Microfluidic system for in-vitro hypoxia assays


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ABSTRACT

Hereby presented is a microfluidic system, including a micro pump, an oxygenator and a cell culture chamber for perfusion controlled hypoxia assays. It consists of laser-structured polycarbonate (PC) foils and an elastomeric membrane which were joined together using thermal diffusion bonding. The elastomer forms an oxygenator element. The microfluidic system is characterized using non-invasive flow measurement based on micro-Particle-Image-Velocimetry (µPIV) and optical oxygen measurement utilizing the oxygen dependent fluorescence decay. Based on those experimental results and mathematical considerations, the oxygenator and mass transport phenomena within the microfluidic system can be described. This oxygen sensor, the micro pump, a controlling device and the gas mixture at the oxygenator forms a regulatory circuit to adjust the oxygen content in the cell culture chamber and helps to produce well-defined hypoxic conditions for the cells.

Keywords: Hypoxia, Cell culture, Lab-on-a-chip, Perfusion, µPIV, Microfluidics

1. INTRODUCTION

Because of its direct and indirect participation in many metabolic processes in cells, tissues and organisms oxygen is one of the most important parameters. Especially in mammalian tissue the adequate supply of oxygen is an essential prerequisite influencing cell viability, proliferation and differentiation. Oxygen shortage is called hypoxia and leads to changes in cellular behaviour. Among others it induces the release of hypoxia inducible factors (HIF) and vascular endothelial growth factor (VEGF) which affect cell metabolism, wound healing, angiogenesis and tumor metastasis. Therefore a deeper understanding of cell response on oxygen limitation is of particular interest in medical basic research. For this issue lab-on-a-chip (LOC) devices have become more and more important for in-vitro cell cultivation. Especially perfused LOCs are able to mimic physiological environment inducing mechanical stimulation and convective mass transfer. Currently most LOC systems are externally perfused. In contrast to that the Fraunhofer IWS developed a multilayer based LOC with on chip actuation. This offers the possibility to create micro circulation systems with a high tissue to cell culture media ratio (volumes in the range of several microliters). Moreover the effect of dilution which appears in flow through LOC is negligible. The mentioned micro circulation approach is superior for the examination of metabolites and their effect on several tissue types. Due to its good optical properties established imaging technologies can be applied throughout the complete microfluidic system. The on-chip micro pump was characterized using micro-Particle-Image-Velocimetry and a mathematic model was developed. In the present study an additional oxygenator element is implemented into the IWS microfluidic platform, allowing automated and reproducible hypoxia assays on the chip. In Figure 1 the principle of the perfusion controlled hypoxia is shown.

Figure 1. Principle of perfusion controlled hypoxia.
2. MATERIALS AND METHODS

2.1 Microfluidic system

One of the main benefits of the presented microfluidic system is the possibility to integrate microfluidic actuators to create completely closed and perfused microfluidic circuits and microvascular systems. This approach allows circulating volumes in the range of several µL. External pumping devices are not needed as it is mostly the case in microfluidic cell cultivation devices presented in literature\(^9\). The microfluidic system is produced with a layer-by-layer manufacturing technology of laser-cut polymer foils\(^{10}\). The mentioned fluidic actuators are pneumatically driven systems\(^{11}\). Therefore a flexible membrane has to be integrated into the microfluidic system, which can be easily displaced with pressure rates up to 100 kPa. A commercially available silicone foil (SILPURAN® FILM, Wacker Chemie AG) with a thickness of 200 microns and high gas permeability is perfectly suited for this application. In Figure 2a an exploded view of the microfluidic system with mounted reservoirs on top, the pneumatic part, the elastomeric membrane in the middle and the fluidic part at the bottom is shown.

![Figure 2](image-url)

Figure 2. Microfluidics used for hypoxia assays: a) exploded view; b) microfluidic layout; c) image of complete system.

The complete system is mounted in a chip-holder with two connection elements which include the pneumatic fittings. In Figure 2b the developed microfluidic layout is shown. It features a micro pump for fluid actuation and a microfluidic valve. With the help of this valve the system can be either operated as flow-through or totally closed fluidic. In previous fluidic designs the oxygen exchange was solely performed by the gas-permeable elastomeric pump membrane. So the pump acted as a combined flow source and oxygenator\(^{12}\). This may be problematic especially when gas bubbles are produced, as reported by Goldowsky\(^{13}\). To enhance the oxygen transport capability an additional helical oxygenator element was placed at the right side of the microfluidic system utilizing the same gas-permeable membrane also used for fluid actuation whereby the gas exchange area of this oxygenator is ten times larger than that of the pump. The process gas flow at the top of the membrane can be adjusted to avoid oxygen depletion over the length of the oxygenator. Two oxygen sensing spots (one in the process gas channel and one in the cell culture chamber) are implemented to measure the oxygen content applied on top of the oxygenator membrane as well as the amount of oxygen applied to the cultivated cells. Flow velocity measurements can be performed easily on each straight channel segment (A, B, C).
2.2 Controlling unit

To control the oxygen content in the cell culture segment a controlling unit based on an embedded Linux device is used. Figure 3 shows a block diagram of the system.

It switches up to 24 pneumatic outputs to actuate the pumps and valves on the chips. Furthermore it is capable to ensure stable temperature conditions and can mix three different process gases (oxygen $O_2$, nitrogen $N_2$ and carbon dioxide $CO_2$) to control the oxygenator. This gas mixing is not performed by mass flow controllers as in medical ventilation systems but utilizes pulsed gas dosing with an electromagnetic switching valve. The process gas is mixed and humidified by a bubbler to prevent pervaporation through the oxygenator membrane and to ensure a constant oxygen concentration in the process gas stream. Several digital interfaces (I²C, ETHERNET, CAN …) give the opportunity to integrate the controller in other laboratory infrastructure or laboratory information management systems.

2.3 Flow velocity measurement with micro Particle-Image-Velocimetry (µPIV)

The micro-particle image velocimetry (µPIV) is a non-invasive flow measurement technique. It is therefore well suited to characterize the transient flow behavior in fully closed microfluidic systems as the presented hypoxia-on-a-chip system. Small particles flowing with the media stream are utilized to observe the fluid movement with a high-speed camera. The experimental setup was described in detail earlier. For image processing an optimized cross-correlation algorithm is used, which can deal with large image stacks in a short time but only works when the flow velocity vector has only one component. This is, for example, the case in the straight channel segments at the outlet of the oxygenator device as well as before and after the micro pump. In Figure 2b those three µPIV measurement spots A, B, C are indicated.
2.4 Optical oxygen sensing

In presence of oxygen, the fluorescence lifetime of several fluorescent dyes is decreased due to quenching. This decay in the fluorescence lifetime can be utilized to measure the oxygen content in the microfluidic system as shown in Figure 4. The experimental setup was described earlier in detail. Commercial available CPOx-beads (Colibri photonics, Potsdam, Germany) are immobilized with Polydimethylsiloxane (PDMS) in the cell culture chamber and the process gas inlet of the oxygenator. The fluorescence signal was measured using a compact optic block coupled to a photomultiplier and the OPAL digital lock-in amplifier (Colibri photonics, Potsdam, Germany) which calculates the fluorescence decay signal and communicates to a host-PC via USB. Each measurement spot can be automatically evaluated using a 3-axis positioning system (Nanoplotter 2.1, GeSiM mbH, Großerkmannsdorf, Germany) controlled by the measurement software. For calibration purpose the microfluidic system was flushed with three different process gases with varying oxygen contents \( w_{O_2} \) compared to the volume fraction of oxygen in atmosphere under standard conditions (\( \phi_{O_2} = 20.9 \text{ vol.\%} \)). Those gases were: Air (\( w_{O_2} = 100 \% \)), reference gas (\( w_{O_2} = 47 \% \)) and nitrogen (\( w_{O_2} = 0 \% \)). Afterwards the microfluidic system was filled with DI water for the oxygenation and deoxygenation experiments. During the experiments pressure and temperature where measured to compensate variations of the oxygen solubility in water.

![Figure 4. Principle of oxygen sensing based on fluorescence lifetime measurement.](image)

3. RESULTS

3.1 Mathematical considerations

A detailed mass transport and flow model of the microfluidic system was presented in a previous work. Modelling was based on a network approach using a special hydraulic library for the simulation tool SimulationX (ITI GmbH, Dresden, Germany). Like in an electronic circuit hydraulic networks consist of several functional blocks (micro pumps and valves, micro channels, reservoirs, exchange membranes, etc.) which were joined together using fluidic connections. A mathematical description of the micro pump was given recently. In each connection both Kirchhoff’s laws have to be fulfilled, whereby the fluid flow is analog to the electrical current and the pressure behaves like the electrical voltage. The complete hydraulic network is then described by a time dependent system of equations with the flow in each functional block as time-dependent variable. Based on the flow distribution in the microfluidic network the mass transport can then be calculated. This mass transport is driven by diffusion and convection processes and can be described by the convection-diffusion equation:

\[
\frac{\delta c}{\delta t} = \nabla \cdot (D \nabla c) - \nabla \cdot (\vec{v} c) + R
\]  

(1)

Whereby \( c \) describes the concentration of the respective substance, e.g. oxygen, \( D \) is the diffusivity of this substance in the used media e.g. water, \( \vec{v} \) is the flow velocity vector and \( R \) is the reaction term. From particular interest for the hypoxia chip is the gas exchange at the oxygenator membrane. In Figure 5 the gas exchange process is shown schematically. The process gas is flushed above the membrane with a constant flow rate and a continuous oxygen...
distribution, which corresponds with a constant oxygen source at the top of the membrane. Below the membrane the liquid is transported by the micro pump contrary to the process gas flow which leads to an oxygen transfer over the length of the oxygenator. The oxygen transfer can be described via the oxygenation coefficient ($K_{O2}$):

$$K_{O2} = \frac{c_B}{\alpha \cdot p_{O2}} = \frac{w_B}{100\%}$$  \hspace{1cm} (2)

Whereby $c_B$ is the oxygen concentration at the oxygenator outlet, $\alpha$ is the oxygen solubility in water and $p_{O2}$ is the oxygen partial pressure applied to the oxygenator. Following mathematical formula gives an approximation for $K_{O2}$ for an rectangular shaped membrane oxygenator using the Gaussian error function $^{22,23}$:

$$K_{O2} = 1 - \text{erf}(f(h, L)) + \frac{2}{\sqrt{\pi h}} \left( 1 - e^{-\left( \frac{h^2 - v_z}{2D} \right)} \right)$$  \hspace{1cm} (3)

With the oxygenator height $h$, length $L$ and the mean flow velocity $v_z$ one can calculate the oxygen transfer capability of the oxygenator. Moreover the transient oxygenation/deoxygenation behavior is influenced by the oxygen storage capability of the used elastomeric membrane material $^{24}$ which leads to a delayed oxygen transport$^{18}$.

![Figure 5. Mathematic model of the oxygenator.](image)

The oxygenator is not the only gas transfer surface in the microfluidic system. As described earlier the pump membrane is permeable for oxygen too and therefore influences the oxygenation/deoxygenation behavior of the system $^{25}$. A mathematical description of the gas exchange at the pump membrane is difficult due to the unknown flow distribution in the pump chamber and the periodically actuated membrane. Therefore the parasitic oxygen input $K_p$ has to be measured. The $\mu$PIV measurements pointed out the unsteady nature of the flow produced by the micro pump. The mass transport under pulsatile flow can be described with the dimensionless Reynolds number $Re$ and the Strouhal number $St$ $^{26}$. For a rectangular channel (width: $1$ mm, height: $0.25$ mm), a kinematic viscosity of $1$ mm$^2$ s$^{-1}$ and a pumping frequency $f$ of $1$ Hz, $Re$ is $6$ and $St$ is $0.08^{18}$. Both values are much lower than the critical values ($Re_{crit} \approx 1400$ and $St_n \approx 1.92$) where turbulence, especially in cavities, can occur, which will enhance mass transport there$^{26}$. Therefore, the flow is laminar and can be seen as quasi static. One can replace the pulsatile micro pump by a constant flow source in the mass transport model$^{18}$. This mean flow rate $Q$ can be calculated from the velocity-time curve shown in Figure 6 by integrating it over the channel cross section area $A$ and a complete pumping cycle (period duration $T$):

$$Q = \int_A \left( \frac{1}{T} \int_0^T v(t) \, dt \right) \, dA$$  \hspace{1cm} (3)
3.2 Flow velocity measurements at different channel positions

The flow velocity was measured at the three measurement spots A, B, C mentioned earlier in the text, to characterize the oxygen transport capability of the microfluidic system and the shear forces applied to adherent cells. Following process parameters were chosen:

- **Pump**: pumping pressure: 50 kPa, filling pressure: -50 kPa, pumping period: 1.25 s (complete cycle)
- **Channel**: width: 1 mm, height: 0.25 mm, length: 4 mm
- **Imaging settings**:
  - Observation window: 30 x 1024 Px (129 x 4403 µm)
  - Framerate: 6622 fps
  - Exposure time: 4 µs
  - Magnification: 4.3 µm / Px
  - Image stack: 25000 frames
- **Particles**: Polystyrene beads with 10 µm mean size diluted in DI-water.

The respective velocity-time curves are shown in Figure 6.

![Figure 6. Measured velocity-time-curve at different spots in the microfluidic system.](image)

The measured velocity-time curves are in good congruence to µPIV measurements performed in other microfluidic systems with integrated micro pump. It can be seen, that the flow is pulsatile with peak velocities up to 1000 mm s⁻¹ and that there are time periods where no fluid movement can be observed at all. Moreover the velocity peaks are strictly connected to position in the microfluidic system. The highest peaks can be measured directly at the filling side of the micro pump. Which is for a clockwise actuation cycle situated left of the pump, at the spot A. The velocity-time curve between oxygenator and cell culture chamber (spot C) is much smoother with a peak velocity of 300 mm s⁻¹. This
smoothing effect can be explained with the fluidic damping of the integrated oxygenator. Due to the high flexibility of the oxygenator membrane it is displaced during the pumping process and acts as microfluidic capacitor which dampens the flow over its length. This is from particular interest if shear sensitive cells are cultivated inside the cell-culture chamber. An evaluation of the shear stress to adherent cells was done with a comparable microfluidic system.\textsuperscript{28}

3.3 Oxygen measurements in the microfluidic system

To characterize the microfluidic system the experimental setup shown in Figure 7 is used.

![Figure 7. Experimental setup used to characterize oxygenation/deoxygenation behavior of the system.](image)

The transient oxygenation/deoxygenation behavior can be determined by measuring the step response of the system when the process gas is changed. Therefore all channels were firstly flushed with DI water and afterwards different gas mixtures where applied to the oxygenator and the oxygen content was measured in the cell culture chamber under perfusion. Following process parameters where chosen for the experiments:

- Pump: pumping pressure: 50 kPa, filling pressure: -50 kPa, pumping period: 0.8 s (complete cycle)
- Gas-mixer: pressure: 50 kPa, gas flow: 0.1 L min\textsuperscript{-1}

In Figure 8 two different oxygen-time-curves where shown. On the left side one can see the measured oxygen content in the chamber when pure nitrogen is applied to oxygenator and micro pump. The right picture shows the oxygen-time-curve for a sequentially application of a mix of 67 % nitrogen and 33 % air and afterwards 100 % air.

![Figure 8. Left: Oxygen-content-curve for a complete media deoxygenation with nitrogen applied to oxygenator and pump; Right: Deoxygenation-oxygenation behavior with a mixture of 33 % air and 67 % nitrogen applied to the oxygenator](image)
Between minute 7 and minute 9 after starting the experiment, the fluorescence signal was disturbed because an air bubble entered the cell culture chamber. Therefore an oxygen concentration of 0 vol. % was detected during that time. It can clearly be seen, that the oxygen content is strictly connected to the process gas applied to the oxygenator. If pure nitrogen is laid out at the micro pump and the oxygenator a minimum oxygen content of 1.8 vol. % can be achieved in the culture chamber. Normally oxygen contents between 1-2 vol. % are required to induce hypoxic conditions and hypoxia inducible factors are released by the cells. So the developed microfluidic system should be well-suited for hypoxia assays. The second experiment pointed out, that the flowing media can be re-oxygenated after hypoxic conditions where applied and that the oxygenation/deoxygenation process is finished within 10 to 20 minutes. Moreover different oxygen contents can be achieved when the process gas is a mixture of air and nitrogen. For a pulsed dosing of 33 % air and 67 % nitrogen the oxygen content in the cell culture chamber reached 8 vol. %, which is really close to the expected value of 7 vol. % (corresponds to one third of the oxygen saturation in water). Although a value of 8 vol. % oxygen content is not connected to hypoxic effects; those intermediate oxygen concentrations can be from particular interest because within our body most organs don’t receive fully oxygen enriched blood. This means that the oxygen level in most tissue is below 21 vol. %, which also influences cell differentiation and maturation processes for example of human cardiomyocytes. So the developed oxygen control system may help to recreate the in-vivo like situation in the microfluidic devices much better. This can be done for example with an oxygen regulation implemented in the controlling unit.

To achieve this goal, the determination of the parasitic oxygen input by the micro pump is necessary. This was done by measuring the oxygen content (in percent) on both spots (w_A, w_B) before and after the oxygenator simultaneously. Following system of equation can then be formulated:

\[
\begin{align*}
w_A &= 100\% \cdot \frac{1}{K_{O_2}} \left( \frac{1}{K_{O_2}} + \frac{1}{K_p} - 1 \right) \\
w_B &= w_A (1 - K_{O_2})
\end{align*}
\]

With an oxygenation coefficient (for the oxygenator) of \( K_{O_2} = 0.4 \) (calculated with equation (3) and a length of \( L = 110 \) mm, a height of \( h = 0.25 \) mm, a diffusivity \( D = 2 \times 10^{-3} \text{ mm}^2 \text{ s}^{-1} \) and a mean velocity of \( v_z = 30 \text{ mm s}^{-1} \) ) a parasitic oxygen input of \( K_p = 0.25 \) (for the micro pump) can be obtained.

4. CONCLUSION

A microfluidic cell cultivation device with integrated oxygenator and micro pump is presented which can be operated with different process gases to generate well-defined hypoxic conditions in one or more cell culture segments. Both, the pneumatic micro pump as well as the oxygenator is operated by a controlling unit. Due to the clearness of the used polymer foils (in the visible wavelength range) which were joined together to form the microfluidic system established imaging technologies can be applied everywhere at the microfluidic system. Therefore the impact of hypoxia can be studied online in perfused 3D cell culture models. Possible applications could be the observation of metastatic spread or wound healing processes under hypoxic conditions. A flow velocity measurement in the microfluidic system was performed using non-invasive micro Particle-Image-Velocimetry (µPIV) on Polystyrene beads at three different spots. The micro pump produces a unsteady flow with peak velocities up to 1000 mm s^{-1}, as described earlier. From particular interest is the fluidic damping by the helical-shaped oxygenator, which acts as fluidic capacitor and reduces the peak velocities to 300 mm s^{-1}. This is from special interest when shear sensitive cells like hepatocytes or cardiomyocytes should be cultivated in the culture chamber later on. So the oxygenator can be used as fluidic damping element even if no gas exchange is necessary for the cell cultivation. Moreover commercial available oxygen sensitive particles are immobilized at different measurement spots to characterize the oxygenation coefficients of the micro pump and the oxygenator. Firstly the oxygenator was operated with pure nitrogen as process gas resulting in a media deoxygenation. Afterwards the process gas was switched to compressed air which induces the oxygenation of the pumped fluid. Subsequent to the characterization of the oxygenator an oxygen regulator should be implemented in the controlling unit allowing the precise adjustment and regulation of oxygen levels even for different oxygen consumers. Moreover the
implementation of the previously developed substance transport model of the microfluidic system in the controller is possible, allowing a “model-in-the-loop” based regulation of the system.

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