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USING AN INDUSTRIAL ROBOT ARM FOR MONITORING CULTIVATIONS OF MAMMALIAN CELLS IN PILOT SCALE

Iris Poggendorf[1], Dirk Lütkemeyer[1], Torsten Scherer[2], Janwei Zhang[2], Alois Knoll[2], Jürgen Lehmann[1]

[1]Institute of Cell Culture Technology, [2]Institute of Technical Computer Science, Technical Faculty, University of Bielefeld, Germany

Abstract: The complete automation of sampling and the subsequent sample management in mammalian cell cultivations is the subject of this paper. To archive this goal an industrial robot arm is used. To be able to perform the tasks the robot arm is equipped with a hand-camera and a force/torque sensor. Additionally to the normal laboratory equipment an computerized automatic sampling station, pipetting device and cell counter had to be employed. The essential steps, preparations and developments, the complete set-up in the laboratory and experiments performed so far are described.

Keywords: robot, automation, sample management, mammalian cell cultivation, automatic cell counter

1. INTRODUCTION

In mammalian cell culture technology the last key issue for a completely automated process is the taking, analysing and storing of a sample from the bioreactor.

The on-line analyser systems existing on the market today are directly coupled to a specific bioreactor (Van de Merbel *et al.*, 1996). Typically, these sealed couplings can not be steam-sterilised which is the proven method for sterile sampling in industry (Larsson *et al.*, 1996; Paliwal *et al.*, 1996). Therefore it was necessary to develop a new device which fulfils this need for an application in industrial production environments. Therefore a reliable online measurement of all the important parameters is not yet possible and samples have to be taken manually on a regular basis.

The status of the culture is derived from the results of the analysed parameters of these samples. The normal procedure includes determination of the total and viable cell densities in relation to the media parameters (glucose, lactic acid, ammonia concentrations). The cell densities are retrieved by counting a stained cell sample. A cell free aliquot is stored in a freezer for further analyses (e.g. amino acid and product concentrations). Information for optimisation of feed and harvest strategies can be retrieved from the analysed parameters additionally.

The set-up in the laboratory (figure 1) includes active components like sampling, pipetting and cell counting equipment and passive components like centrifuge, barcode scanner, scales and freezer. To enable a completely automated sample management a communication network had to be established between the devices in the laboratory. All active devices used in the process have to be computer controllable. The status of passive devices has to be computer detectable.

The first step in the procedure is retrieving a sample from the bioreactor. A computerized sampling device, that is directly coupled to the bioreactor was developed. It allows the sterile taking of a sample, which is filled into a 50 mL tube. After sampling the

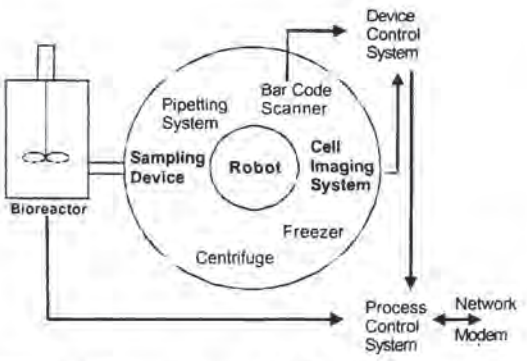

Fig. 1. Schematic view of the whole set-up. The machines marked in boldface are described detailed in this paper. The robot takes the sample tube filled by the sampling device to the different machines. The cell imaging system counts the cells. The whole system is controlled by the device control system. Barcode scanner and cell counter deliver the off-line data to the device control system. After processing, off- and on-line data are stored and visualised in the process control system which is connected to a network and modem.

device is steam sterilised. Cells are automatically stained and counted using an imaging system (Cedex, innovatis, Germany). After a centrifugation step a cell free aliquot is taken from the supernatant and filled into a barcode marked tube which is stored in a freezer.

During the off-line treatment the robot takes the tubes to the different machines involved. Visual and tactile information from a hand-camera and force/torque sensor is used to guide the robot. This is necessary to deal with uncertainties of goal position and fine manipulation. Due to some limitations of the robot slight modifications of the devices used had to be performed. Still all equipment are standard devices with only slight modifications as coloured markings or additional tube racks. All devices can be used by human operators for other tasks.

The whole set-up is controlled by software (device control system) which triggers the single actions. Data such as cell count and barcode readings are processed prior to their storage in the process control system. History plots of the on- and off-line data are possible. A connection via network and/or modem allows monitoring the cultivation from the office or at home. Usage of the equipment will be supervised by the device control system. This will restrict parallel handling of the devices at the same time by the robot and humans for safety reasons.

This automation strategy will reduce manpower in routine laboratory procedures to be more efficiently used at other tasks and maintain the cultivation process on a high quality standard.

2. LABORATORY SET-UP

The robot has a fixed position in the laboratory. Because of its limited range all equipment has to be positioned within a distance of about 60 cm. Figure 1 displays not only a schematic view of the laboratory set-up but gives also an approximate representation of the spatial arrangement of the devices. For the experiments and testing of the system a standard set-up was used but all procedures and devices were designed to be very flexible and easily adaptable to other situations and tasks. Table 1 shows the general actions that are performed during the sample management.

Table 1 General actions during sample management

Action	Device	Description
Sampling	Sampler	Automated, sterile sampling from the bioreactor
Moving sample tube	Robot	Taking sample to the pipette system
Pipetting	Pipette	Taking an aliquot of cell broth
Moving sample tube	Robot	Inserting sample tube into centrifuge
Moving Cedex tube	Robot	Fetching cell broth aliquot from the pipette system and taking it to the Cedex
Cell counting	Cedex	Automatic cell density determination
Centrifugation	Centrifuge	Pelleting of the cells
Moving sample tube	Robot	Removing the sample tube from the centrifuge and taking it to the pipette system
Pipetting	Pipette	Taking of supernatant
Moving storage tube	Robot	Moving storage tube to the pipette system to store aliquots of supernatant
Moving storage tube	Robot	Taking storage tube to the freezer

2.1 Material and equipment

Cell Culture The cultivations were done in a 100 L (UD100, B. Braun Biotech International, Germany) and in a 20 L bioreactor (Diessel, Germany) which can be run in batch as well as in continuous mode. Both bioreactors were aerated bubble free (Büntemeyer *et al.*, 1987). Standard culture conditions were used (Heidemann *et al.*, 1998). The cell lines were recombinant CHO cell lines and a recombinant human leukaemia cell line. All cultivations were done with a serum free standard medium using human transferrin, bovine insulin and albumin as protein supplements (Jäger *et al.*, 1988). Amino acids and glucose were supplemented as needed (Büntemeyer *et al.*, 1991).

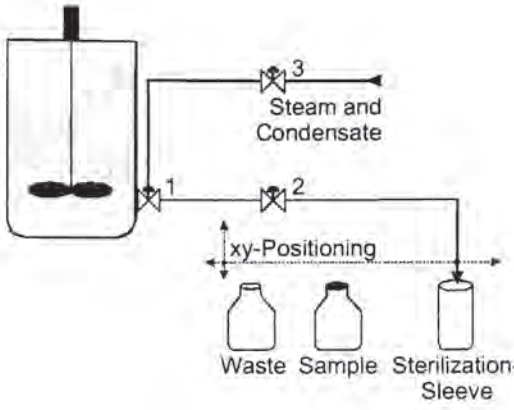

Fig. 2 Diagram of the steam sterilisable sampling device. The sampling valve (1) is inserted into the bioreactor vessel. Steam sterilisation is done via valve 2 and 3. The xy-positioner places the needle into the sterilisation sleeve, sample tube or waste container. The sample tubes are covered with a septum to insure aseptic aerosol free sampling. Using the computerised sampling device the number of samples and the volume are variable.

With both methods (Neubauer chamber and automatically) single cells were counted using the trypan blue exclusion method (Tennant, 1964). The total cell density of cells which tend to aggregate was determined after osmotic shock of the cells and staining of the nuclei (0.1 M citric acid plus 0.8 % crystal violet, (Sanford *et al.*, 1950). To count aggregating cells automatically the aggregates have to be broken up. To do so the sample was diluted 1:1 with Accumax (PAA Labaratories GmbH, Germany) and incubated 10 min at 37 °C (shaken).

The concentrations of glucose and lactate were measured with the automatic analyser YSI 2700 S (Yellow Springs Instruments, USA). The lactate dehydrogenase (LDH) activity was determined by measuring the reduction of pyruvate to lactate via photometry (Wagner *et al.*, 1992). The activity data is expressed in kat/L. 1 Katal (kat) converts 1 mol of substrate per second.

Device Control System The whole system is computerized using a real time operating system (QNX, QNX Software Systems, Canada) as the centre part for controlling the performance and distribution of tasks. The applications are programmed with a C++-software including a graphical interface in the final version.

To communicate with the laboratory devices and to control the actions different interfaces are used. The sampling device is controlled via parallel port. The signals for the valves are transferred via TTL-interface. Pipetting device, barcode scanner, centrifuge and scales are connected via serial port, the cell counter via network.

Robot system The robot (PA-10, Mitsubishi Heavy Industries, Japan) is a redundant 7-joint arm. It is equipped with a hand mounted colour micro camera (JAI M1250, Stemmer Imaging, Germany) and an electrical parallel yaw gripper (PHD, Germany). It is controlled at joint level via ARCNET interface by the software RCCL (Robot Control C-Library) from a PC running the Linux operating system.

Sampling device The main components of the sampling device (figure 2) are silicone tubing, a movable stainless steel needle and a few valves. Two pneumatic valves (605, Gemü, Germany) and one pneumatic sampling valve (A907-T, Südmo, Germany) inserted directly in the bioreactor are used to control the fluid and steam flow and to maintain the sterility in this special set-up. The assembly can be modified so that up to eight valves can be used for different tasks and purposes.

During the sampling procedure the needle punctures the septum and the sample gets injected into a 50 mL sample tube (Nunc, Germany). The movement of the needle is done using a xy-positioner (ELVamat, ELV, Germany) The tubes are sealed with a special plastic film (ELAS, Zinsser Analytic, Germany).

The procedure to take a sample is divided into three steps. To prevent dilution of the sample the tubing system is flushed with cell broth at first, then the xy-positioner moves to the tube and the sample gets injected. The third step includes flushing of the system with condensate and steam sterilisation of the tubing system. For this purpose the needle is moved to a sterilisation sleeve. The condensate is collected in a waste pot.

Cell counting system The Cedex cell counting system (innovatis, Germany) is based on the standard trypan blue dye method for living/dead cell determination combined with an advanced pattern recognition system for the evaluation procedure (Gudermann *et al.* 1997, Wehn *et al.* 1999). A 1 mL sample has to be inserted, mixing of sample and dye and injection into the counting chamber is done automatically. Starting of an analysis is done via the graphical user interface or a text based network connection. By default, twenty images are evaluated in about four minutes. This results in a lower standard deviation compared to the manual cell count.

Peripheral devices For further handling of the sample additional laboratory equipment is needed. The *pipetting system* (PSD/2 and MVP, Hamilton, USA) allows the computerised pipetting of cell broth and supernatant and the filling of storage and Cedex tubes by the robot arm.

A *centrifuge* (Megafuge 1.0, Kendro, Germany) is used to separate the cells from the broth. For failure proof performance the centrifuge is equipped with an additional interface. Through the interface status information gets retrieved. Especially the status of the lid and rotor speed have to be queried by the device control system. With a *balance* the weight of the sample tube gets detected so the centrifuge can be operated.

In the *process control system* (MFCS 2.0, B. Braun Biotech, Germany) the on- and off-line data is stored. The data from the cell counter and the *barcode scanner* (DS1100, Datalogic, Germany) is automatically transferred and stored in the process control system. Figure 1 illustrates the entire set-up.

3. RESULTS

The results for the main components of the automation strategy are described. The sampling device as the first key component, the cell counter which enables direct determination of living and dead cells, and the robot system as the connection between the different devices. All systems were tested and optimised during the cultivation of mammalian cells in a 20 or 100 L bioreactor.

3.1 Sampling Device

Since there is the danger of an infection taking a sample from a bioreactor is a crucial operation for the system. There are two different methods to prevent an infection of the bioreactor – sterilisation of the tubing system with a disinfectant or with steam. The sterilisation with a disinfectant bears the risk of residues in the system which might have influences on the sample. For that reason steam sterilisation was chosen, it is the proven method in industry. Anyway this method bears the risk of influencing the sample as well. After the steam sterilisation condensed water accumulates in the tubing system and will have a dilutive effect but there aren't any chemical substances which could have a direct influence on the sample.

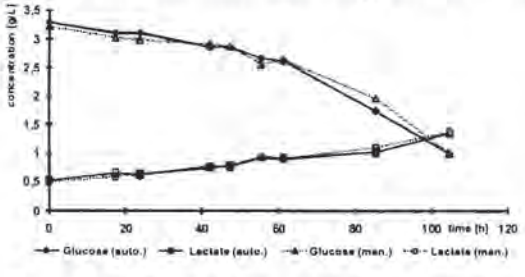

Fig. 3 Comparison of manual and automatic sampling during a batch cultivation of Hybridoma cells in 20 L scale. The analysed concentrations of glucose and lactate do not show any significant differences. Therefore, the flushing of the tubing system with cell broth prior to sampling is sufficient.

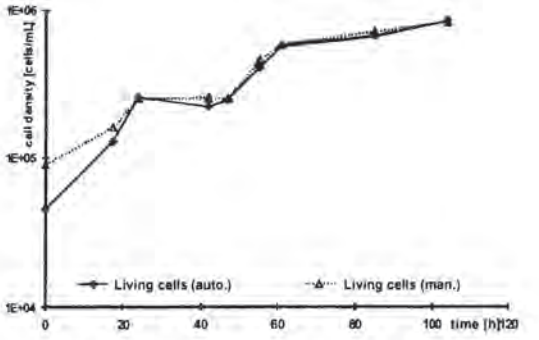

Fig. 4 Comparing the cell count during the described 20 L cultivation. The manual and automatic sampling show the same living cell density. Each sample was counted three times, the error was calculated to 5 %.

The injection needle is inserted into the tube, which is sealed with a septum, therefore a pollution of the surrounding environment with aerosols is minimised and the samples kept aseptic. During sterilisation, the injection needle is pressed down in the sterilisation sleeve which is sealed by its special design and an o-ring.

The reliability of the xy-positioning tool was evaluated through automatic injection of the needle 25 times in succession; there was no deviation measured. A 10 day sterile testing of the system with daily sampling showed no contamination in the connected bioreactor. The further evaluation of the sampling device was carried out during several cultivations. As an example, the results from a 20 L batch cultivation are presented. The manual sampling is compared with the sampling device. The comparison of manual and automatic samples does not show significant differences in lactate and glucose concentrations (Fig. 3). Therefore it can be concluded that there is no dilution of the sample. There are also no differences between the manual and automatic cell density determination (Fig. 4). It is concluded that there are no sedimentation effects during the movement of the injection needle. Looking at possible cell disruption, the LDH activity - as a rate for the damage of the cells - was analysed. In all measurements the LDH activity was slightly lower in the automatic samples than in the manual samples.

Considering these results, it can be concluded that the new sampling device is a reliable means of sampling mammalian cell cultivation bioreactors.

3.2 Robot

The PA-10 robot by Mitsubishi Heavy Industries, which is used to operate the different devices and to transport the samples between them, requires - like

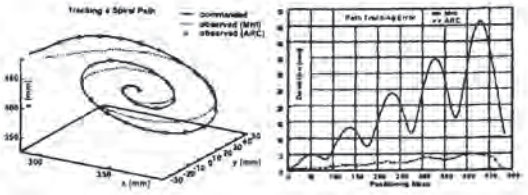

Fig. 5 The left image shows the commanded and observed path for a sample motion when controlling the robot via the original MHI hard/software and RCCL using the ARCNet interface. The right image shows the path error for both cases. The error has been reduced by one order of magnitude.

any other robot - a very high precision in measuring the positions of the objects to be manipulated. This poses the following problem. Since the system should be both easily adaptable to a modified spatial arrangement of the set-up and tolerant against human interference on that arrangement, this precision can neither be initially achieved nor maintained in the long term. Since all devices are standard equipment only marked with coloured symbols and explicitly meant to remain usable by human operators they cannot be arbitrarily modified to ease this problem. Instead all position data used in the system is treated as potentially inaccurate and each operation dealing with a specific device is divided into a direct motion to its coarse position, followed by several fine motions taking sensor information into account (Zhang *et al.*, 1999).

The action following the object recognition is context-depending. This means that, after recognition of the sample tube in the sampling device, the movement of the hand is fixed but different from the movement after recognition of the pipetting system. Therefore, the robot system needs to know the approximate position of the devices and, after a visually-guided fine positioning, uses a library of commands for each device.

Robot Control C-Library A complex problem like this needs a language to program the robot with a flexibility that proprietary robot manufacturer programming languages usually lack. In order to use RCCL (Lloyd and Hayward, 1992) - which offers this flexibility - an interface to access and control the robot at joint level via an ARCNet network has been built. This solution also improved the path tracking accuracy at joint control level, as can be seen in figure 5.

Visually Guided Fine Positioning After approaching a device, the robot takes an image, recognises objects and their positions in that image, and then centres itself above the desired object using an uncalibrated, iterative, positioning loop.

Colour Based Object Recognition The object recognition is based on a 2d colour recognition of the images, discarding the intensity component. Based on the goal to detect an object of at least approximately known colour, the distribution of the colour components of the image is projected into a 1d measure of how closely a pixel's colour matches the searched colour using a co-ordinate transformation similar to the principal component analysis. In this distance measure, contiguous pixels of sufficiently matching similarity are merged to regions, which in turn are identified or rejected as objects by their form factors.

Illumination Tolerance A vision system operating in an environment which is primarily focused on humans must not be disturbed by typical human behaviour interfering with the system, like humans switching the light on and off. This approach is much more tolerant against varying external illumination conditions than the well known greyscale based edge detecting approaches.

Force Sensing Since the accuracy of even the best visually guided fine positioning is limited by at least the resolution of the camera providing the images, the robot will additionally use force control strategies to eliminate remaining uncertainties and to increase robustness when manipulating objects.

Manipulations The robot is able to load the sampling device with tubes. During the filling procedure, the robot holds the tube to prevent the needle from lifting the tube when pulling out of the septum. Then the robot takes the sample tube to the pipetting system and afterwards inserts it into the centrifuge. When the centrifugation is finished the robot picks up the tube and takes it to the pipetting device again. An aliquot gets filled into the storage tube and deposits it in the freezer. The next step will be the handling of smaller tubes like storage container and Cedex tubes by the robot.

3.3 Automated cell density determination

With the new Cedex system the procedure of the trypan blue dye exclusion method (sample preparation and cell counting) is carried out automatically. The only task that has to be performed by the robot is the insertion of a Cedex tube into the device.

In several cultivations at the 20 and 100 L scales the characteristics of the Cedex have been tested. In all cases, the sample was taken with the sampling device. In suspended single cell cultures the results are very satisfying. In cultivations with cells that tend to aggregate, the automated cell density determination yields in deviating results. In these cases the parameters of the device restrict it from counting clusters. An adaptation of the sample treatment in regards to the needs of the cell counter (clusters with less than ten cells) is necessary. An enzymatic treatment of the sample solves this problem. An additional

pipetting and mixing step followed by a short incubation breaks up the clusters. An automation of this procedure will be done by the computerized pipetting system. This allows the Cedex to determine the exact cell density and viability during all stages of the cultivation.

External triggering of the measurement and query of the results will be done by the device control system. Then the results will be forwarded to the process control system from there the results are remotely accessible.

4. CONCLUSION

The main components of this automation strategy have been successfully tested. The sampling device and the cell counter were used during several cultivations in pilot scale. There were no sterility problems and the automatic sampling showed the same results as a manual sample. After an optimisation of the disintegration of possible aggregates, the cell counter was able to analyse such cell samples.

The direct control of the robot at joint level showed a satisfying accuracy during arm movement. Only this accuracy enables the combination of arm movement and sensor information. Controlled by the colour-based object recognition, the robot arm showed its ability to reproducibly find and grasp sample tubes under a wide range of different illumination conditions. It loaded the sample device and took the sample to the laboratory devices.

The next step will be the completion of the communication between cell counter, centrifuge and bar code reader with the device control system and the process control system for creation of history plots and storage of data. An intensive testing of the robustness of the entire system during cultivations will be the last step of this investigation.

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