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An operational concept for long-term cinemicrography of cells in mono- and co-culture under highly controlled conditions – The SlideObserver

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ABSTRACT

Cell morphology, proliferation and motility, as well as mono- and heterotypic cell-to-cell interactions, are of increasing interest for in vitro experiments. However, tightly controlling culture conditions whilst simultaneously monitoring the same set of cells is complicated. Moreover, video-microscopy of distinct cells or areas of cells over a prolonged period of time represents a technical challenge. The *SlideObserver* was designed for cinemicrography of cells in co-and monoculture. The core elements of the system are the *SlideReactors*, miniaturised hollow fibre-based bioreactors operated in closed perfusion loops. Within the *SlideReactors*, cells can be cultured under adaptable conditions as well as in direct- and indirect co-culture. The independent perfusion loops enable controlled variation of parameters such as medium, pH, and oxygenation. A combined automated microscope stage and camera set-up allows for micrograph acquisition of multiple user-defined regions of interest within the bioreactor units. For proof of concept, primary cells (HUVEC, human hepatocytes) and cell lines (HuH7, THP-1) were cultured under stable and varying culture conditions, as well as in mono- and co-culture. The operational system enabled non-stop imaging and automated control of process parameters as well as elective manipulation of either reactor. As opposed to non-perfused culture systems or comparable devices for cinemicrographic analysis, the *SlideObserver* allows simultaneous morphological monitoring of an entire culture of cells in multiple bioreactors.

1. Introduction

Over the past two decades, the exact morphological observation of cultivated cells in vitro has become of increasing interest in many experimental setups. Cell migration, proliferation, differentiation and cell–cell interactions are vital to tissue (re)generation and organisation, and deregulation of these processes often cause dysfunction and disease. Cinemicrography of cultured cells and tissues is currently widely used to study such cell behaviours over time.

Various approaches have been conceived for time-lapse microscopy of living cells. A simple method is the repeated manual or automated image acquisition of the sample (Gogolla et al., 2006). Automated systems often consist of an incubation chamber mounted on a microscope utilizing standard cell culture materials (Ozturk and Erdogan, 2004; Wick et al., 2003). Microfluidic devices that enable video-microscopic monitoring offer the possibility to culture low numbers of cells, even down to the level of single cells, and retain good control of culture parameters (Ho et al., 2005; Petronis et al., 2006).

Primary cells require well-defined culture conditions in order to maintain physiological morphology and function. Standard systems subject cells to a changing gradient of metabolites and oxygen and require the exchange of media for long-term culture. Directly perfused systems meet cellular requirements more closely, but often necessitate additional cell protection (e.g., coating, sandwich layers) to minimise shear stress effects. In addition, automated observation of large numbers of identical sets of cells within these

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systems is not possible. The simultaneous conduction of experiments with various culture groups, i.e., with primary cells of the same origin, poses another limitation on the use of such systems.

Bioreactors based on hollow fibres provide a continuous supply of oxygen and nutrients and the removal of cell metabolites. At the same time, shear stress on the cells is minimised due to diffusive mass exchange across the membranes. Clinically, large-scale hollow fibre-based bioreactors have been studied as bioartificial liver support devices (Jauregui et al., 1995; Liu et al., 2001; Sauer et al., 2001). Continuous perfusion allows for extended culture periods and suggests that membrane-based bioreactors may be effectively used for long-term culture. However, on-line analysis of cell physiology is only possible via the monitoring of metabolites or oxygen and glucose consumption, but not by the observation of cell morphology.

The hollow fibre-based *SlideReactor* (Schwartlander et al., 2007), although suitable for light microscopy, is limited to the observation of one region of interest within a single perfusion system. For optimal exploitation of cinemicrographic analysis, a complete overview of all cultured cells is desirable. Furthermore, hypothesis-driven experiments necessitate the operation of multiple independent perfusion loops in parallel for control and experimental groups. At the same time, process parameters within all bioreactors have to be monitored and controlled independently from each other to warrant identical conditions apart from the experimental treatment. Here we present the *SlideObserver* as an operational concept for meeting these requirements.

2. Materials and methods

2.1. Bioreactor construction

The *SlideReactor* is a miniaturised cell culture device for continuous microscopic observation. Components and construction of the *SlideReactor* have been described in detail previously (Schwartlander et al., 2007). Briefly, silicone frames are cast on an adhesive cell-culture plastic slide to form two medium compartments for in- and outflow on each side of a cell compartment. Hollow fibre (HF) membranes (Micro PES TF 10, Membrana, Wuppertal, Germany) connecting the medium compartments cross the cell compartment and constitute it as extracapillary space. Two silicone tubes with Luer Lock-to-tube connectors are inserted to provide direct access to the extracapillary space. The cell compartment has an average volume of 2.5 mL, and the medium compartments hold 0.25 mL each. The HFs ($n = 16$) integrated into each *SlideReactor* have a diameter of 500 μm and a length within the cell compartment of 52 mm, forming an intracapillary space of 163 μL. The extracapillary surface area for cell adhesion averages 10 cm^2. After assembly, each bioreactor is tested for shell integrity and membrane permeability using a pressure sensor. The reactors are then gamma sterilised according to a DIN certified protocol (BBF sterilisation service, Kernen, Germany).

2.2. SlideObserver setup

The *SlideObserver* setup comprises a microscope for image acquisition, devices for the control of process parameters and data storage, and analysis and perfusion circuits for the continuous operation of the bioreactor units (Fig. 1A).

One single perfusion system consists of four main components connected via standard polyethylene tubes: the bioreactor, the oxygenator, a peristaltic pump for recirculation of the medium (IPC 12 peristaltic pump, Ismatec, Wertheim, Germany) and a medium reservoir in which the combined pO_2 and temperature probe (D100OxyProbe, Broadly James, Irvine, USA), a pH-probe (SteamLine SL 80-120pH, Schott Instruments, Mainz, Germany) and a custom-made gas sparger are inserted.

For the operation of multiple parallel loops, the bioreactors are positioned on a microscope (Axiostar plus, Zeiss, Jena, Germany) equipped with a custom-made motorised stage and fine focus (Märzhäuser, Wetzlar, Germany). Micrographs are acquired using a digital camera (SPOT Insight Color Mosaic, Diagnostic Instruments Inc., Michigan, USA) with an adapted implement of the VisiView software (Visitron Systems, Puchheim, Germany). Perfusion loops and microscope are placed in a temperature-controlled incubator (KB 720, Binder, Tuttlingen, Germany). pH and pO_2 of the circulated medium is altered by the composition of the process gas provided by a multichannel gas mixing unit and applied directly via the sparger and/or indirectly via the oxygenators. Together with a sensor module, each perfusion loop can be addressed separately (DasGip, Jülich, Germany). Camera, microscope stage, incubator, gas mixing unit and sensor module are interconnected and controlled by one standard PC equipped with additional com-ports.

2.3. Control of process parameters in multiple reactor systems

Process benchmarks of the system were tested in two independent perfusion loops. Thirty milliliters of DMEM was reperfused in each loop and the entire system was pre-warmed to 37 °C until both the incubator sensor and the probes stably showed the same value. The system was then subjected to two gassing protocols. Firstly, physiological pH was maintained whilst inducing hypo- and hypoxia. Secondly, air ambient pO_2 was maintained and pH was altered via maximum/minimum CO_2 percentages. All protocols were repeated 4 times.

2.4. Image acquisition and processing

Micrographs were acquired using an adapted VisiView software implement, which controls both the automated microscope stage and the digital microscope camera. Regions of interest within the reactors were focussed, and the coordinates in *X*, *Y* and *Z* direction were stored for each point. Depending on the experimental setup, recording intervals ranging from 1 to 10 min were programmed. The automated image acquisition was then monitored on a daily basis until the end of the experiment.

Images were processed into time-lapse micrographic videos using Graphic Converter (Lemke Software GmbH, Peine, Germany) and iMovie (Apple, Cupertino, CA, USA). ImagePro (MediaCybernetics, Bethesda, MD, USA) was used for in depth morphological analysis of cell size and tracking of motile cells.

2.5. Cinemicrographic analysis of morphologically altered HUH7 cells

For proof of concept, identical cells that were morphologically modified prior to inoculation and native control cells were examined. HuH7 cells labelled with microscopically visible particles of iron oxide were compared with unlabelled cells under identical conditions in both perfusion loops. Labelling of cells with MPIO is described in detail elsewhere (Raschzok et al., 2008). Labelled and control cells were trypsinized, and 4×10^5 viable cells were inoculated into each unit. Cells were allowed to adhere for 1 h before reperfusion and image acquisition. In each bioreactor, five areas were observed and images were taken every 10 min for 24 h. Processed cinemicrographs were visually analysed for cell adherence, particle load, mitosis and cell death.