end of the cell accumulation phase shown above. Over a period of more than two weeks, no cells or debris were found to permanently attach to the sapphire windows.

Conclusions

The *in-situ* microscope was successfully tested in a CHO-cultivation and the results correlate very well with the results of hemocytometer hand counts and Cedex counts. The system also showed good stability and proved to be robust enough for the application in an industrial process.

The next phase of image processing software development will also address the automation and interfacing with the control software. Another goal of the next project phase is to extract information on cell viability from the cell morphology using a higher magnification objective.

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