Chapter 9

General discussion and future outlook

Trends in science nowadays show great similarity to developments in the global economy: quantity over quality, short-term satisfaction over long-term goals, competition instead of collaboration, and buying instead of making. While researchers all around the world are aware of the flaws in our current model for performing research, it seems impossible to reverse [1]–[3]. Despite its many negative implications, there are some positive outcomes of this fast-science approach as well. The main advantage is that we have been forced to become more effective, and technological developments to support scientists have been rapid. In the case of life sciences it means that we can obtain more (detailed) information with less effort. Currently, researchers in all fields have methods at their disposal which yield and analyze tremendous amounts of data from a single experiment. The most important enabler in this regard has been the digital revolution (improved computing power, computer memory, fast Internet connection, constantly growing databases with information about every topic). Advances in the field of electronics also contributed to the appearance of a new field in science, namely microfluidics, in the early 90’s, in which microchip technology is used to make fluidic rather than electronic conduits. This particular field is focused on the miniaturization and integration of laboratory equipment, and is becoming more present in commercially available products [4]–[6]. Microfluidic tools/devices are expected to improve the throughput of experiments, to keep up with the increasingly powerful computers responsible for data processing from those experiments. Microfluidics, since its introduction in 1990, has contributed to the miniaturization and integration of laboratory equipment into chip format, which in some cases literally fit in one’s hand [4], [7], [8]. Additionally, microfluidic technologies were positively received in the field of cell biology due to the possibility of flexible geometry design and the possibility of application of physical cues affecting cells in vivo [7]–[11]. Moreover, microfluidics seems an ideal solution to accommodate the modern-day scientific trends where we want to do more experiments, in less time, with as much automation as possible.

This thesis describes two major topics that relate to the above, namely, the development of a miniaturized system to better mimic mammalian vasculature than in well-plates for cultivating cells (Chapter 3 and 4), and the development of an optical chip with integrated platform for real-time and label-free cell culture monitoring in microfluidic experiments (Chapter 5, 6, and 7). In essence, all parts of this thesis are tethered by microfluidics to one interdisciplinary entity, which attempts to develop a novel approach for label-free, real-time cell monitoring in a device with a channel geometry that better resembles those occurring in vivo. To facilitate our scientific endeavors we exploited 3D-printing technology to improve user-friendliness of our experimental setups and accelerate progress in our research (Chapter 8).

9.1. Optical methods for real-time cell monitoring

In Chapter 2 of this thesis, we discussed different methods for real-time monitoring of cell and tissue cultures. We reviewed three different optical approaches which are commonly applied in cell culture experiments and indicated possible directions for future developments, which should include further development of real-time measurement methodology. The latter should preferably be done in a label-free fashion, as every addition of labelling molecules can influence the biological component of the experiments, and in consequence influences the measurement and thus the obtained data. Currently, the most powerful method for the observation of cells is microscopy, but, due to the development of approaches such as evanescent waveguide sensing it will become possible to monitor cell cultures in
real-time with other techniques. An additional advantage of this approach is the possibility to carry out multiple parallel experiments in a single cell incubator, whereas microscopy does not offer such solutions. Furthermore, different sensors provide information about additional aspects of the biological system, and thus can be used in a complementary fashion to microscopy. In terms of experimental throughput, it will be difficult for other optical methods to compete with flow cytometry, which means that future developments will be focused on improvement rather than replacement of this method. In conclusion, future developments of such approaches can undoubtedly benefit from the application of microfluidic solutions towards next generation instrumentation. As mentioned before, microfluidics can help in the miniaturization of the cell culture and sensing devices. Additionally, it is possible to automate experimental setups by installation of flow chambers in the cell culture platforms, which help in dosing of chemicals and medium refreshment.

9.2. Toolbox for work with microfluidic endothelial cell cultures

In research laboratories, we often work with in vitro cell or tissue cultures, which act as models for certain organs or systems. Results obtained from those models serve as a proxy for the situation in an entire organism. Such experiments are performed in well plates, which currently are the most popular format for in vitro cell experiments. They offer straightforward handling, but usually poor resemblance of the physiological situation of cultured cells. Therefore, cell culture platforms have been adapted to mimic the physiological situation of the cultured biological model by means of microfluidics. However, multiple channel designs used for cell culture experiments make it difficult to compare experimental data obtained in similar experiments carried out in different channel designs, whereas this issue does not exist for experiments carried out in standardized well plates. Therefore, microfluidic cell culture devices require standardization in terms of dimensions, geometry, and fabrication materials of the device.

In working with endothelial cells, which line all blood vessels in the body, the most important natural factor to include is the flow of fluid past them (shear stress). The concept of endothelial cell cultures in microchannels is not new, as it was introduced in 2000 [12]. However, less attention has been paid to the fact that endothelial cells are lining blood vessels with diameters ranging from 3 cm down to 4 µm. The latter has substantial impact on the shear forces that are exerted on the endothelium, and blood flow velocity in the body. The smallest blood vessels, capillaries, are responsible for the transportation of nutrients to different organs, yet are studied in devices with dimensions that do not match their dimensions. To bridge this gap, we developed a microchannel geometry which allows efficient cell seeding and cultivation in channels as narrow as 60 µm, which represents the next step towards a cell culture platform for small blood vessels. Obviously, further development is required to culture cells in channels with even smaller dimensions. However, this presents additional challenges in terms of device fabrication and cell loading into the device. Our work puts emphasis on operation simplicity of the cell culture system. Additionally, in future experiments, this channel geometry can be easily implemented in more complicated devices with integrated sensors. Therefore, the contents of Chapters 3 and 4 should be viewed as a toolbox with protocols for people interested in endothelial cell studies in channels with different widths and shear stress.

9.3. The optical chip for cell monitoring

In the chapters dedicated to the development of a new photonic tool for real-time, label-free, few-cell cytometry of adherent cell cultures, we monitored cellular micromotion locally in just a few cells in the context of the entire cell culture. For this purpose, the light was introduced directly to a few cells in the context of a cell layer and forward-scattered light was collected from the cell layer for further
analysis. Thus, this approach is different from other optical sensor-based approaches, which monitor individual cells from beneath (e.g. evanescent waveguide sensors) or above (e.g. microscopy). In the future, methods for the observation of cell-cell interactions with the optical chip can be developed, which would be a valuable method for studies of molecule trafficking through or between the endothelial cells (e.g. drug transport). Moreover, cells monitored with this method do not require labelling, thus the probed cells do not exhibit the negative effects of labelling, such as photo-oxidation of dyes to forms that can be toxic for cells. It is important to mention that with this method, cells can be monitored continuously from initial seeding until the end of the experiment. Overall, our approach is distinctive from those currently available (e.g., impedance or surface plasmon resonance measurements) in that it exploits nanoscale light scattering of only a few cells to obtain information on cellular micromotion. Importantly, motion behavior may serve as a readout for changes in the cytoskeleton caused by chemicals. Observation of cytoskeletal changes can be related to different parameters, including inflammatory changes in endothelium. This might be important in the investigation of endothelial cells, which play a crucial role in the onset of cardiovascular diseases (CVD). Therefore, this new approach for non-invasively monitoring real-time changes in micromotion as a result of chemical challenges provides valuable insight into the cytoskeletal processes underlying these changes.

The data obtained in the experiments shown in Chapter 5 and 6 show patterns, that can be ascribed to changes in the cytoskeleton in tested cell cultures. However, to fully explore the potential of the device, we need to develop a method for data analysis. Currently, this analytical approach is applicable only when both the biological effect and the time of its occurrence are known from the literature, upon exposure to a stimulus. This means that we can link effects that we observe visually to patterns found in the registered forward-scattered light signal. However, when a thus-far unknown effect takes place, we are not yet capable of discovering that event from the power-trace alone. We are aware of this issue and are currently pursuing ways to address data analysis. Different methods based on Fourier transform were tested for datasets obtained for observation of cellular micromotion, but none of them showed any significant trends, which could be used for data interpretation. As an example of an analytical approach using Fourier transform, we used Power Spectral Density (PSD) analysis, which describes the distribution of power into frequencies composing the signal. This was applied for data analysis obtained from an experiment where cellular micromotion was observed. However, this method did not deliver conclusive results, and we continue our search for an appropriate analytical method to process our datasets for the optical chip.

The logical next step in experiments with endothelial cells monitored with the optical chip is the exposure of the cells to complex molecules (proteins) which induce postponed cellular responses in time, generally hours after the moment the drug is added. Delayed cell response is caused by the fact that these added molecules affect treated cells by binding to specific receptors and induce or modify cellular metabolic pathways. Therefore, cellular response costs more time than the action of molecules capable of migrating through the cell membrane and binding directly to their targets in the cytoplasm, as is the case with latrunculin A and jasplakinolide (Chapter 6). An example of a complex molecule for modifying the endothelial cytoskeleton is tumor necrosis factor α (TNF-α), because of its involvement in the induction of an inflammatory status in endothelium, which is an important topic in cardiovascular research, and in cancer metastasis as well [13]. However, to conduct a thorough study of TNF-α effects on endothelial cells with the optical chip, we need to implement better and robust data analysis in the experimental protocol. The latter is dictated by the complex nature of the endothelial cells response to TNF-α, of which cytoskeletal rearrangement is merely one. Therefore as a continuation of this work, it is important to develop an analysis protocol for collected data.
Furthermore, attention needs to be paid to improving the lifespan of the optical chips (currently usable in 5 - 7 experiments), which will contribute to a broader adaptation of micromotion optical measurements in medical and pharmaceutical *in vitro* research. Another exciting opportunity for application of the optical chip is to study cells which exhibit naturally occurring regular motion (e.g. cardiomyocytes) [14]. In this context it would be interesting to see how “beating” is reflected in the monitored signal and also how it is affected by addition of xenobiotics, such as beta-blockers.

### 9.4. Setup optimization

In Chapter 7, the Integrated Cell Cultivation Platform (ICCP) was presented as an example of a culture platform incorporating the capabilities of an incubator, microscope and pumping system in order to automate the experiments with the optical chip from Chapter 5 and 6. Although the ICCP is a great help in carrying out microfluidic experiments with the optical chip, the setup is not perfect yet. Both the optical chip and the ICCP require a number of improvements if a user without microfluidic background is to apply them. The most urgent issues were observed in the optical device, where the fragile fiber array unit (FAU) easily breaks upon contact. Additionally, the humid environment of cell incubators tends to weaken FAU attachment to the optical chip, due to glue softening at 37°C and humidity in the incubator. This in return causes fiber misalignment towards the waveguiding structures on the optical chip or fiber detachment from the chip, and in consequence failure of the experiment. The culture conditions in the ICCP significantly improve the optical chip lifespan in comparison to the optical chips used in a standard cell incubator, because inside the ICCP casing no humid air is present. Humid air normally ensures that medium does not evaporate. In the ICCP, there is no need to humidify the air, since all the cell cultivation actions are performed in an enclosed system driven by a pump, thus the cell culture is not endangered by drying out. Additionally, the automation of manipulations on the chip significantly improved the number of successful experiments performed with a single device.

One of the possible solutions to solve the fragility issue of the FAU is the development of a setup which would remove the need of the permanent fiber attachment to the optical chips altogether. An FAU would be installed on a XZ-stage (in an improved version of the ICCP on a XYZ-stage). The XZ stage is connected to a chip-holder, which has alignment spots on it, on which a chip can be placed. The alignment spots ensure identical placement of the chip every time. The fiber array installed on this alignment unit needs to provide the optical connections associated with the intended functioning of the optical chip. Next to these connections, three extra connections will be used with which the simple alignment routine can be executed to find the correct alignment of the fiber array to the chip. The stability over the measurement period and alignment reproducibility of such a solution has to be ensured. The variability of the collected light from the experiments with the optical chip was caused by random cell positioning in the nanocuvette, which occurs during cell seeding. However, if an experiment with the optical chip is established according to the developed protocol, it is possible to achieve reproducible data in terms of signal fluctuation, rather than light intensity.

In all the chapters, the aspect of user-friendliness was addressed by customization of the setup with 3D-printed parts, simplification of experimental protocols, and automation of the experimental setup. All these efforts were made with the idea that a next generation of young researchers can take this thesis and use it as a guide in their microfluidic experiments.
9.5. References


