First Steps in Robot Automation of Sampling and Sample Management during Cultivation of Mammalian Cells in Pilot Scale

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The robot automation of sampling and the subsequent treatment and storage of aliquots during mammalian cell cultivations was investigated. The complete setup, the development and testing of the sampling device, the robot arm, and the cell imaging system are described. The developed sampling device is directly coupled to a pilot bioreactor. It allows the computerized sterile filling of cell broth into 50 mL sample tubes. After each sampling the whole tubing system is steam sterilized. For further off-line treatment a robot takes the sample to the different devices. This robot is equipped with a camera and a force/torque sensor. A color-based object recognition guides the arm in a complex surrounding with different illumination situations, enabling the robot to load the sampling device with tubes and take the sample to further devices. For necessary pipetting and refilling we developed a computerized device. Cells are automatically stained and counted using an imaging system. The cell number and viability is automatically saved in a process control system together with the on-line parameters. During several cultivations in 20 and 100 L scale these main components of the automation strategy were successfully tested.

Introduction

The automation of obtaining, analyzing, and storing samples from a cell cultivation process is the last key issue that enables complete automation in a production environment. During the cultivation of mammalian cells, samples have to be taken regularly in order to monitor the status of the cultivation. Counting a stained cell sample, analytical determination of some components (glucose, lactate, etc.), and the storage of a cell-free aliquot is the normal procedure for the operators. With today's technology, all other procedures can be automated. The physical parameters in a bioreactor (pH, pO_2, temperature, stirrer speed) are maintained automatically by digital controllers. Sensors are continuously monitoring these parameters, and all on-line data are stored in a process control system together with the off-line data (cell count, glucose concentration, etc.).

The on-line analyzer systems existing on the market today are directly coupled to a specific bioreactor (Van de Merbel et al., 1996). Typically, these sealed couplings are not steam-sterilized, 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 that fulfils this need for an application in industrial production environments.

In this approach, a set of standard equipment is used instead of developing a specially adapted apparatus, thereby keeping a high degree of flexibility to allow easy adaptation to other production plants.

Figure 1 shows a schematic setup of all devices involved in the automation strategy. The central component is a sensor-guided robot that takes the sample from the sampling device to the different machines. Currently the entire setup is connected to one pilot bioreactor. In the future it should be possible to disconnect the robot cell from the devices needed for sample treatment. The samples from the different bioreactors would then be brought to the robot by autonomous vehicles or by conveyors. For further off-line treatment, this robot takes the sample to the different devices. To compensate for uncertainties in the positioning of these devices, both

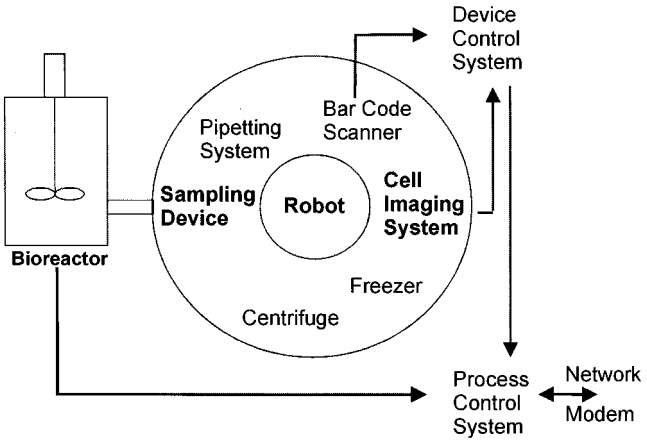

Figure 1. Schematic view of the whole setup. The machines marked in boldface are described in this paper. The robot takes the sample, filled up by the sampling device, to the different machines. The cell imaging system counts the cells. The whole system is controlled by the device control system. Barcode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

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Table 1. General Action Course for Sampling and Subsequent Treatment[a]

action	apparatus	description
sampling	sampling device	automated, sterile sampling from the bioreactor
moving sample tube	robot	taking sample to the pipette system
pipetting	pipette device	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 device	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

[a] After finishing the investigation three different types of sample tubes will be handled by the robot: sample tubes (50 mL), Cedex tubes (1 mL), and Eppendorf containers (1.5 mL). Movements of the robot to dispose or fetch tubes are not listed.

visual (obtained by a hand-mounted camera) and tactile information (obtained by a wrist-mounted force/torque sensor) are used to guide the robot. Therefore, the sensor-based robot is the active manipulation component of the automation system. A general action description is given in Table 1.

All devices involved are standard equipment with only slight modifications to cope with some of the robot's limitations. They still can be used by human operators for other tasks. Pipetting and refilling of cell broth or supernatant is done by a computerized pipetting system. Cells are automatically stained and counted using an newly developed automated imaging system (Gudermann et al., 1997). After a centrifugation step, cell-free aliquots are taken and filled into bar code marked tubes, which are stored in a freezer.

The whole setup is controlled by software (device control system) that triggers the single actions. Data such as cell count and bar code 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 of the cultivation from the office or from home. This automation strategy will reduce labor cost and maintain the high standard of the cultivation process.

This paper describes the main components of the automation strategy: the sampling device as the first key component, the cell counter that enables direct determination of living and dead cells, and the robot system as the connection between the sampling device and the machines. All systems were tested and optimized during the cultivation of mammalian cells in a 20 or 100 L bioreactor.

Material and Equipment

Cell Culture, The cultivations were done in a 100 L bioreactor (UD100, B. Braun Biotech International, Germany) and in a 20 L bioreactor (Diessel, Germany) that 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 leukemia 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). Total number of cells, which tend to aggregate, were 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). Single cells were determined in a Neubauer chamber or automatically (Cedex, Innovatis, Germany) using the trypan blue exclusion method (Tennant, 1964). To break up aggregates the sample was diluted 1:1 with Accumax (PAA Laboratories GmbH, Germany) and incubated for 10 min at 37 °C (shaken). The concentrations of glucose and lactate were measured with the automatic analyzer YSI 2700 S (Yellow Springs Instruments, U.S.). The lactate dehydrogenase (LDH) activity was determined by measuring the reduction of pyruvate to lactate, which was coupled to oxidation of NADH to NAD (Wagner et al., 1992). The activity data are expressed in kat/L. One Katal (kat) converts 1 mol of substrate per second.

Sampling Device. The sampling device (Figure 2) consists of a silicone tube with a stainless steel needle. To control the fluid/steam flow and to maintain the sterility, pneumatic valves (605, Gemü, Germany) and a pneumatic sampling valve directly in the bioreactor (A907-T, Südmo, Germany) were used. The sampling procedure is done using an *xy*-robot (ELVamat, ELV, Germany) to inject the sample into 50 mL tubes (Nunc, 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 first step is to flush the tubing system with cell broth, then the *xy*-robot moves to the tube and the sample is poured in. The third step includes a flushing of the system with condensate and the steam sterilization of the tubing system. For this purpose the needle is moved to a sterilization sleeve. The condensate is collected in a waste pot. The whole system is computerized using a real time operating system (QNX, QNX Software Systems, Canada) and C++-software including a graphical interface.

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

Cell Counting System. This system (Cedex, Innovatis, Germany) uses 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).

Peripheral Devices. For further handling of the sample other peripheral devices are needed**.** The pipetting system (PSD/2 and MVP, Hamilton, U.S.) allows the computerized pipetting of cell broth/supernatant and the filling of Eppendorf tubes and Cedex tubes by the robot arm. The cells are separated from the broth by centrifugation (Megafuge 1.0, Kendro, Germany). For safety reasons, this centrifuge is equipped with an additional

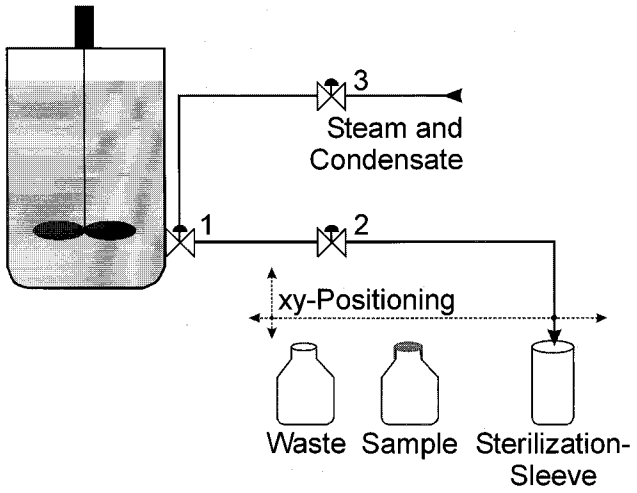

Figure 2. Diagram of the steam sterilizable sampling device. The sampling valve (1) is inserted into the bioreactor vessel. Steam serilization is done via valves 3 and 2. The *xy*-robot places the needle into the sterilization sleeve, waste, or a sample tube. The sample tubes are covered by a septum to ensure aseptic aerosol-free sampling. Using the computerized sampling device the number of samples and the volume are variable.

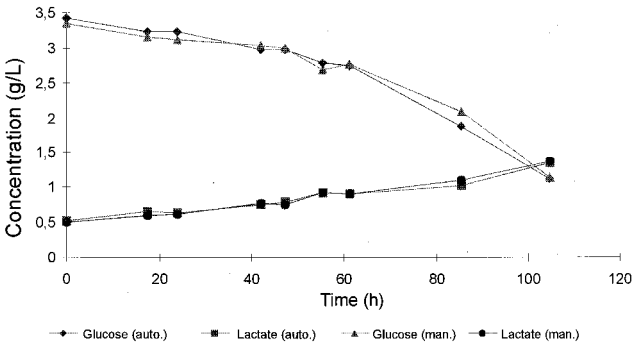

Figure 3. Comparison of manual and automatic sampling during a batch cultivation of hybridoma cells in 20 L scale. The analyzed concentrations of glucose and lactate show no difference. Therefore, the flushing of the tubing system with cell broth prior to sampling is sufficient.

interface showing the device control system the status of the lid. The whole automation procedure is controlled by the device control software running on a standard IBM-compatible PC with Linux as operating system.

A process control system (MFCS 2.0, B. Braun Biotech, Germany) stores the on- and off-line data. The data from the cell counter and the barcode scanner (Tc2100, Datalogic, Germany) are automatically transferred and stored into the MFCS. Figure 1 illustrates the entire setup.

Results

Sampling Device. Taking a sample from a bioreactor is a crucial operation for the system, because of the danger of an infection. There are two different methods to prevent an infection of the bioreactor, sterilization with a disinfectant and steam sterilization of the tubing system. The sterilization with a disinfectant bears the risk of residues in the system, which might have influences on the sample. For that reason, steam sterilization was chosen, the proven method in industry. Of course this method also bears the risk of influencing the sample. After the steam sterilization condensed water accumulates in the tubing system, which will have a dilutive effect, but there are no chemical substances that could have a direct influence on the sample.

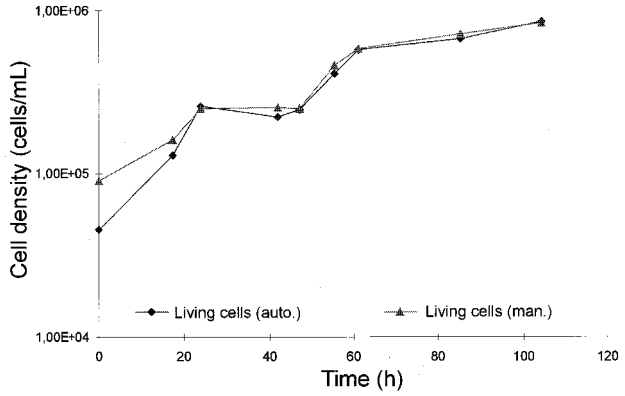

Figure 4. Comparing the cell count during the described 20 L cultivation. The manual and automatic sampling show the same living cell density. Each sample was counted three times, and the error was calculated to 5%.

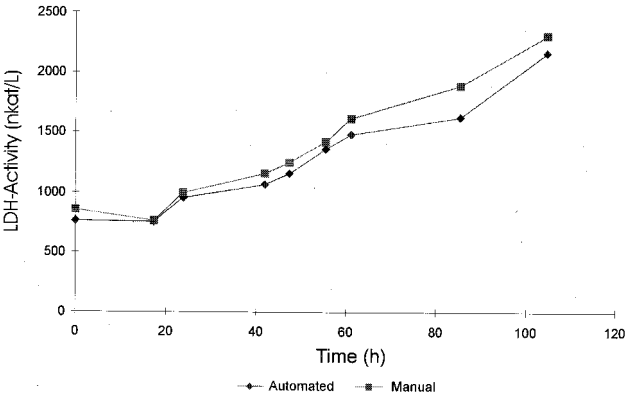

Figure 5. To reviewing possible cell disruption caused by the automated sampling device, the LDH-activity was determined. Comparison of manual and automatic sampling during the 20 L hybridoma cultivation the automatic sampling shows a slightly lower activity, which means that there is no cell disruption during automatic sampling.

In the newly designed sampling device, an injection needle is inserted into the tubes. The tube is sealed with a septum; therefore pollution of the surrounding environment with aerosols is minimized and the samples kept sterile. During sterilization, the injection needle is pressed down in the waste and sterilization sleeve, which are sealed by its special design and O-rings.

The reliability of the *xy*-positioning tool was evaluated by automated injection of the needle 25 times in succession; there was no deviation measurable. 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 any differences in lactate and glucose concentrations (Figure 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 (Figure 4). It is concluded that there are no sedimentation effects during the movement of the injection needle. Looking at possible cell disruption, the LDH (lactate dehydrogenase) activity was analyzed. In all measurements the LDH activity, as a rate for the damage of the cells, was slightly lower in the automatic samples than in the manual samples (Figure 5).

Considering these results, it can be concluded that the

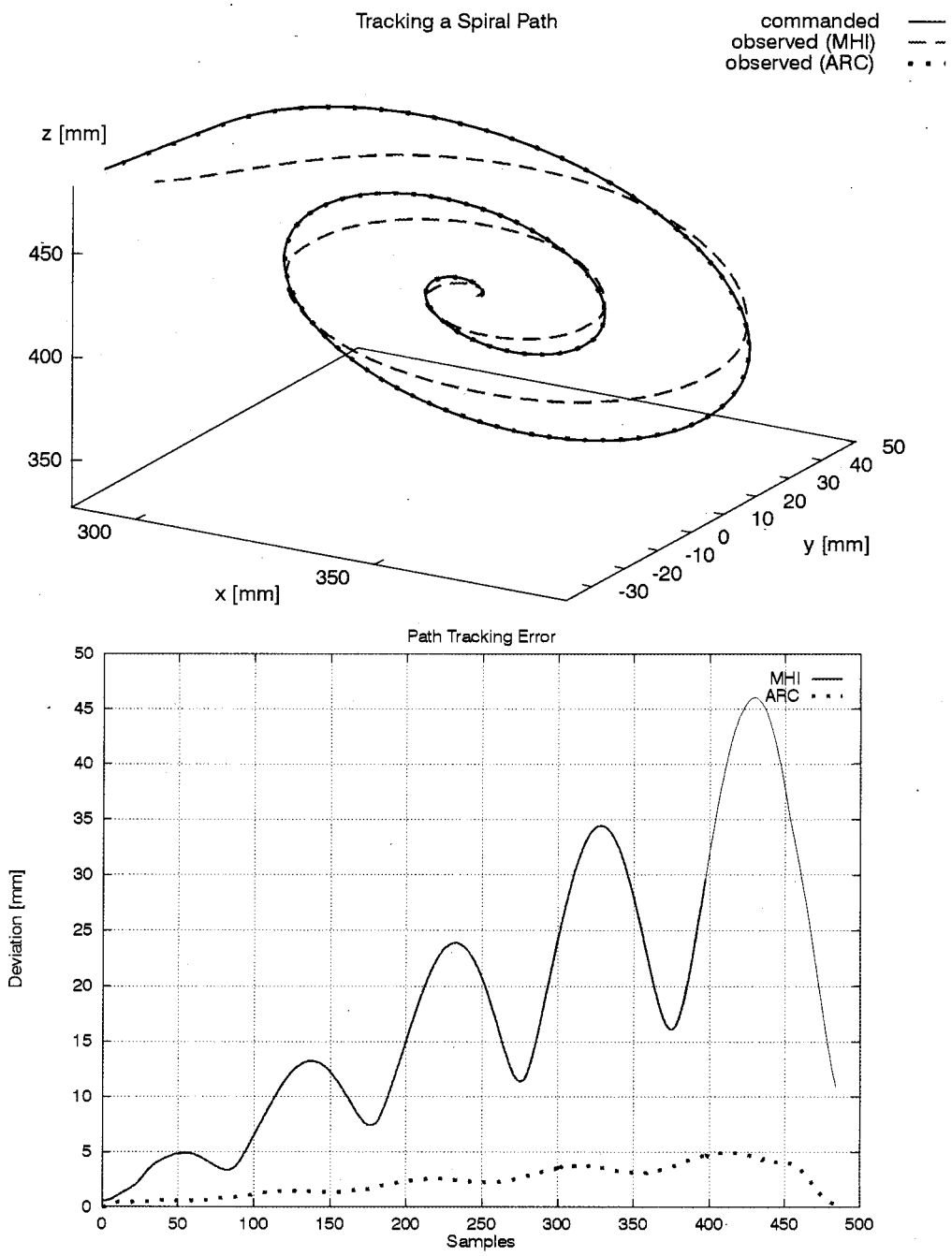

Figure 6. Path tracking accuracy. The upper figure shows the commanded and observed path for a sample motion when controlling the robot via the original MHI hard/software and RCCL using the ARCNet interface (ARC). The lower figure shows the path error for both cases; the error has been reduced by 1 order of magnitude.

new sampling device is a reliable means of sampling mammalian cell cultivation bioreactors.

The Robot. The PA-10 robot by Mitsubishi Heavy Industries, which is used to operate the different devices and to transport the samples between them, like any other robot requires a very high precision in measuring the positions of the objects to be manipulated. This poses the following problem: Because the system should be both easily adaptable to a modified spatial arrangement of the setup and tolerant against human interference on that arrangement, this precision can neither be initially achieved nor maintained in the long term. Because all devices are standard equipment only marked with colored 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 are 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-dependant. 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 orders 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 program-

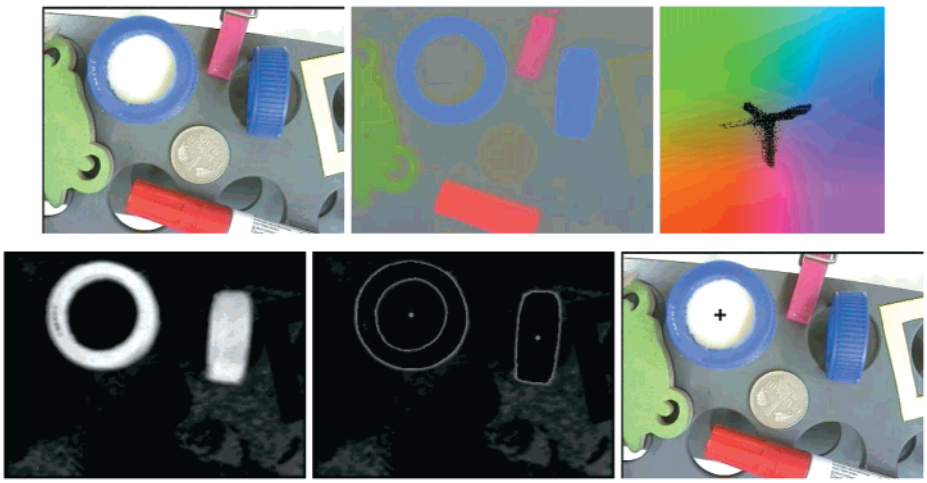

Figure 7. Color-based object recognition. The images from the upper left to the lower right show (a) the original image, (b) the color image, (c) the color distribution, (d) the distance measure computed from that distribution, (e) the filled distance measure, and (f) the recognized objects.

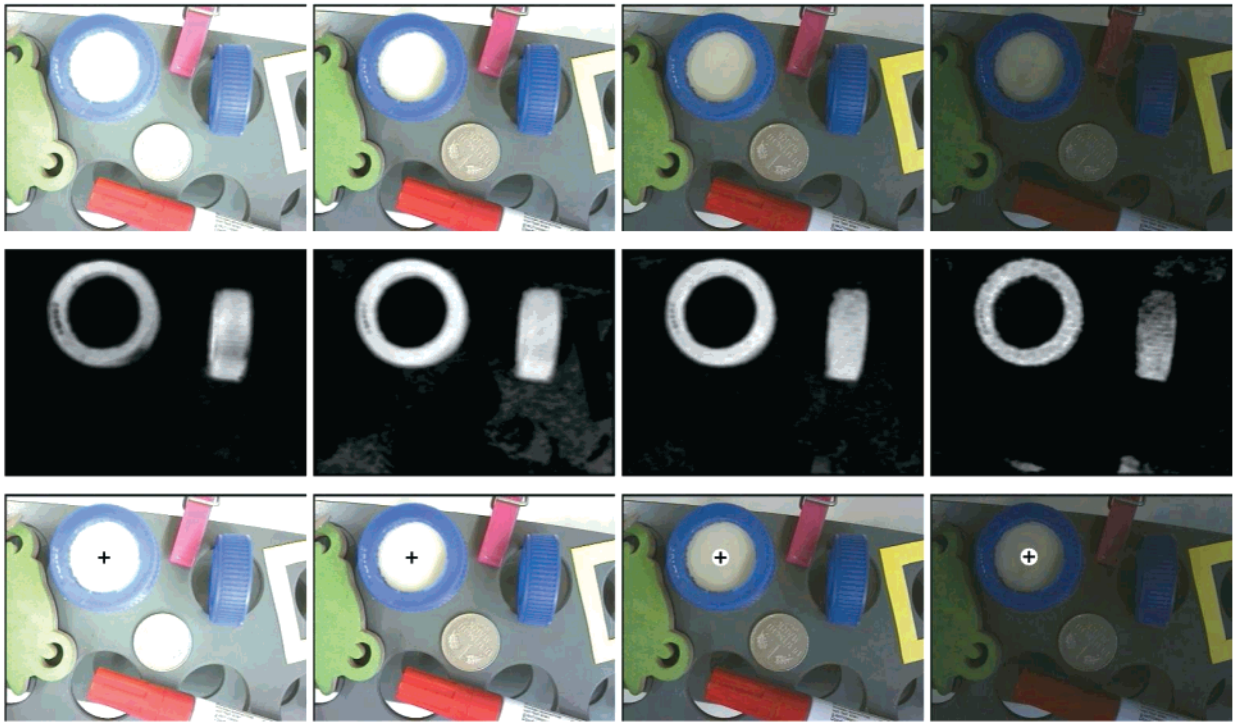

Figure 8. Illumination tolerance. The columns from left to right show the original image, the distance measure, and the recognized objects of an overexposed, a normally exposured, and two underexposed situations. In each situation the tube is correctly recognized.

ming languages usually lack. 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 improves the path tracking accuracy at joint control level, as can be seen in Figure 6.

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

Color-Based Object Recognition. The object recognition is based on the *2d* color component (CCIR-601 YUV, International Telecommunication Union) of the images, discarding the intensity component. On the basis of the goal to detect an object of at least approximately known color, the distribution of the color components of the image is projected into a *1d* measure of how closely a pixel's color matches the searched color using a coordinate transformation similar to the principal component analysis (PCA). 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. A sample situation can be seen in Figure 7, where the system reliably detects the blue cap of a tube despite numerous objects intentionally disturbing the scene.

Illumination Tolerance. A vision system operating in an environment that is primarily focused on humans must not be disturbed by typical human behavior interfering with the system, such as humans switching the light (partially) on and off. This approach is much more tolerant against varying external illumination conditions than the well-known grayscale-based edge detecting

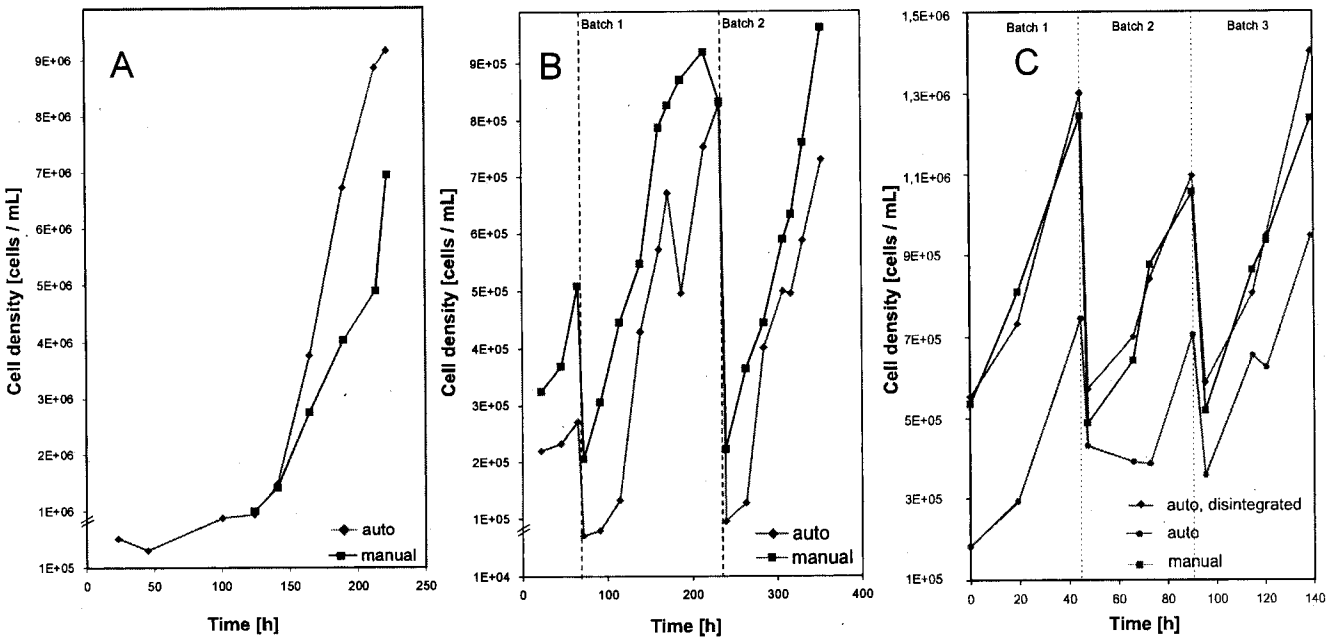

Figure 9. Adaptation of the pretreatment of the sample for cell counter. Comparison of automated and manual counting during a continuous cultivation of human leukemia cells in 20 L scale (A) and of rCHO cells in 100 L (B) and 2 L scale (C). Depending on the aggregation of the cells for the manual count the nuclei were counted; therefore those cells were not suitable for the cell counter. For a reliable analysis the aggregate size should not exceed 10 cells. However, after an optimized disintegration procedure the cell counter was able to accurately count living and dead cells (C).

approaches. A sample set of situations can be seen in Figure 8, where the system reliably detects the blue cap of a tube despite substantially varying illumination conditions.

Force Sensing. Because 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.

Currently, the robot can 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 it. Afterward, the robot takes the sample tube to the pipetting system, which is recognized by the visual system by its blue mark. The next step will be the handling of smaller tubes such as Eppendorf container and Cedex tubes by the robot.

Automated Cell Density Determination. In mammalian cell cultivation, the success of the cultivation process is monitored by various on- and off-line parameters such as pH, pO_2, and nutrient and metabolite concentrations. The most important parameter, however, is the actual cell density and viability.

The standard trypan blue dye exclusion method is time-consuming. The sample has to be prepared and evaluated by means of a Neubauer chamber. In the image of the microscope, the tinted and non-tinted cells have to be counted manually. The accuracy is highly dependent on the evaluating person and the number of squares counted.

With the new system (Cedex, Innovatis, Germany) 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, already filled by the pipetting system. By default, 20 images are evaluated in about 4 min. This results in a lower standard deviation compared to the manual cell counting.

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 (data not shown). In cultivations with cells that tend to aggregate, the automated cell density determination yields in deviating results (Figure 9A,B). 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 10 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 (Figure 9, C).

The external triggering of the measurement and polling of the results will be the final step for the integration of the Cedex into the automated sample management. With these features included, the entire process from sample acquisition to the determination of the cell density and viability is remotely controllable and ready for a "round-the-clock" service.

Conclusion

The main components of this automation strategy have been successfully tested. The sampling device and the cell counter were used during several cultivations at the pilot scale. There were no sterility problems and the automatic sampling showed the same results as a manual sample. After an optimization of the disintegration of possible aggregates, the cell counter was able to analyze 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 color-based object recognition, the robot arm showed its ability to repro-

ducibly find and grasp sample tubes under a wide range of different illumination conditions. It loaded the sample device and took the sample to the pipetting system.

The next step will be the establishment of the communication between the cell counter, centrifuge, and bar code reader with the device control system and the process control system for 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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