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Automatic Production of Induced Pluripotent Stem Cells

Ulrich Marx[a,*], Friedrich Schenk[a], Jan Behrens[a], Ulrike Meyr[a], Paul Wanek[b], Werner Zang[c], Robert Schmitt[a,e], Oliver Brüstle[d], Martin Zenke[b], Fritz Klocke[a,e]

[a]*Fraunhofer Institute for Production Technology IPT, Steinbachstr. 17, 52074 Aachen, Germany*
[b]*Institute for Biomedical Engineering, Department of Cell Biology, RWTH Aachen University Hospital, Pauwelsstr. 30, 52074 Aachen, Germany*
[c] *FH Aachen Campus Jülich, Heinrich-Mußmann-Str. 1, 52428 Jülich, Germany*
[d] *Institute of Reconstructive Neurobiology, University of Bonn, Sigmund-Freud-Straße 25, 53127 Bonn, Germany*
[e]*Laboratory for Machine Tools and Production Engineering WZL, RWTH Aachen University, 52056 Aachen, Germany*
* Corresponding author. Tel.: +49-241-8904-418; fax: +49-241-8904-6418.*E-mail address*: ulrich.marx@ipt.fraunhofer.de.

Abstract

Induced pluripotent stem cells (iPS cells) exhibit unique properties similar to embryonic stem cells (ES cells). A central feature of iPS cells is their pluripotency: they have the potential of growing indefinitely and differentiating into all cell types of the human body. Therefore, essentially unlimited numbers of somatic cells might be generated. Until now, there is a paucity of industrial processes for large scale production of human iPS cells and their differentiated progeny. This is mostly due to the high complexity of the manual processes, which are involved in the production of these cells. To take full advantage of the potential of iPS cell technology there is the need to produce iPS cell lines in high numbers, by employing automation, high throughput techniques and standardized protocols. Therefore we set up a large scale research project to develop a novel automatic production unit for reprogramming, cultivation and differentiation of iPS cells. A key step within the process chain is the automatic selection of primary iPS cell colonies in the culture dish. Within this study central elements of the automation process and quality control are presented.

Keywords: Induced pluripotent stem cells; iPC cells; iPSC; laboratory automation; measurement technology

1. Introduction

The potential of induced pluripotent stem cells (iPS cells) provides novel opportunities for generating essentially unlimited numbers of patient and disease-specific cells. Thus, iPS cell technology opens up new perspectives to test and validate novel drugs for hitherto incurable neurodegenerative or cardiac diseases directly in human cells. In the long term, iPS cell-based neuronal and cardiac cells may also be used as autologous source in cell replacement therapy. These exciting new biomedical perspectives demand for technologies to generate iPS cells and their differentiated progeny, such as neuronal and cardiac cells, in large quantities in a standardized and industrial format.

Until now there is a deficiency in industrial processes for large scale production of human iPS cells for drug development. One reason is the high complexity of the manual processes involved and the need for highly qualified and experienced laboratory staff. The adaptation of iPS cell production to industrial formats and automatic process steps for high throughput screening (HTS) has remained a major challenge. Established biotechnological methods aim for a large scale production of one specific cell type. The iPS cell technology however demands the production, generation and differentiation of large numbers of individual iPS cell clones with the respective quality control. The

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transfer of manual laboratory processes for reprogramming, selection and expansion to an automatic production process requires innovative solutions when it comes to the system control unit. A major challenge is the systematic parallelization of all biotechnological production steps.

A critical step for the entire process chain for generation of clonal iPS cell lines is the automatic selection of primary iPS cell colonies in the culture dish. Frequently, morphology of the colonies are analyzed by phase contrast microscopy. Additionally, fluorescence-based life cell imaging methods are used to detect pluripotency-associated markers. The robotic units being developed allow the purification and sorting of the detected iPS cells and are fully integrated into the production system. Within the framework of this project, we present initial data on how to automate the complex manual process steps for reprogramming, cultivation and differentiation of human iPS cells.

2. Methods

2.1. Reprogramming of primary fibroblasts to iPS cells

iPS cells exhibit unique properties similar ES cells. iPS cells are pluripotent: they have the ability to grow indefinitely and differentiate into all cells of the human body [1,2]. Frequently, iPS cells are generated by reprogramming of somatic cells, such as fibroblasts, towards pluripotency. This is achieved by targeted delivery of a specific set of reprogramming factors, including the transcription factors Oct4, Sox2, c-Myc and Klf4. 25 days later emerging iPS cells are isolated by mechanically picking individual colonies, which are further expanded under specific culture conditions with the growth factor bFGF. As shown in Figure 1, iPS cells show ES cell-like morphology, express a panel of specific pluripotency markers and exhibit an ES cell-like gene expression program. Hence, iPS cell quality is assessed by a panel of criteria prior to their expansion and use.

The reprogramming process described here employs Sendai virus vectors for delivery of reprogramming transcription factors. This delivery system was chosen to ensure efficient delivery and expression of reprogramming factors without modifying the genome of target cells. A specific Sendai virus variant is used to allow complete removal of virus vector after successful reprogramming [3,4]. The temperature sensitive virus variant can be removed from target cells by shifting the temperature to 38°C for 5 days. iPS cells are expanded under serum free conditions on specifically coated tissue culture surfaces (e. g. Matrigel). With time in culture the pluripotent iPS cell phenotype stabilizes, thereby yielding homogenous, fully reprogrammed pluripotent cell populations.

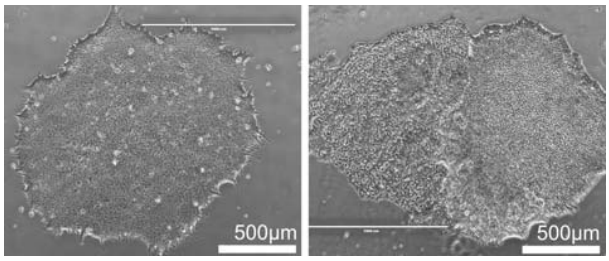

Fig. 1. iPS cell colonies are characterized by a distinct morphology, including a “smooth” colony rim. iPS cell differentiation is characterized by the loss of rim integrity, gross heterogeneity of cell morphology within the colony and the emergence of more differentiated cells with alternate cell shapes. (a) Human iPS cell colony with ES cell-like morphology grown on matrigel exhibits a well-defined rim; (b) Human iPS cell colony with large area of differentiated cells in the left part of the image.

When viewed by phase contrast microscopy undifferentiated human iPS cells grow as compact, multicellular colonies, as shown in Figure 1. Cells also exhibit a high nuclear-to-cytoplasm ratio and prominent nucleoli. These colonies are characterized by a distinct ”smooth” rim. iPS cell differentiation is associated with loss of rim integrity, gross heterogeneity of cell morphology within the colony and an increase of differentiated cells, exhibiting a large variety of cell morphologies.

2.2. Automation of the iPS cell culture process

The challenge in automation of the iPS cell culture process lies in the complexity and the long cultivation periods [5]. Starting point of the process analysis are flow charts describing the (i) manual process, (ii) the materials that are used and (iii) the general requirements. The next step is to merge the workflows and select devices for each process-cluster that are suited for the automation. After defining all disposable formats, a draft of the machine concept, including all automation steps, is designed. Additionally, non-deterministic processes, like iPS cell generation, require highly flexible handling systems, such as articulated robots. In an iterative procedure, process and machine concept are optimized in detail.

The next phase of developing the automated system for iPS cell generation is the detailed design of the machine including main frame and enclosure. Based on the layout of the lab devices all handling systems are specified. Collision analysis of all handling operations is essential to avoid extensive corrections during the implementation. To ensure the effectiveness of these methods the machine design follows hygienic guidelines

and is optimized for targeted air flow. To handle the complexity of the production unit, a customized control software is being developed. The key to reduce complexity is to divide the process into small unique workflows that are handled without interaction. To allow efficient automated scheduling, process data, e.g. duration, material or, devices, is added to each individual workflow.

2.3. Criteria for iPS cell quality

Frequently, iPS cell quality is assessed by a panel of criteria: ES cell-like morphology, expression of pluripotency markers by immunofluorescence (Tra-1-60, Tra-1-81, SSEA-4, Oct4), alkaline phosphatase staining, differentiation potential in embryoid body (EB) assays and teratoma formation in immune-deficient mice. These criteria have been extended by evaluating the genome-wide gene expression profile of iPS cells by DNA microarrays and specific software packages (e. g. PluriTest; [6]).

In the automated production system for primary iPS cells a high quality standard is maintained by several measurement technologies to monitor cell growth and to detect potential contaminations as early as possible. Further live cell imaging techniques are linked with customized image processing algorithms to allow efficient quality control processes.

3. Results

3.1. Automated reprogramming of primary fibroblasts

The prototype production unit is designed to generate up to 60 different iPS cell lines in parallel. From donor material to end product the reprogramming process takes up to ten weeks. To allow the necessary flexibility the operator can either start all cell culture processes manually or use a scheduler. The machine includes following main modules.

The border between operator and machine is a 5.30m x 2.60m x 2.80m housing, as seen in Figure 2, with 18 electrically lockable doors for good operator accessibility. Via the laminar flow system in the ceiling of the housing sterile air is continually blown downwards to ensure biological product and operator safety.

For fast and flexible handling, a KR5 sixx 850 CR Kuka (Germany) robot is used, which is installed on a linear transport slide to cover the whole operating area with a seize of 4m x 1.5m as seen in Figure 3. With a servo-electric gripper and a collision sensor from PTM (Germany), reliable material transport is guaranteed. The robot is not only suited for transport but also to perform complex movements, like pivoting of tubes and plates for liquid mixing. To ensure correct handling of tissue culture plates a 1D / 2D barcode scanner is integrated.

Material transfer between machine and lab-environment is realized via aerodynamically separated locks. These locks with integrated cooling systems are loaded by the operator with up to 2600 disposable pipette tips, 75 multi well plates, 100 centrifuge tubes and 16 media bottles. Liquid waste and used disposables are automatically disposed in a separated area, which has an additional aspiration system to avoid contamination.

All liquid handling tasks are executed by a gantry robot from HiTec Zang (Germany), which uses disposable pipette tips and detects fluid levels via an ultrasonic sensor. For automated media refill, pressure based fluidics and a heating system are integrated.

Fig. 2. Machine enclosure with laminar flow (right), external control system (left) and external incubator (left back)

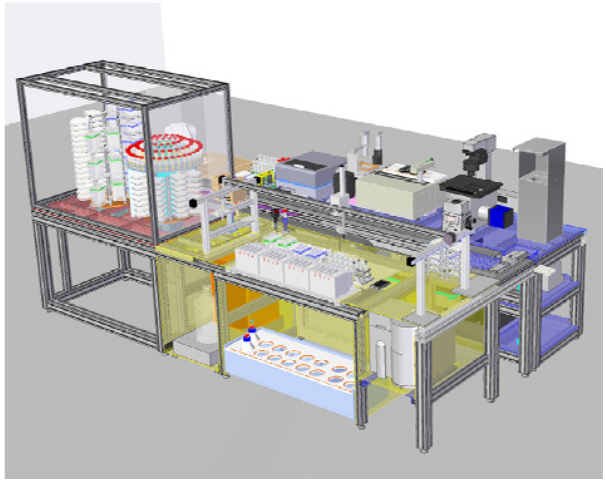

Fig. 3. View without enclosure: liquid handling area (yellow), lock area (red), process and quality control area (blue)

3.2. Automated quality control

For process and quality control several measurement devices are integrated into the automated production system. Both, commercially available devices as well as customized and specifically developed systems are integrated to provide an effective process and quality control.

3.2.1. Automated microscope

The main instrument for evaluation of cell colonies throughout the process is an inverted research microscope (Nikon Ti-E, Japan). The microscope is fully automated and provides bright-field, phase contrast and fluorescence imaging with magnifications ranging from 4x to 20x. The microscope automation includes (i) choice of objectives, (ii) setting of required phase rings, (iii) controlling of intensity of light sources and (iv) setting of wavelengths and filters required for fluorescence imaging. For fluorescence measurements three wavelengths (365, 470 and 565 nm) together with ET filter sets for EGFP, Texas Red and DAPI are implemented. LED light sources are used due to the longer life time.

The graphical software environment LabVIEW is employed to control the microscope and to communicate with the overall control level by means of web services (RESTful architecture) [7]. This communication scheme is used in a similar manner with the other measurement devices, which are integrated into the production system. The microscope images are analyzed by image processing methods (e.g. determination of confluence or colony morphology)

3.2.2. Cell counting device

For determining cell numbers and assessing the overall state of cell culture, we use the automated, image-based Cedex HighRes (Roche, Switzerland). The measurement method of this device is based on Trypan Blue Exclusion [8] and provides information about cell density, viability, aggregation rate, morphology and amount of cell debris in cell samples.

3.2.3. Camera station

For a macroscopic overview of cell cultures a camera station was developed, which allows precise positioning of microtiter plates in a LED background illumination and high resolution image acquisition. Photos are used for documentation to detect abnormalities, like yeast or fungi contaminations. Furthermore, the pH of the culture medium is read out by processing the color change of the phenol red indicator.

3.2.4. Photometer

Potential contaminations in cultures are detected by measurement of turbidity. This is achieved by measuring the absorbance with the spectrometer based microplate reader FLUOstar Omega (BMG Labtech, Germany).

3.2.5. Cell harvesting device

A key process of the automation is the harvesting of pluripotent cell colonies, which are subsequently further expanded to establish iPS cell lines. For automated colony detection and harvesting the AVISO CellCelector (Germany) is used [9]. This device is a freely configurable tool for automated transfer of single cells and cell colonies, which employs a harvest process with a gentle cell uptake directly from culture plates without enzymatic pre-treatment. Using this technology, high survival rates and cell integrity are achieved. The harvesting process is supported by an inverted microscope that offers phase contrast, bright field observation and fluorescence illumination. The selection process is based on image processing algorithms that detect the colonies to be picked and transferred.

4. Conclusion & Discussion

In this study we present data on developing an automatic production process for human iPS cells. Technological challenges are addressed by integrating smart monitoring solutions for detecting the cellular conditions and quality throughout the entire production process. By developing a transfer method from manual to automatic process protocols, a systematic approach is achieved. The project is currently at an early stage of its development. Reports on process and biotechnological efficacy are planned once the sterile cell culture process is running.

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