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ABSTRACT

Microfluidic technologies are widely used for manipulation and precise control of the environment of cells, tissues and organisms. Integration of electrical impedance measurements in the microfluidic platforms can provide label-free, non-invasive means to achieve a multi-parametric readout. In this thesis, two microfluidic platforms with integrated impedance readouts for (1) growth rate monitoring of yeast cells, and (2) viability detection of parasitic worms are described. The objective of each platform is described below:

1. Impedance-based growth-rate monitoring of yeast

An integrated microfluidic platform for long-term culturing and imaging of non-adherent cells as well as for continuous monitoring of growth rates of cells by means of impedance measurements is detailed in the first part of the thesis. Yeast colonies were grown in a 2D monolayer under culturing pads enabling high-resolution microscopy at single cell level, as all cells were in the same focal plane. Upon cell growth and division, cells leaving the culturing area passed over a pair of electrodes and were counted through impedance measurements. As the number of cells in the culturing area remains constant, the number of washed-out cells was used to measure the growth rate of the cells in the chip by using impedance. The developed platform was used to for parallel growth-rate measurements of different engineered yeast strains by using impedance, and the obtained results were similar to those observed by using standard culturing and analysis methods.

2. Impedance-based viability detection of parasitic worms

Impedance-based readout can also be used for the assessment of multicellular organisms. In the second half of the thesis, the design and development of a microfluidic platform with impedance-based viability detection of *Schistosoma mansoni*, a human parasitic worm for drug screening application is presented. Schistosomiasis is a neglected tropical disease,
caused by parasitic worms, which affects almost 200 millions of people worldwide. Currently, the drug-screening process is based on the visual evaluation of drug effects on worm larvae, also known as Newly Transformed Schistosomula (NTS), in vitro by a trained operator. This manual process is extremely labor-intensive, has limited throughput and may be affected by subjectivity of the operator evaluation. The developed microfluidic platform overcomes these limitations by using integrated electrodes to measure the viability of NTS when exposed to different test compounds. The microfluidic analysis unit consists of two sets of electrodes and a channel of variable geometry to enable counting and size detection of single parasite larvae, while the collective evaluation of the motility of the larvae is used as an unbiased estimator for their viability. The developed platform was used to record size and motility variations of S. mansoni larvae, exposed to different concentrations of mefloquine, a drug with established in vitro antischistosomal properties.

The presented platforms showcase novel tools for automated and parallelized readout methods. The technology further allows for integration with other readout methods, such as microscopy to enhance the information that can be obtained from a single device. Impedance-based systems show a great potential in increasing the throughput and temporal resolution of the analysis, which are pivotal features to improve the current compound screening process and to gain insights into drug dynamics.
La microfluidica trova ampia applicazione nella manipolazione e nel controllo delle condizioni di coltura di cellule, tessuti e organismi. L’integrazione di misure di impedenza elettrica all’interno di piattaforme microfluidiche permette di ottenere misure multi-parametriche in maniera non invasiva e senza l’utilizzo di marcatori, che possono modificare le condizioni di crescita. In questa tesi presenterò due piattaforme microfluidiche con sensori integrati di impedenza elettrica per (1) misurare la crescita di popolazione di lieviti e (2) per rilevare la sopravvivenza di vermi parassiti. L’obiettivo delle due piattaforme è il seguente:

1. Misurazione di crescita di popolazione di lieviti

La prima parte della tesi presenta una piattaforma microfluidica per il mantenimento e la caratterizzazione ottica di cellule non aderenti, e per la continua rilevazione della crescita della popolazione delle cellule attraverso misure di impedenza. La crescita di colonie di lieviti in un monostrato all’interno di zone specifiche di coltura permette microscopia ad alta risoluzione di singole cellule, grazie al mantenimento delle cellule su un unico piano focale. A causa della crescita e della divisione cellulare, le cellule fuoriescono dalla zona di coltura. Le cellule che abbandonano la zona di coltura sono trasportate sopra una coppia di elettrodi e vengono contate attraverso misure di impedenza. Dato che il numero di cellule nella zona di coltura rimane costante, il numero di cellule rimosse può essere usato per stimare il tasso di...
crescita della popolazione nel dispositivo via misure di impedenza. La misura di impedenza in parallelo di diverse popolazioni ingegnerizzate di lieviti nel chip ha riportato valori comparabili a quella osservata usando le normali condizioni di coltura, quindi confermando che la piattaforma qui presentata non modifica le condizioni di crescita del campione e risulta essere un valido metodo di rilevamento.

2. Rilevamento della sopravvivenza di vermi parassiti

Misure di impedenza possono anche essere usate per monitorare organismi multicellulari. Nella seconda parte della tesi vengono presentati la progettazione e lo sviluppo di una piattaforma microfluidica con sensori di impedenza integrati per rilevare, attraverso misure di impedenza, la sopravvivenza di *Schistosoma mansoni*, un parassita umano, al fine di identificare nuovi medicinali. La schistosomiasi è una malattia tropicale causata dall’infezione di vermi parassiti, la quale colpisce quasi 200 milioni di persone nel mondo. Attualmente il processo di identificazione di nuovi medicinali è basato sulla valutazione visiva *in vitro* dell’effetto del medicinale sulle larve del parassita, chiamate Newly Transformed Schistosomula (NTS), da parte di un operatore specializzato. Questo processo presenta notevoli rischi di oggettività nella misura, e un basso livello di rendimento. Queste limitazioni possono essere superate utilizzando dei sensori di impedenza integrati all’interno di una piattaforma microfluidica per misurare la sopravvivenza delle NTS esposte a diversi medicinali. L’unità di analisi microfluidica è composta da due coppie di elettrodi e un canale a geometria variabile che permette di misurare il numero e la dimensione delle larve sotto analisi, e di valutarne la loro motilità come indicatore, oggettivo, delle loro condizioni. La piattaforma qui sviluppata è stata usata per misurare la dimensione e le variazioni di motilità delle larve dopo essere state esposte a diverse concentrazioni di Meflochina, un medicinale con note proprietà antiparassitiche *in vitro*.

Le piattaforme qui presentate dimostrano la possibilità di usare la microfluidica per sviluppare nuovi metodi di misura automatizzati. Questa tecnologia può essere integrata con altri metodi di analisi, ad esempio la microscopia, per aumentare la quantità di informazioni
che possono essere ottenute con un singolo dispositivo. Le misure basata su variazioni di
impedenza mostrano un grande potenziale per incrementare la produttività della misurazione
e la risoluzione temporale dell’analisi, le quali sono caratteristiche necessarie per migliorare
la ricerca di nuovi medicinali e per ottenere informazioni sulla loro dinamica.
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1 INTRODUCTION

Monitoring of growth rate and viability are critical to many areas of basic and biomedical research both, from a mechanistic perspective to understand the molecular and biochemical pathways regulating cell growth and viability, and from a therapeutic angle to find compounds, which modulate cell viability. Growth rate and viability of cells or organisms are known to be correlated to the environment. Study of these parameters can provide insights into understanding the influence of an environment on cells or organisms, how they interact with the environment and respond to it. Over the years, more and more sophisticated technologies, such as microfluidics, have been developed that allow to precisely control the environment. Integration of sensors in microfluidic systems can further be used to increase the number of the parameters that can be extracted per experiment. Moreover, a simple and straightforward sensor integration can offer great potential to scale, automate and parallelize experiments. In this thesis, two different types of integrated microfluidic platforms that rely on impedance-based readouts were developed to (1) monitor growth rate of yeast and (2) detect the viability of parasitic worms.

1.1 Microfluidics

Microfluidics is the process by which small amounts of liquids can be manipulated using channels with dimensions ranging from ten to hundreds of micrometers. Micro Total
Analysis Systems (µTAS) sometimes denoted “lab-on-chip” methodologies are based on microfluidics and serve a large variety of biological and biochemical applications. One of the early prominent examples for such a system is the development of a capillary-electrophoresis chip by Manz et al. The channels in this device were etched in a silicon substrate and sealed with a glass slide to allow optical access for microscopy. Introduction of simple fabrication methods, such as “soft lithography” techniques using poly(dimethylsiloxane) (PDMS), a soft and optically transparent elastomer, rendered the use of microfluidics more appealing to a broad range of laboratories, which fueled the development of various components for liquid manipulation such as valves, pumps and mixers.

Miniaturized microfluidic systems were used to develop complex assays, to reduce sample volume and costs, to maximize information obtained from a sample, as they provide scalability for increased throughput. The fact that microfluidic systems feature precise spatial and temporal control of a cellular environment has made those systems attractive for cell-based applications in comparison to conventional techniques. Microfluidic cell culture platforms have been designed for adherent and non-adherent cells. Adherent cells attach to the surface of a given substrate and naturally grow as a monolayer making it easy to image single cells at high resolution. To achieve high-resolution imaging of non-adherent cells, such as yeast, under continuous perfusion conditions, the cells need to be confined, while growth must be restricted to a two-dimensional (2D) plane. Existing approaches include growing of cells in low-height micro chambers, clamping cells between PDMS and cellulose membranes or clamping cells between glass and PDMS micropads.

Over the years, the use of microfluidic systems has also gained popularity for the precise manipulation of multicellular organisms, such as C. elegans nematodes, for fundamental studies in biology, chemistry, and physiology. Microfluidic platforms were developed to study the worm behavior and sensory response due to locomotion, mechanosenstivity, thermosensitivity and chemosensitivity. Different techniques for precise immobilization, trapping, imaging and sorting of single worms have also been implemented.
The readout for most microfluidic systems relies on light and fluorescence microscopy to visually inspect cells or organisms and to identify mechanisms at work. This readout typically requires a label, such as a fluorophore, to tag the molecule or unit of interest. However, only a low number of parameters per cell can be extracted, which is limited by the availability of suitable fluorophores and the occurrence of phototoxicity\textsuperscript{21}. Integration of label-free techniques in the microfluidic platform can provide a complementary readout to microscopy. Moreover, such approach may feature better temporal resolution and yield enhanced information on the dynamic behavior of cells and organisms.

1.2 Electrical Impedance Spectroscopy

Electrical impedance spectroscopy (EIS) is a non-invasive, label-free technique used to probe the dielectric properties of a biological sample. The use of this technique dates back as early as 1910 to Hoeber, who investigated the internal conductivity of erythrocytes\textsuperscript{22}. Pioneering work to characterize cell and tissue dielectric properties was carried out by Fricke, Curtis, Cole and Schawn\textsuperscript{23–25}.

1.2.1 Impedance-based readout for single cells

An early example demonstrating the use of impedance for single particle analysis was the Coulter counter with two large electrodes placed on either side of a small orifice\textsuperscript{26}. As a particle passed through this orifice, it displaced the conductive fluid, which resulted in a change in the current. The magnitude of the change in current is dependent on the volume of the displaced fluid, which is proportional to the size of the particle.

With the advent of microfluidics, more sophisticated devices, called impedance cytometers, were developed. These devices were realized by integrating a pair of electrodes within a microfluidic channel to perform measurements on single cells. One of the first such microdevices was demonstrated by Ayliffe et al. to differentiate between human polymorphonuclear leukocytes (PMNs) and fish red blood cells (RBC)\textsuperscript{27}.
The use of impedance cytometers for qualitative analysis to distinguish between erythrocytes and ghost cells was shown by Gawad et. al\textsuperscript{28}. The cytometer consisted of three co-planar electrodes on a glass substrate and a microfluidic channel made in PDMS on the top. A differential measurement scheme was used to characterize the cell properties against the cell medium and to calculate the velocity of the cells by measuring the transit time between peaks. Fitting of impedance models, developed for cells, to the experimentally obtained spectra, recorded with impedance cytometers, was used to characterize and differentiate cells\textsuperscript{28}. It was shown that, at lower frequencies (less than 1 MHz), the cell membrane acts a barrier for the current flow so that the impedance signal provides information on cell size and volume. At high frequencies (above 1 MHz), information on the membrane and internal properties of the cell can be obtained\textsuperscript{28,29}. Impedance cytometers with integrated coplanar electrodes were also used for identification of the state of stem cells\textsuperscript{30}, high speed particle detection in a micro-coulter counter\textsuperscript{31}, and in point-of-care diagnostic devices to count CD4 and CD8 T cells in whole-blood samples\textsuperscript{32,33}.

Another approach to integrate electrodes in a microfluidic channel is a configuration with sets of electrodes at opposing faces or walls of a microfluidic channel or container. This configuration, though more complex to fabricate, ensures a more homogenous electric field around the cell. An impedance cytometer with facing electrodes, developed by Cheung et al., was shown to provide discrimination of beads, RBCs, ghosts, and RBCs that were fixed in glutaraldehyde\textsuperscript{34}. A similar electrode configuration was used in different micro-cytometers to analyze and distinguish various cells types, such as yeast\textsuperscript{35}, bacteria\textsuperscript{36,37}, platelets\textsuperscript{38}, leukocytes\textsuperscript{39}. Due to their complexity, most of the impedance cytometers are stand-alone devices, which can be used downstream of a cell-culturing unit to assess suspensions of cultured cells.

1.2.2 Impedance-based readout for multicellular systems

Multicellular model systems have gained significance for drug screening applications over the last years due to the intention to better understand the effects of cell-cell and cell-matrix
interactions. Electrical cell–substrate impedance sensing (ECIS) was used to measure the bioimpedance of cellular monolayers using a set of interdigitated planar electrodes. The cells were seeded over the planar electrodes, and the impedance spectrum was measured continuously. Such systems provided information on confluency of the cells, proliferation, viability and cytotoxicity effects on cells. ECIS was also used as readout to study migration of cancer cells inside a 3D matrix within a microfluidic device.

3-dimensional cellular models, such as microtissues, are being considered a better model system as they more closely mimic the in vivo environment. Electrical impedance spectroscopy was demonstrated as a label free method to detect the effects of drugs on morphology, size, and viability of the microtissues. Realizations of impedance-based readouts in microfluidic devices, such as hanging-drop arrangements or microfluidic devices based on liquid flow through tilting proved to be successful in 3D cell-culture systems.

Integrated microfluidic platforms with electrodes were also used to perform whole-organism drug screening of C. elegans using a non-invasive electrophysiological readout, to guide the movement of worms and for neurobiology studies. A commercially available platform featuring interdigitated microelectrodes at the bottom of a 96-well microtiter plate was also used to measure the motility of and drug effects on helminths by means of electrical impedance spectroscopy.
1.3 Scope and structure of this thesis
The focus of this thesis is on the application of impedance–based readout methodologies to study the growth rate of non-adherent cells and the viability of parasitic worms. In Chapter 2, a microfluidic platform that comprises features for long-term cell culturing, imaging and growth-rate-monitoring of yeast, *S. cerevisiae*, is presented. In Chapter 3, a different microfluidic platform with integrated impedance readout is described that has been used for viability detection of parasitic worms, *S. Mansoni*. Chapter 4, finally includes the conclusion and a brief outlook.
1.4 Summary of major results and findings

1.4.1 Development of an integrated microfluidic platform combining long-term culturing and imaging of yeast cells with EIS in a single device

The device is a combination of a microfluidic chip, realized in PDMS, with a glass substrate featuring co-planar electrodes. Cell colonies grow below clamping pads and form 2D monolayers to enable time-lapse analysis of the cells by means of high-resolution microscopy. Each cell that leaves the limited-size colony under a pad is directed via flow over a set of electrodes and analyzed through impedance measurement upon passage.

1.4.2 Automated and parallelized growth-rate monitoring of yeast using impedance

The device enables parallel growth-rate measurements of different engineered yeast strains in multiple culture chambers using impedance. The growth rates of these strains were modulated by switching between standard medium and calcium-rich medium. The changes in the obtained growth rates were determined by impedance measurements and were similar to those observed by using standard culturing and analysis methods.
1.4.3 Viability detection of parasitic worms through impedance measurements

Schistosomiasis is a neglected tropical disease, caused by parasitic worms, which affects almost 200 million people worldwide. The current in-vitro drug screening process for this disease is extremely labor-intensive, subjective, and has limited throughput. To overcome these drawbacks, a microfluidic platform with integrated electrodes for viability detection of the parasitic worms is demonstrated. The impedance measurements enable counting and size detection of single parasite larvae (NTS) and the collective evaluation of the motility of the larvae as an unbiased estimator for their viability.

1.4.4 Impedance-based size and motility readouts of parasitic worms for a drug dose-response assay

The worm larvae were exposed to different concentrations of the antischistosomal drug mefloquine. An on-chip assay was performed after 24 hour and 72 hour exposure of the larvae to the drug compound. Variations in size were observed, which correlated well with visual observation results. Differences in the motility upon dosage of different drug concentrations were detected and were used to extract IC\textsubscript{50} values for the dose-response assay.
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INTEGRATING IMPEDANCE-BASED GROWTH-RATE MONITORING INTO A MICROFLUIDIC CELL CULTURE PLATFORM FOR LIVE-CELL MICROSCOPY

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2.1 Author contributions

KC: Designed and fabricated the chip, planned and performed experiments, data analysis and wrote the manuscript

SCB: Designed the PCB, software and provided experimental advice

GWS: Provided training on yeast cell culturing and the use of earlier versions of the chip

HMK: Provided feedback with statistical analysis

FR: Conceived the project, provided samples and feedback on manuscript

OF: Conceived the project, provided guidance, experimental advice and feedback on manuscript

AH: Responsible for planning the overall research activities, funding, ideas and critically reviewed all sections of this thesis and corresponding manuscripts
2.2 Abstract
Growth rate is a widely studied parameter for various cell-based biological studies. Growth rates of cell populations can be monitored in chemostats and micro-chemostats, where nutrients are continuously replenished. Here, we present an integrated microfluidic platform that enables long-term culturing of non-adherent cells as well as parallel and mutually independent continuous monitoring of (i) growth rates of cells by means of impedance measurements and of (ii) specific other cellular events by means of high-resolution optical or fluorescence microscopy. Yeast colonies were grown in a monolayer under culturing pads, which enabled high-resolution microscopy, as all cells were in the same focal plane. Upon cell growth and division, cells leaving the culturing area passed over a pair of electrodes and were counted through impedance measurements. The impedance data could then be used to directly determine the growth rates of the cells in the culturing area. The integration of multiple culturing chambers with sensing electrodes enabled multiplexed long-term monitoring of growth rates of different yeast strains in parallel. As a demonstration, we modulated the growth rates of engineered yeast strains using calcium. The results indicated that impedance measurements provide a label-free readout method to continuously monitor the changes in the growth rates of the cells without compromising high-resolution optical imaging of single cells.
2.3 Introduction

Cells regulate their growth rate in response to external signals, and as cells grow, their metabolism, macromolecular synthesis and the processes included in cell division must be coordinated\textsuperscript{1-4}. This coordination of different processes, the way in which cells monitor their nutritional environment, how they integrate this information into the cell cycle, how they regulate their cell cycle, as well as whether and how these regulatory processes change during a cellular life cycle still include many open issues\textsuperscript{5-7}. The investigation of these open issues requires a well-developed and broadly understood model system, such as budding and fission yeast\textsuperscript{8,9}, and an experimental setup that can be used to perform such investigations. The chemostat provides a powerful method to systematically study the coupling between growth rates and cellular processes: It allows for experimentally controlling the growth rate of a cell population by adjusting the nutrient supply into a defined culture vessel volume, thereby providing a stable and defined environment for cells\textsuperscript{10}. In a chemostat, the growth kinetics, i.e., the relation between cell growth rate and substrate consumption is controlled by manipulating the medium addition to the culture vessel.

Micro-chemostats rely on microfluidics technology for culturing cells in a constant and defined environment under continuous perfusion. The cells in these devices grow in chambers or channels of defined size, and their growth rates are usually determined by using microscopy\textsuperscript{11-15}. In contrast to conventional chemostats, the growth rates in these microfluidic platforms are defined by the composition of the supplied media. An advantage of microfluidic devices is that they do allow for monitoring of individual cells over an extended period of time. However, associated growth rate measurements are often limited by the field of view or the overall size of the culture chamber or pad and require dedicated software for cell segmentation and tracking. Detailed cell tracking requires high-temporal-resolution optical measurements, which limits the number of positions that can be imaged by the microscope in a single experiment due to the required stage movements. The limited number of imaging positions considerably reduces the throughput and detracts from the possibility to parallelize experiments under similar or identical conditions. Additionally, the
use of fluorescence microscopy for measuring cell growth rates limits the number of fluorophores that are available for tracking other specific events and processes in the cells. Moreover, photo-toxic effects may be induced upon frequent imaging\textsuperscript{16} so that additional control experiments become necessary to assess such phototoxicity effects, which are tedious to perform. Phototoxicity effects can be obviated by the use of label free techniques, such as measuring the optical density of the cell solution in microfluidic platforms\textsuperscript{17,18}. Unfortunately, suitable devices are not amenable to high-resolution optical imaging and to obtaining information at single-cell resolution.

Electrical impedance spectroscopy (EIS) is a label free, non-invasive method for cell or particle counting and analysis\textsuperscript{19–22}. Impedance cytometers, microfluidic devices with impedance measurement features offer the capability to characterize and analyze cell populations without the need for fluorescent labels\textsuperscript{23–26}. A common implementation of microfluidic impedance platforms consists of simple microfluidic channels with single or multiple facing electrodes to perform the impedance measurements. Most of these flow-through platforms are stand-alone devices that can be used downstream of cell culture reactors or with cell suspensions, and are not easy to parallelize. Growth rate measurements in cell cultures using electrical cell – substrate impedance sensing (ECIS) were demonstrated for adherent cells\textsuperscript{27,28}. Impedance-based measurements of viable biomass in micro titer plates were also performed for non-adherent cells\textsuperscript{29,30}. However, to the best of our knowledge, there is currently no integrated platform, which features continuous and parallel execution of (i) cell culturing under controlled perfusion conditions, (ii) monitoring of the cell growth rate, and (iii) high-resolution imaging of non-adherent or suspended cells in the same single device.

Therefore, our goal was to develop a platform that allows for long-term culturing as well as continuous parallel and mutually independent monitoring of (i) growth rates by means of impedance measurements and of (ii) specific other cellular events by means of high-
resolution optical or fluorescence microscopy. To this end, we used the concept of a previously developed microfluidic device to culture yeast cells in a defined volume underneath pads in a defined environment under continuous perfusion\textsuperscript{31}. We then developed a new device, which, besides some other features, included an impedance readout to count the number of cells leaving culturing areas under the pads. This new microfluidic platform enables continuous impedance-based monitoring of the growth rates of populations of non-adherent cells, such as yeast cells, while it enables simultaneous long-term, high-resolution imaging of cellular events. Combined microscopy and impedance measurements can be conducted in an array-based format, as the device features multiple culturing chambers and sets of sensing electrodes. Multiple yeast cell colonies in different defined culturing environments can be analyzed in parallel. The setup is straightforward and simple to use on an automated microscope including all electrical connections for impedance measurements. Parallel growth-rate measurements of different engineered yeast strains over two days have been conducted with the device. The growth rates of these strains were modulated by switching between standard medium and calcium-rich medium. The changes in the obtained growth rates were determined by impedance measurements and were similar to those observed by using standard culturing methods. In contrast to microscopy, impedance assessment enables high-throughput monitoring of growth rates without imposing additional strain on cells as a consequence of light exposure. The developed platform enables the user to employ the full repertoire of microscopy methods for tracking a wide range of cellular events, while the growth rate of the cells is continuously and independently monitored through an impedance readout.
2.4 Materials and Methods

2.4.1 Device fabrication

The device consists of two parts: a poly(dimethylsiloxane) (PDMS) layer containing the microfluidic structures, and a glass slide with a patterned metal layer. The PDMS layer was cast from a mold, fabricated in a three-layer microfabrication process on a 4-inch silicon wafer (Figure S 2.1a). The first layer for the clamping pads was obtained through selective ion-beam etching of a silicon substrate using a photoresist mask. This process allows for precise adjustment of the height of the first layer, which defines the clamping gap below the pads to be 4 µm ± 0.3 µm. The next two layers for the cell culture chambers and for the microfluidic channel structures were fabricated by two SU-8 photolithography steps. SU-8 25 and SU-8 100 (MicroChem Co., USA) were sequentially spin-coated to obtain layers of 20 µm and 200 µm height; the layers were then exposed to UV light through transparency masks for cross-linking. The mold was silanized for 2 h with trichloro(1H,1H,2H,2H-perfluoro-octyl)silane (Sigma-Aldrich, Switzerland) in a vacuum desiccator. A mixture of silicone and curing agent (10:1 w/w, Sylgard® 184, Dow Corning, Germany) was poured on the SU-8 mold and cured for 2 h at 80°C. The PDMS was then peeled off the mold and cut into single chips. Holes were punched for the device inlets and outlets.

The electrodes were fabricated on 4-inch 500-µm- or 200-µm-thick glass wafers using a lift-off process (Figure S 2.1b). The wafer was spin-coated with lift-off resist (LOR3B, Microchem Corp., Newton, USA), followed by a positive photoresist (S1813, Rohm-Haas, Schwalbach, Germany) and patterned using photolithography. A sputtering process was applied to deposit a platinum film of 200 nm thickness on top of a 20 nm-thick W/Ti adhesion layer. Metal lift-off was carried out using mr-Rem 400 remover (Micro Resist Technology GmbH, Berlin Germany). A 500-nm passivation layer of silicon nitride was then deposited on the fabricated metal layer using plasma-enhanced chemical vapor deposition. The passivation layer was removed in the areas of electrodes and contacts pads (using a
S1813 positive resist mask) by means of reactive-ion etching. After fabrication, the glass wafer was diced into individual glass slides (70 mm x 32 mm).

Cell loading and device assembly

The cell loading procedure is schematically shown in Figure S 2.2. The assembly and precise alignment of the structures in the PDMS layer and the electrodes on the glass slide was carried out during the cell loading procedure with a custom-made alignment tool (Figure S 2.2a). The PDMS layer and glass slide were placed on the top and bottom holder of the alignment tool. The glass slide was brought into close proximity to the PDMS layer without touching it. The PDMS structures were aligned with the electrodes on the glass (X, Y, and theta) by using micrometer screws and a hand-held microscope (Dino-Lite digital microscope, Netherlands). Once aligned, the top holder with the PDMS layer was removed and placed on the table with the culture chambers facing upwards. 0.5 µl of cell suspension at a concentration of $3 \times 10^7$ cells/ml was pipetted into each chamber. As PDMS is hydrophobic in nature, the liquid did not spread. The top holder was again placed on the alignment tool. The four cone-shaped pins ensured that the top holder re-centered at the same position as aligned before removal. The glass slide was then moved upwards and brought in contact with the PDMS layer. The assembled device was afterwards removed from the alignment tool.

2.4.2 Setup

2.4.2.1 Impedance measurements

The experimental setup is shown in Figure S 2.3a. After cell loading, the device was placed in a custom-made PCB (115 mm x 75 mm), which was used to switch between the sensing electrodes of different analysis units in an automated way (Figure S 2.3b). The PCB was connected to the impedance spectroscope (HF2, Zurich Instruments AG, Switzerland) and to a custom-made, automated, multiplexed EIS (AMEIS)$^{32}$ controller board, which controlled the digital signal to switch between analysis units and provided the interface to a PC. Custom-made software was used to select the analysis units and to program the switching
protocol and recording duration. The sensing electrodes of selected analysis units were connected to the impedance spectroscope for defined recording durations. Each analysis unit included two electrodes – a stimulating and a recording electrode. An AC signal with an amplitude of 2 V and frequencies of 1.12 MHz and 1.5 MHz was applied to the stimulating electrode. At the recording electrode, the current was transformed into a voltage using a trans-impedance amplifier (HF2TA, Zurich Instruments AG, Switzerland), which was then measured using the impedance spectroscope. The data was stored on the PC for later analysis. The phase signal of the output voltage was recorded and analyzed. The obtained data was bandpass-filtered using MATLAB (The MathWorks Inc., USA) using a frequency range of 0.1 Hz – 30 Hz. The peaks were extracted from the filtered data by applying a threshold to minimize the number of false positives and false negatives. The threshold values varied, depending on the medium conditions and frequency used for recording. The threshold values ranged between $2 \times 10^{-3} – 6 \times 10^{-3}$ degrees for 1.5 MHz and $0.5 \times 10^{-3} – 0.8 \times 10^{-3}$ degrees for 1.12 MHz.

2.4.2.2 Microscopy
The PCB hosting the device was placed in a custom-made holder, which fits onto the stage of an automated, inverted microscope (Figure S 2.3b). The images were obtained using inverted microscopes (Olympus IX 81 and Nikon Ti Eclipse microscope) placed in an environmental control box, which maintained a stable temperature of 30°C. Fluorescence images were captured on the Nikon microscope using a Nikon Plan Fluor 40X objective (NA 0.75, WD 0.66). The microscope was controlled using Youscope$^{28}$, and offline image analysis was performed using ImageJ$^{33}$ and CellX$^{34}$. Syringe pumps (neMESYS, Cetoni GmbH, Germany) were connected to the microfluidic chip for media supply (flow rate 10 µl/min, 15 ml of medium are required for a 24-h experiment) and were controlled using Youscope.

Experiments to assess the medium exchange characteristics (Figure S 2.4) were carried out on the Olympus microscope, and images were taken every second with a CMOS camera.
(Hamamatsu ORCA Flash 4.0 camera). Syringes were filled with de-ionized water and a solution of Amaranth (4 mg/ml, Sigma-Aldrich, Switzerland) in de-ionized water. Switching between the different syringes was controlled using Youscope. The calculation of the average intensity of the images and further analysis was performed using ImageJ.

2.4.3 Cell culture
*S. cerevisiae* strains – *vph1Δ* (BY4741, Euroscarf, FRY2033) and wild type strain (BY4700, FRY1398) were used for the experiments to measure growth rate dynamics in different medium conditions. These strains were cultured overnight in shaking flasks with YPD (Yeast extract Peptone Dextrose) containing 1% yeast extract (BD Biosciences, Germany), 2% peptone (BD Biosciences) and 2% glucose. The overnight culture was diluted and grown for a few hours in YPD to have the cells in the exponential phase before loading them into the device. Cells were grown in YPD with 100 mM CaCl$_2$ to observe changes in the growth rate. Yeast strains carrying Vph1-Citrine::KanMX (FRY 2011), Whi5-Citrine::KanMX (FRY 1935) and Cdc12-Citrine::KanMX (FRY 2145) fluorescent fusion protein constructs were used to perform fluorescence microscopy. These strains were tagged with Citrine fluorophore but at different locations in the cell and cultured in synthetic defined (SD) media. Growth rate measurements in a plate reader (Infinite M200 PRO, Tecan Group Ltd., Switzerland) were carried out for *vph1Δ* and wild-type strains in YPD and YPD with 100 mM CaCl$_2$. The absorbance of the cells was recorded at 600 nm every 6 min over a period of 72 h at 30°C. The growth rate of the cells was measured as an increase in the absorbance over time, and was extracted using MATLAB.
2.5 Results & Discussion

2.5.1 Device design and function
The device consists of two parts, the PDMS layer containing the microfluidic structures and the glass slide patterned with co-planar platinum electrodes (Figure 2.1a). The central part of the assembled device is schematically shown in Figure 2.1b. Up to 4 different medium solutions can be infused through the four inlets and can be mixed in the subsequent meandered channel structure\textsuperscript{35–37}(a feature, which has not been used in this paper) to study the effects of medium variation on the cell growth rate. The medium channels around the chamber are 1 mm wide and 200 µm high. The medium is then guided through the three microfluidic cell culture chambers with identical flow rates, as indicated by the white arrows, and is collected at the outlet. Two flow resistors (30 µm wide) are connected in parallel to the culturing chambers. They ensure a controlled, parallel and unidirectional flow through the chambers and ensure rapid nutrient availability as well as continuous cell removal. The three chambers (4.5 mm long, 5 mm wide, and 20 µm high) allow for simultaneous culturing of three different strains under identical medium conditions.
Figure 2.1: (a) Photographs of the PDMS layer and the glass slide with the electrodes. For device assembly, the PDMS layer was aligned with and sealed onto a glass slide. The glass appears yellowish due to the silicon nitride insulation layer. (b) Schematic illustration of the central part of the device consisting of 3 chambers, each having 5 analysis units. The 15 analysis units can be used with automated microscopy and feature integrated impedance read-out. The channel for the medium is depicted in dark blue with white arrows indicating the flow direction. The vacuum channel to seal the PDMS structure against the glass and to remove bubbles in the liquid is shown in red. (c) Enlarged sketch of one analysis unit. Yeast cells are clamped under 4 PDMS pads and proliferate under constant media perfusion. The blue arrow indicates the flow direction of the medium, and black arrows illustrate the cell trajectories. Cross-section AA’ illustrates the clamping of yeast cells are between the square PDMS pads and the glass substrate. Cross-section BB’ shows cells passing through the electric field lines of the co-planar electrodes for impedance measurements.

Each chamber has 5 identical analysis units (Figure 2.1c). An analysis unit includes four clamping pads (150 µm in diameter), two hexagonal support and guiding pillars, and one pair of sensing electrodes, placed between the guiding pillars downstream of the clamping pads. The vertical distance between the clamping pads and the glass is 4 µm ± 0.3 µm, which is sufficient for growing yeast cells in a monolayer under flow conditions. Once the space under
the pads is completely filled, cells start to outgrow the pads. These cells are entrained in the flow around the pads and guided over the electrodes for impedance measurement. As the number of cells under the pad remains constant, the number of washed-out cells per time can be used to measure the growth rate of the cells in the chip by using impedance. The sensing electrodes are 300 µm in length, 20 µm in width, 20 µm apart and located between two hexagonal pillar structures that focus the medium flow. The hexagonal structures also provide support to the chamber ceiling and prevent collapsing. Once the cells have passed the electrodes, they leave the chamber and are removed through the outlet. This prevents clogging of the device and allows for long-term culture.

The device accommodates a total of 15 electrode pairs, 5 replicates in three chambers, which are all routed to the connection pads along the side of the glass slide. The glass slide is plugged into a PCB that allows for automated sequential impedance recording through the different electrode pairs. The device with the PCB is placed in a custom-made holder mounted on a microscope stage.

2.5.2 Cell culturing and cell flow in the microfluidic device

A flow diagram of a standard experiment is shown in Figure 2.2a. After loading, cells are allowed to grow under the pads and impedance measurements of the outgrowing cells passing the electrodes are possible once the pads are filled. The setup allows for simultaneous optical imaging of the cells under the pads throughout the duration of the experiment.
Cells were loaded by pipetting the selected cell suspension (0.5 µl) into each chamber of the PDMS layer, which were then sealed with a glass slide hosting the electrodes by applying vacuum through the vacuum line. During sealing, the electrodes have to be precisely aligned with respect to the hexagonal structures in the PDMS layer. The alignment and sealing has to be quick, so that evaporation of the small volume of the cell suspension is minimized. To this end, an alignment machine that enables a simple and quick loading procedure was developed in-house (Figure S 2.2). The loaded volume is low enough to fill only the cell culture chamber; neither overflows of the cell suspension into neighboring chambers nor cross contamination were observed.

After sealing, the PDMS layer and glass slide were held together only through vacuum, applied to the vacuum channel that surrounds the medium microchannel inside the PDMS
layer (red feature in Figure 2.1b). This feature is different from what has been previously presented by Frey et al.\textsuperscript{31} and enables a comfortable use of the device also in laboratories lacking plasma bonding equipment. The device can be perfused for the duration of the experiment at flow rates up to 500 µl/min without leakage. The non-permanent bonding between PDMS and glass enables the facile re-use of both parts of the device. As there is a substantial risk of compound carry-over due to the absorbing nature of PDMS, only the glass slide was reused. The second function of the vacuum channel was the removal of any bubbles formed in the device after loading or during the experiment, thus ensuring robust long-term cell culturing under constant medium perfusion.

After loading, only cells below the pads were clamped. All unclamped cells outside the pads were washed away by the medium flow. The cells clamped between the PDMS and glass slide grew only in a 2D plane forming a monolayer of cells, as shown in time-lapse images of one of the pads in Figure 2.2b. Cell counting can be carried out by using bright-field imaging with 500-µm-thick glass slides. Clamping was found to cause no stress for the cells, as was shown previously using a Msn2 reporter\textsuperscript{31}.

A key issue in designing the microfluidic device was to ensure that all the cells growing out of the clamping pads were guided over the sensing electrodes. Figure 2.2c shows a picture of the analysis units with the four pads, placed in a diamond-like configuration, and the hexagonal pillar structures further downstream. Loading of the chip leads to stochastic distribution of cells under the pads. The arrangement of the four pads in each analysis unit and their geometric area (150 µm side length) provides a sizeable number of clamped cells per analysis unit. The diamond-like shape of the pad minimizes the area of stagnant flow and prevents cell accumulation. The cell occupancy of the pads and the flow paths of the cells leaving the pads in Figure 2.2c were extracted from a video. The hexagonal structures were designed to act as a funnel for collecting all cells growing out of the pads (dotted line in Figure 2.2c). A finite-element model of the analysis unit evidences that the flow lines around
the culturing pads pass through the hexagonal structures (Figure S 2.4c). It also shows the fluid velocity around the analysis unit (Figure S 2.4c). At the same time, the channel between the two hexagonal structures was designed sufficiently large so that all cells pass over the pair of electrodes due to the laminar-flow conditions in the chamber. A narrower channel would have entailed the risk of either clogging or of losing cells that pass outside the channel owing to the increased fluidic resistance. The five analysis units are arranged at sufficient distance within the culture chamber to completely obviate cross-talk under the given laminar-flow regime.

2.5.3 Impedance measurements of cells
The electrodes downstream of the clamping pads were used to perform impedance-based counting of the cells coming from the pads. An AC voltage signal was applied to the stimulating electrode, and the current flowing through the system was converted into a voltage by using a trans-impedance amplifier. When a cell moved over the electrodes, it caused a transient variation of the intra-electrode impedance, which, in turn, produced a transient change in the recorded current and, hence, the output voltage $^{38,39}$. A characteristic peak was observed upon passage of a cell over the electrodes in the phase component of the output voltage at 1.12 MHz and 1.5 MHz. Impedance measurements of the cells correlated well with optical monitoring of the cells flowing over the electrodes (Figure 2.3a). The impedance peaks were then used to count the cells and quantify the growth rate.
Figure 2.3: (a) Impedance phase signals measured at 1.5 MHz, recorded while cells pass over the electrodes. Figures a1 – a5 show snapshots of events, which yielded two consecutive peaks. The first peak occurred, when the cell marked with a green circle passed over the electrode, followed by the one marked with a red circle. The blue arrow indicates the direction of the medium flow. (b) Number of peaks counted during 4-min windows through impedance measurements, plotted versus time for two different analysis units having 2 and 3 filled pads. Each circle represents the number of peaks counted during the respective 4-min measurement window. $t_{EIS}$ denotes the time interval from the beginning of the experiment until impedance values were recorded, which was 13 h in this case. (c) Peak rate$_{norm}$ plotted for both analysis units obtained by normalizing the peak count (obtained from impedance data) for the average cell number and the recording window duration (in hours). The green and red line by and large coincide and indicate the mean growth rate for a given cell type extracted from all the measurement windows for a given analysis unit.

We examined the effect of the electrode area, confined by the hexagonal structures, on the impedance signal. The detection volume is defined by the width of the channel segment above the electrodes, the electrode spacing, and the height of the chamber, which is within the reach of the electric field lines. This volume defines the magnitude of the impedance change upon passage of cells. Minimization of this detection volume increased the
measurable impedance signal, so that the passage of cells could be detected. With larger detection volumes, for example upon misalignment of PDMS layer and glass slide, a detection of cells was no more possible (Figure S 2.5).

To enable a multiple use of the glass slide hosting the electrodes, the stability of the electrodes was analyzed. A frequency sweep on all electrodes of the device evidenced low variation in phase and magnitude signal between the beginning and the end of an experiment, which indicated the robustness of the electrodes (Figure S 2.6a). The low variation also reflected the uniformity of the fabricated electrode pairs across the chip. Further, the signal-to-noise ratio obtained from a re-used electrode pair at identical medium conditions and measurement frequency was measured for two separate experiments (Figure S 2.6b). Signal peaks could be detected in both experiments, and there was no significant difference in the observed signal-to-noise ratios of the two independent experiments. These findings illustrate that a glass slide with electrodes can be used for multiple experiments.

Cells flowing over a pair of co-planar electrodes show a characteristic signature, which depends on both, cell size and cell position and speed. The convolution of these effects currently limits the ability of the system in distinguishing between single or budded cells, and small cell clusters. Other studies have shown that optimized geometries and setups, featuring multiple facing and focusing electrodes, provide the capability to distinguish between different cell clusters or to analyze intra-cellular features. However, integration of complex sensing structures, such as sandwich structures or electrode posts perpendicular to substrate plane, into the current setup would increase device complexity and compromise ease of cell loading, culturing, and imaging. The current setup meets the requirements for measuring cell growth rates and changes in those, as the passage of clustered cells over the electrodes is happening very rarely, as has been confirmed with microscopy observations. This statement is supported by the fact that the growth rates determined in our system coincide with those measured by other methods. In order to compensate for not being able to measure budding cells, a correction factor (mentioned later in the paper) that is commonly used in yeast biology and includes budded cells was applied to the measured growth rates.
2.5.4 Measuring growth of cell colonies
The loading process of the cells into the chamber did not produce a homogeneous cell
distribution over all pad regions. Upon using low concentrations of cells in the loaded
suspension in order to only clamp a few or a single cell under each pad, there is a high
probability that some pads remain empty. After cell loading and starting medium perfusion,
clamped cells grew and formed a 2D colony under the respective pads (Figure 2.2b). Figure S 2.7a displays the number of cells under the pad over time, while the pad was getting
filled. Once the pad was full, cells started to outgrow and were continuously removed from
the colony through the medium flow around the pads. From this time on, the colony size
under the pad remained stable and a counting of the outgrowing cells with respect to time
could then be used as a direct measure for the growth rate of the cells under the pad.

Figure 2.3b presents peak counts (cells passing over the electrodes) within 4-min time
windows for two different analysis units once the space under the pads was fully occupied.
The analysis units featured 3 and 2 pads that had been filled with the same yeast strain. As
expected, peak counts were lower for the analysis unit with 2 filled pads compared to that
with 3 filled pads. To compare the measurements of the different analysis units, peak counts
by the electrodes were normalized with regard to the number of cells under the respective
pads. This was possible because the device allowed for simultaneous imaging of the pads
during recording of the impedance signals.

Once the area under the pad was fully occupied, the variability of the cell number under the
pad was low. Relative inter-pad variability in the number of cells under filled pads (in the
same experiment and between experiments) was found to be approximately 8% for a given
cell type (Figure S 2.7b). Averaged cell counts under the pads at the beginning and at the end
of the impedance recording sessions were used for normalization. Further, the impedance
peak count was also divided by the elapsed time of the recording window (4 min), which
yielded the normalized peak rate (peak rate_{norm}). The peak rate_{norm}, calculated for both
analysis units, was proportional to the growth rate of the cells under the respective pads. As shown in Figure 2.3c, the calculated mean value of the peak rate$_{\text{norm}}$ was found to be the same for the two analysis units (0.288 ± 0.009 h$^{-1}$ and 0.288 ± 0.015 h$^{-1}$) in which the same strain had been cultured.

2.5.5 Parallel growth rate measurements of multiple strains
The setup allows for automated switching between the sensing electrodes of different analysis units and for sequential impedance recording in a round-robin configuration during the experiments (Figure S 2.3). We used this feature to analyze variations in the growth rates of different yeast strains cultured on the same device by using the impedance readout. Two strains ($vph1\Delta$ and wild type) were loaded and cultured in different chambers in parallel. Both strains were first grown in yeast extract peptone dextrose (YPD) until the pads were filled. The medium was then switched to YPD with calcium and switched back to solely YPD after approximately 10 hours. The strains are known to have different growth rates when calcium is added to the medium$^{43}$. The switching time between the two media was ~40 s at a flow rate of 10 µl/min (Figure S 2.4) for all analysis units, such that recordings in all analysis units under new media conditions could be performed after less than a minute. Switching time was therefore not a limiting factor in the experiments.

Figures 2.4a and 2.4b show the normalized peak rates for the strains under different medium conditions. The average cell numbers under the pads at the beginning and at the end of the experiments for each medium condition were used for normalizing the peak rates. As expected, the mean peak rates for $vph1\Delta$ and the wild-type strain, grown in YPD, were similar (0.32 ± 0.02 h$^{-1}$ vs. 0.30 ± 0.01 h$^{-1}$). When the media was switched to YPD containing calcium, the $vph1\Delta$ strain grew slower. The mean peak rate calculated for this condition was 0.196 ± 0.004 h$^{-1}$ for $vph1\Delta$ versus 0.25 ± 0.01 h$^{-1}$ for the wild type. The cells fully recovered to their faster growth rates upon switching back to YPD without calcium (t = 20 - 26 h). We observed a higher variability in the peak rate for $vph1\Delta$ in YPD at the beginning of the experiment (t = 0 - 6 h) as compared to the end (t = 20–26 h). This finding can be attributed
to the fact that the pads were initially not completely occupied, which resulted in a higher variation in the cell number under the pads and the related peak count.

Figure 2.4: (a) Normalized peak rates of three replicates (represented by different symbols) of the Vph1Δ strain in YPD with calcium (100 mM) and in YPD without calcium. The line indicates the mean peak rate for each of the replicates. \( t_{EIS} \) denotes the time from the beginning of the experiment until the respective impedance values were recorded, which was 13 h in this case for both strains. (b) Normalized peak rates of the wild type strain (replicates represented by different symbols) in YPD with calcium (100 mM) and in YPD without calcium. (c) Growth rates as obtained from the plate reader (filled circles) and the impedance device (empty circles) under different medium conditions (YPD with and without calcium) for Vph1Δ (green) and wild type cells (red). Each circle indicates one replicate. Squares are means, error bars represent standard deviations.

The absolute growth rate of a monitored cell colony was obtained by taking into account that a fraction of cells passing over the sensing electrodes was in a budded state but yielded a single peak (Figure 2.4c). As mentioned before, the current device design does not allow for differentiating between single and budded yeast cells. Therefore, the absolute growth rate was obtained after accounting for budded cells in the obtained peak rates. A budding index (percentage of budded cells) of \(-0.6\) (60% of all are budded) was measured for the cells used in these experiments through image analysis of the cells flowing over the electrodes.
value is comparable to the budding index value of 0.68 obtained from FACS analysis of the same cells grown under identical medium conditions in a shaking flask.

The growth rate values measured with the impedance device have also been compared to the ones obtained by using an automated plate reader for both strains under the different medium conditions. Figure 2.4c displays the growth rates obtained from the plate reader and obtained from the impedance device for both media: YPD with and without calcium. After adjusting the values of the impedance measurement with the budding index, the determined growth rates were comparable yet slightly lower than those obtained from the plate reader. The relative change in growth rate upon switching the medium from YPD with calcium to YPD for the \textit{vph1Δ} strain in the plate reader and in the impedance device amounted to 58% and 63%. For the wild type strain, the relative changes were 29% and 20%, respectively.

In summary, the developed platform enabled monitoring relative changes in growth rates of different strains in parallel upon changing the medium conditions. The change was more pronounced for \textit{vph1Δ} as compared to the wild type. Moreover, we showed that replicates of the same strain yielded similar mean peak rates under the respective medium conditions, which demonstrates that the results of the impedance measurements for a given strain of cells were reproducible and consistent under identical conditions in the different analysis units. Additionally, we verified that the growth rate changes monitored in our platform were similar to those obtained with classical analysis methods.

2.5.6 Optimization of the multiplexed impedance readout

The presented device features a serial readout of 15 analysis units in a sequential way. A computer-controlled switch-board was used to route the selected electrode pair to the impedance analyzer for a defined time window. During this time, the cells passing the electrodes were counted according to the obtained impedance peaks. To maximize the information that can be obtained from multiplexing several analysis units, the recording time for each analysis unit needs to be optimized. This optimization can be achieved by defining a
minimal time that is required per analysis unit to obtain an accurate estimate of the cell growth rate.

We continuously recorded from a cell colony of a single analysis unit over 8 hours to analyze the factors that may define a suitable recording time. Figure 2.5a shows the peak counts for time windows of 60 s and 240 s. More variability in the peak count per cycle was observed for a window of 60 s as compared to 240 s. In both cases, however, the mean peak count over all measurement cycles was identical within measurement errors. Figure 2.5b depicts the peak count in dependence of the time window. The variability in the peak number per cycle decreased with increasing time window, as the number of peaks was averaged over a longer duration, and was found to be relatively constant for measurement windows of 300 s or more.
Figure 2.5: (a) Peak counts per second plotted for window lengths of 60 s (blue) and 240 s (red). The black line indicates the mean of the peak count for both window lengths. $t_{EIS}$ denotes the time from the beginning of the experiment until the respective impedance values were recorded, which was 24 h in this case. (b) Peak counts per second for window lengths varying from 1 s to 600 s. (c) Mean values with error bars indicating SEM values for 60 s window length for different numbers of cycles. Below is the same standard error of the mean (SEM) for 60 s window length for different numbers of cycles. The x-axis at the bottom indicates the total measurement time in seconds (cycles × window length). (d) Mean values with error bars indicating SEM values and SEM values for 240 s window length.

Larger time windows, however, reduce the sampling frequency of the analysis units during an experiment and, therefore, the temporal resolution of the obtained data (fewer red dots as
compared to the blue dots in Figure 2.5a). The window length and number of cycles ultimately define the obtainable measurement precision. Figure 2.5c and 2.5d show mean and standard error of the mean (SEM) for a window length of 60 s and 240 s in dependence of the number of measurement cycles. As an example, an SEM of 0.004 was obtained, when an analysis unit was sampled ~85 times with a 60 s window but only ~22 times with a 240 s window. It can be inferred that for obtaining a certain precision, fewer cycles are required for recording with longer time windows from a given analysis unit. The total recording time was similar (5100 s and 5280 s) for the two example detailed above.

The number of cells passing over the electrodes is a stochastic process that can be modeled by a Poisson distribution. One feature of the Poisson model is that the precision of the estimate only depends on the total recording time, irrespective of the length of each recording interval. Consequently, it does not matter if several longer-interval or many short-interval recordings are conducted, as long as the overall recording duration is the same. It has to be noted that the window size per analysis unit should be chosen with respect to the growth rate of the cells – an analysis unit featuring slowly growing cells should be assigned a longer recording window as compared to a unit with rapidly growing cells to achieve, in both cases, good precision of the relative growth-rate measurements.

Cell imaging in the microfluidic device

The advantage of the “micro-chemostats” is that they are amenable to high-resolution imaging. We tested the imaging capabilities in the devices by using a Citrine tag endogenously fused to different yeast proteins. For high-resolution fluorescence imaging, the electrodes were patterned on a 200-µm-thick glass. The fluorescent fusion proteins localized to the vacuolar membrane (Vph1-Citrine), at the bud neck (Cdc12-Citrine) and shuffled between the cytoplasm and the nucleus in a cell-cycle-dependent manner (Whi5-Citrine). All three cell types were inoculated separately, mixed in equal ratios, and cultured in the device.
The different cell types and their growth over time were clearly distinguishable according to their different levels of fluorophore signals (Figure 2.6a). The intensity of the Citrine tag was highest for Vph1 tagged cells (~2940 copies/cell), followed by Cdc12 (~680 copies/cell) and Whi5 (~120 copies/cell) tagged cells. Figure 2.6b shows images of single cells featuring the different tags. The appearance of the Cdc12 marker (in yellow) at the bud neck and the localization of Whi5 in the nucleus shortly before cell division were clearly visible in the device (see also video S1). The use of a thin glass slide makes it possible to image intracellular components and enables high-resolution microscopy comparable to that with commonly used glass slides.

Figure 2.6: (a) Time-lapse images showing the growth of different cells under one of the pads (40X NA 0.75). The three strains are indicated through different colors in the bright-field images. The
strains were tagged with the Citrine fluorophore at the bud neck (Cdc12; yellow), in the vacuole (Vph1; blue) and in the nucleus (Whi5; green). The pad hosts all three strains that grow, divide and feature different fluorescence intensities of Citrine (video S 1). (b) Enlarged images of cells with the different tags. Arrows indicate the cellular distribution and transitional localization of Whi5 in the nucleus of a cell.

2.6 Conclusion
We described a platform enabling long-term culturing and high-resolution imaging of yeast cells, while the growth rates of populations of cells could be assessed in parallel and label-free by means of impedance measurements. The platform allows for simultaneous culturing of up to three strains under different microfluidic-flow and medium conditions, and features five sets of analysis units per strain for parallelized in situ impedance analysis of the growth rate. The availability of multiple analysis units enables the user to conduct experiments in the same platform in parallel in several replications and to include all necessary control experiments, while having the capability to perform optical imaging. Importantly, the use of non-permanent vacuum-based bonding between the microfluidic structure and the glass slide hosting the electrodes allows for simple sample loading into the device and multiple usage of the glass slides.

The yeast cells grow in a monolayer under the clamping pads, they divide and develop - potentially from a single cell - to a cell colony of constant size, which is defined by the clamping pad dimensions. We validated all functions of the platform by continuously monitoring the changes in the growth rates of differently engineered yeast strains, loaded in separate culturing compartments of the device, upon changing media conditions by using the impedance readout. The observed growth rates and their changes coincide with those recorded by using standard methods. We also confirmed the quality of the optical imaging by using a set of endogenously tagged proteins that are frequently used in yeast research. This
experiment shows that continuous bright-field and fluorescence imaging of the cells at sub-cellar resolution were possible with our platform.

Growth rate measurements were based on impedance and complementary to measurements performed by microscopy. Furthermore, the automated recording and switching between several impedance analysis units was completely independent of the position, temporal resolution or field of view of the microscope used in parallel. Such a platform could serve as a useful experimental setup for nutrition and cell metabolism studies that require a continuous monitoring of growth rates in a steady and controlled environment along with tracking of fast cellular processes by means of high-resolution time-lapse imaging.

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2.8 References


2.9 Supplementary Information

2.9.1 Fabrication process

Figure S 2.1: Fabrication steps of the device. (a) Fabrication steps of the PDMS layer using SU-8 photolithography and PDMS molding. The silicon etching is for the clamping pads, followed by multilayer SU-8 photolithography for the chamber and microfluidic channels. After development, the three layered SU-8 mold is silanized. Regular PDMS casting is done to obtain the PDMS layer of the device. (b) Fabrication steps for patterning the electrodes on the glass using the lift-off process. The
glass is spin-coated with lift-off resist (orange) followed by a positive photoresist (red). After patterning these layers, the metal is deposited in the required areas. The metallic layer is insulated with silicon nitride except for the open electrode areas needed for measurements.

2.9.2 Loading procedure

![Alignment tool with an alignment stage to adjust the X,Y,Z and Theta (R) directions; top holder used to place the PDMS layer; bottom holder used for the glass with pins to position top holder.](image)

Figure S 2.2: (a) Picture of the alignment tool with an alignment stage to adjust the X,Y,Z and Theta (R) directions; top holder used to place the PDMS layer; bottom holder used for the glass with pins to position top holder.
electrodes. (b) Schematic showing the loading procedure. The PDMS layer and glass are brought to close proximity and aligned in X,Y,R direction (b1,b2). The top holder is removed, and cell suspension is pipetted in the chamber of the PDMS layer (b3). The top holder is placed back on the alignment machine and glass is moved upward to make contact with the PDMS layer (b4). Vacuum is then applied to the vacuum channel to seal the PDMS against the glass.

2.9.3 Experimental setup

![Diagram of experimental setup](image)

**Figure S 2.3:** (a) Schematic of the setup. Enlarged image of the device in the holder on the stage of the microscope. The device is placed in the PCB, which is used for automated switching between the sensing electrodes of different analysis units and which routes the selected electrodes to the impedance spectroscope for measurements. The AMEIS controller communicates to the PCB the switching protocol and recording duration of the electrodes. This information has to be entered previously by the user into the custom-made software. (b) Top and bottom of the PCB with the device in the holder which can be placed in the automated stage of a microscope.
2.9.4 Fluidic characterization

**Figure S 2.4:** (a) Medium exchange dynamics were characterized in the culture chambers by first infusing non-colored liquid (at t = 0 s) and then switching to colored liquid (t = 125 s) while performing continuous imaging. Intensity changes as a result of infused colored liquid at different positions in the device at a flow rate of 10 µl/min. Switching time (Δts) denotes the time required to exchange the liquid at a particular position (time for the decay of the relative intensity from 90% to 10% at the respective position due to color infusion). The switching delay (Δtd) is the time required for the medium to reach the analysis unit from the inlet (time from starting the infusion until a relative intensity level of 90% of the initial value is reached). (b) Table listing the switching time and delay for the three positions indicated in (a) for different flow rates. (c) Finite-element modeling of the analysis unit. The streamlines (in red) indicate the media flow inside the chip. The streamlines closer to the pads pass through the hexagonal structures, where the sensing electrodes are located. Cells growing out of the clamping pads are directed and focused towards the sensing region with the electrodes between the two hexagonal pillars at the bottom. The gray-scale color map represents fluid velocity (for an inlet flow rate 10 µl/min) throughout the analysis unit.
2.9.5 Impedance characterization

Figure S 2.5: Loss of signal due to misalignment of the electrodes. (a) The figure shows the peaks when cells pass over the electrodes while those are aligned with the hexagonal structures. (b) The figure shows the loss of the signal upon misalignment, which entails an increased detection volume and less sensitive measurements.
2.9.6 Electrode characterization

Figure S 2.6: (a) Sweep of phase and magnitude of the electrodes in a frequency range between 50kHz and 2MHz before the experiment and at the end of the experiment. (b) Mean of signal-to-noise ratio plotted for the same electrode pair, which was used for two different experiments. The mean signal-to-noise was calculated over the duration of the impedance recording under identical medium conditions and the same frequency for both experiments. The error bars represent the standard deviation. It should be noted that the glass substrate with the electrodes from experiment 1 was cleaned and aligned with a new PDMS layer and then used for experiment 2.
2.9.7 Cell numbers under the pad

Figure S 2.7: (a) Cell number under a pad plotted versus time for different media conditions. Cell numbers were counted after 13 h from the beginning of the experiment. The pad was not completely occupied at this time point. The arrow indicates the point when the pad was fully occupied. The mean cell number after the pad was full (22 h - 38 h) was 1192 ± 37 (CV 3%). (b) The mean and standard deviation of cell numbers for WT and Vph1Δ cells under a full pad, calculated from two different experiments. The mean value for WT was 1167 ± 81 (CV 6%, n = 17) and was 950 ± 76 (CV 8%, n = 6) for Vph1Δ. Error bars represent standard deviations.
3 IMPEDANCE-BASED MICROFLUIDIC ASSAY FOR AUTOMATED ANTISCHISTOSOMAL DRUG SCREENING

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3.1 Author contribution

KC: Designed and fabricated the chip, planned and performed experiments, data analysis and wrote the manuscript

MMM: Provided experimental advice, designed a part of data analysis algorithm

PSR: Designed the PCB and assisted during some experiments

FCL: Provided samples and visual score assessment for drug dosing experiments

ML: Designed software to control the setup and PCB

GP: conceived the project and provided experimental advice

SCB: conceived the project and provided experimental advice

JK: Provided samples, drugs used for experiments and provided feedback

AH: responsible for planning the overall research activities, funding, ideas and critically reviewed all sections of this thesis and corresponding manuscripts
3.2 Abstract
Schistosomiasis is a neglected tropical disease, caused by parasitic worms, which affects almost 200 million people worldwide. For over 40 years, chemotherapeutic treatment has relied on the administration of praziquantel, an efficacious drug against schistosomiasis. However, concerns about developing drug resistance require the discovery of novel drug compounds. Currently, the drug-screening process is mostly based on the visual evaluation of drug effects on worm larvae \textit{in vitro} by a trained operator. This manual process is extremely labor-intensive, has limited throughput and may be affected by subjectivity of the operator evaluation. In this paper, we introduce a microfluidic platform with integrated electrodes for the automated detection of worm larvae viability using an impedance-based approach. The microfluidic analysis unit consists of two sets of electrodes and a channel of variable geometry to enable counting and size detection of single parasite larvae, and the collective evaluation of the motility of the larvae as an unbiased estimator for their viability. The current platform also allows for multiplexing of the analysis units resulting in increased throughput. We used our platform to record size and motility variations of \textit{Schistosoma mansoni} larvae exposed to different concentrations of mefloquine, a drug with established \textit{in vitro} antischistosomal properties. The developed platform demonstrates the potential of integrated microfluidic platforms for high-throughput antischistosomal drug screening.
3.3 Introduction

Schistosomiasis is one of the major neglected tropical diseases and affects just under 190 million people worldwide, predominantly children living in rural areas with poor sanitary conditions. This disease is caused by parasitic worms (helminths) of the genus *Schistosoma*. If left untreated, the infection slowly becomes chronic, resulting in fibrosis of the liver, intestines and/or bladder, anemia, growth and cognitive stunting, malnutrition, urogenital cancers and, eventually, death. Preventative chemotherapy is recommended by the World Health Organization as the most cost effective control strategy for schistosomiasis. For over 40 years, therapeutic treatment has relied on the administration of praziquantel due to its high safety and efficacy against the adult worm infection. However, the use of a single compound to treat millions of people annually raises concerns with respect to the emergence of drug-resistant worms.

The use of abundantly available larval-stage worms or Newly Transformed Schistosomula (NTS) of *Schistosoma mansoni* for pre-screening of drug candidates has been established a decade ago. The ability to acquire large sample numbers of these larvae in a cost-effective way and the concurrent reduction in the use of live animals to obtain relatively low quantities of adult worms have further promoted the development of automated and high-throughput approaches for antischistosomial drug screening. Fluorescence- and luminescence-based assays have been developed for automated detection of larvae viability. In addition, automatic image acquisition and image analysis using bright-field microscopy have been used for evaluating worm viability, based on morphology and motility, for hit/non-hit screening of drug compounds. However, the need for massive computational power and high-cost equipment ultimately limits the application of microscopy-based systems for dose-response assays and continuous viability monitoring. Moreover, both fluorescence/luminescence-based approaches and image-based detection require a relatively large number of larvae to provide reliable viability detection, which reduces the actual throughput that can be attained with these methods. As an alternative method, microcalorimetry was shown to be a suitable method for the real-time monitoring of adult-
stage schistosomes; however, this approach was not sensitive enough to measure NTS heat production\textsuperscript{15}. As a consequence, the current gold standard for antischistosomal drug screening still consists of a phenotypic evaluation of the worm larvae by a trained operator using manual microscopy. This procedure is extremely labor intensive, and the operator assessment may be affected by a high level of subjectivity and, therefore, feature low reproducibility\textsuperscript{16,17}. Consequently, there is an urgent need for identifying novel methods for replacing the manual microscopic evaluation of NTS larvae viability to advance the current drug screening pipeline.

Electrical impedance spectroscopy (EIS) is a non-invasive and label-free method for the investigation of the dielectric properties of samples. Integration of electrodes in microfluidic devices has enabled highly sensitive low-volume impedance measurements for a wide variety of biological samples in solution, ranging from single cells\textsuperscript{18} to multicellular aggregates\textsuperscript{19}, and to multicellular organisms\textsuperscript{20}. Impedance cytometers, realized by integrating a set of electrodes within a microfluidic channel, were used for multi-parametric assessments across different frequencies for differentiation of single cells, based on cell size, membrane integrity, and internal properties of cells\textsuperscript{21–25}. The use of microfluidic cell traps or immobilization of cells enabled long-term, real-time EIS measurements of cells\textsuperscript{26–28}. Integrated microfluidic devices have been used for parallelized measurements of microtissue spheroid size\textsuperscript{29}. Microfluidic methods also have been used to study multicellular organisms. Microstructures enabled the detection, trapping and on-chip fluorescence characterization of \textit{S. haematobium} eggs in urine\textsuperscript{30}. Microfluidic devices for real-time drug screening of \textit{Caenorhabditis elegans} nematodes based on worm locomotion\textsuperscript{31} and with electrophysiological and impedance readouts have also been developed\textsuperscript{32–34}. Platforms, based on electrical cell-substrate impedance sensing (ECIS) methodology, have been used to monitor cell growth, spreading and cell attachment to the surface of electrodes\textsuperscript{35}. The xCelligence system (ACEA Biosciences), a commercially available platform, based on the ECIS principle and featuring interdigitated microelectrodes at a bottom of a 96-well
microtiter plate, was used to measure the motility of helminths. This system was also adopted to study different aspects of the schistosomal life cycle (eggs, cercariae, adults). However, the large sensing volume requires a relatively large number of samples as compared to the standard method and is not sensitive enough to detect the small movements of NTS larvae of *S. mansoni*.

In this paper, we present a parallelized and integrated microfluidic platform for the automated assessment of the viability of NTS by means of EIS. The platform was used to detect variations in size and motility of the larvae after being exposed to different concentrations of a test compound at two time points. The use of a small detection volume (6 nL) for measuring larvae motility enabled viability detection of NTS with only a low number of worm larvae (~10 NTS). The platform was operated via gravity flow to reduce the complexity of the experimental setup and to avoid the use of external pumping systems. The chip comprises four analysis units to allow for simultaneous execution of measurement replicates and to enable increased throughput. We recorded size and motility variations of NTS after 24-hour and 72-hour exposures to mefloquine, a rapidly-acting antimalarial compound, known for its antischistosomal efficacy *in vitro*. The obtained results indicate that impedance-based size and motility analysis can provide dose-dependent viability and activity patterns of the larvae and can be used for drug screening applications.
3.4 Experimental section

3.4.1 Device design and loading

The platform consists of two parts: a top poly(dimethylsiloxane) (PDMS) layer, containing the microfluidic structures that define the four analysis units, and a bottom glass slide with patterned platinum electrodes (Figure 3.1a). The two parts are bonded together using plasma bonding. The fabrication process of the chip is shown in Figure S3.1.

A schematic of one analysis unit is shown in Figure 3.1b. An analysis unit consists of an inlet port for loading the NTS solution and two medium reservoirs on either side. The sensing part of the analysis unit is formed by a channel with a funnel-like constriction (50 µm) to allow the passage of single NTS larvae, and by two pillar structures to retain the NTS within the channel but to enable a flow of the solution between the medium reservoirs and the inlet region. An electrode pair around the funnel constriction (electrode pair 1) is used to count the number of loaded NTS larvae and to measure the relative size of single NTS, while a second electrode pair (electrode pair 2) is used to measure the combined motility of NTS that have been loaded into the sensing region of the analysis unit as a proxy for their viability.

Figure 1c shows the experimental procedure. Each analysis unit of the device is loaded with 20 µL of NTS suspension (~10 NTS per 20 µl) through the inlet port. After loading the NTS in the chip, 5 µL of the medium is removed from medium reservoir 2 to generate a flow towards the reservoir and to keep the NTS away from the sensing region of the analysis unit. The platform is then tilted to generate a hydrostatic pressure difference between the medium reservoirs. This movement enables single NTS to pass through the funnel constriction and over electrode pair 1. The NTS are then stopped by the pillar structure between the sensing region and reservoir 1 and are then kept between the electrodes pair 2 for measuring their motility. The sensing region between electrode pair 2 can accommodate up to 15 NTS. Size and motility measurements were performed at three tilting angles (10°, 15°, 20°) with the same NTS sample (Figure S2). No significant changes in both, size and motility recordings

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were observed. An angle of \(\sim 15^\circ\) was used for the majority of experiments. The developed loading methodology is simple, requires a small volume of NTS solution, and can be performed without additional fluidic systems.

Figure 3.1: (a) Photograph of the microfluidic platform, which consists of a top PDMS part, aligned and bonded to a platinum-patterned glass slide; (b) schematic of the analysis unit marked in (a). The unit is composed of two medium reservoirs, separated from the sensing region by pillar structures. The sensing region is composed of: a funnel constriction, aligned with an electrode pair (electrode pair 1), which allows for the passage of single NTS and is used for counting the number of loaded larvae; a larger sensing area, aligned with a second electrode pair (electrode pair 2), which is used to measure the collective motility of the NTS. (c) Loading and operation of the chip (c1- c4). First, 20 µL of NTS suspension are loaded through the inlet with a pipette; 5 µL of medium are removed from medium reservoir 1 to generate a hydrostatic pressure and ensure that no NTS are in the sensing area before starting the measurement. The platform is then tilted to drive the NTS through the funnel constriction for counting and EIS size estimation, and to subsequently measure their motility.
3.4.2 Sample preparation

*S. mansoni* cercariae were harvested from infected intermediate host snails (*Biomphalaria glabrata*) and transformed into NTS using a transformation method described previously. The resulting NTS suspension was adjusted to a concentration of 500 NTS/mL, and 20 µL of NTS suspension were added to each analysis unit to perform the measurements. We used standard samples as they also are used for visual inspection. Culture medium components for NTS were obtained as follows: Medium 199 RPMI 1640, supplemented with 1% penicillin /streptomycin, purchased from Lubioscience (Lucerne, Switzerland), and 5% fetal calf serum (iFCS), which was purchased from Connectorate AG (Dietikon, Switzerland). No additional purification or modification of the samples was performed.

For the experiment with exposure of the NTS to DMSO, the larvae were incubated with 30% DMSO at 37°C and 5% CO\textsubscript{2} and measured after 30 minutes of exposure. For the drug dose-response experiments, the NTS were incubated in culture medium with serially diluted concentrations (0.78, 1.56, 3.3, 6.25, 12.5, 25 µM) of mefloquine (Sigma-Aldrich, Buchs, Switzerland) in a 96-well plate in triplicates for 72 hours. The viability of the NTS was assessed microscopically and with EIS after 24 and 72 hours after addition of mefloquine. The visual scores were given based on a previously described viability scale (3 = motile, no changes to morphology; 2 = reduced motility and/or some damage to tegument noted; 1 = severe reduction to motility and/or damage to tegument observed; 0 = dead).

3.4.3 Data acquisition and analysis

Detailed information on the experimental setup is reported in Figure S3.3. Briefly, the microfluidic platform was placed on a custom-made PCB, which was used to switch between the electrode pairs of different analysis units in an automated way. A custom-made software was used to select the analysis units and to program the switching protocol and recording duration for the electrode pairs for each unit. The sensing electrodes of the selected analysis units were connected to an impedance spectroscope (HF2, Zurich Instruments AG, Zurich,
Switzerland) for user-defined recording durations. The system allowed for short recording durations at each electrode pair (2ms) and fast switching between three analysis units in a round-robin fashion. All the recordings were then combined to reconstruct the total signal for each analysis unit for both, counting and motility data. The total time required for parallelized size and motility measurements for a given condition in all three analysis units amounts to 5 min on average.

A multi-frequency AC signal with an amplitude of 300 mV at 48 kHz and 333 kHz carrier frequencies was applied to the center electrode (Figure 1a). The circuit was then closed by connecting either the right or the left electrode (electrode pair 1 or 2, respectively) to a trans-impedance amplifier (HF2TA, Zurich Instruments AG, Zurich Switzerland) for signal acquisition through current-to-voltage conversion. The magnitude signal of the output voltage, recorded at electrode pairs 1 and 2, was then used for further analysis.

The recorded data was filtered in MATLAB (The MathWorks Inc., Natick, USA) using a band-pass filter with 0.2 - 4 Hz cut-off frequencies for the signal trace, acquired with electrode pair 1, and a 0.5 Hz high-pass filter for the traces acquired with electrode pair 2. As different samples might exhibit varying baseline values, due to differences in the conductivity of the solution or variations in the electrode alignment with the fluidic structure, the acquired traces were normalized with the signal mean to reduce the influence of any of these effects. The location and count of the transient negative peaks in the signal, which were evoked by the passage of NTS and recorded with the electrode pair 1, were extracted from the filtered data by applying a threshold greater than -0.1 mV. The absolute peak-amplitude was then calculated from the raw data to minimize signal distortion caused by the filter. To quantify the fluctuations induced by motion of the larvae between electrode pair 2, we calculated the power of the filtered and normalized signal in a 1-3 Hz bandwidth. This approach minimizes the effect of readout noise, which is present at higher frequencies, while it preserves the signal power that is related to the contraction and expansion of the NTS between the electrodes. The calculated power of the measured fluctuations was normalized by the number
of loaded larvae to be able to compare measurements with a different number of NTS in the analysis units.

3.5 Results & Discussion

3.5.1 Count and motility measurement
A representative output signal, recorded from a single analysis unit, is shown in Figure 3.2a. The passage of a NTS over the electrode pair 1 induced a transient reduction of the voltage-converted current between the electrode pair resulting in a peak. Figure 3.2a-1 shows the peaks generated by the passage of five individual NTS through the funnel structure. Electrode pair 2 was then selected to record the motility of the NTS. The movement of the NTS between the electrodes caused fluctuations in the signal around the mean value (Figure 3.2a-2). DMSO was added to the device to detect the difference in the motility signal recorded from alive and dead NTS. As the NTS stopped moving, the signal stabilized around the mean value, and no further signal variation was detected (Figure 3.2a-3).

The multiplexing capability of the platform allowed us to record from three analysis units simultaneously. Therefore, it was crucial to test that the signal of each analysis unit could be reconstructed without cross talk. Figure 3.2b shows the counting and motility recordings of three different analysis units. Analysis unit 1 and 3 were loaded with NTS, whereas analysis unit 2 contained only medium. Peaks and motility signals were only detected in analysis units 1 and 3 hosting NTS, which indicates that there was no cross talk between the chambers as there were no signals detected in unit 2, which did not contain any larvae.

To compare motility recordings from measurements with different numbers of loaded NTS, the calculated signal power was normalized by the number of NTS in the respective analysis unit. The number of NTS in the sensing region was obtained in an automated way by counting the number of peaks recorded with electrode pair 1 prior to the motility measurement by using a peak detection algorithm. The normalized signal power of live and
dead NTS as a function of the number of NTS in the chip is shown in Figure 3.2c. Signal power values of the live (-16.5 ± 3.4 dBµ) and dead NTS (-35.8 ± 1.1 dBµ) fall in two narrow ranges for different number of worms. The two mean values were significantly different from each other (at least 4 times standard deviation), which evidences that a robust and accurate estimation of viability can be obtained even by using a small number of NTS. A lower limit of 3 NTS in the chip was chosen to reduce the dependence of the motility estimation on the behavior of individual larvae.

Figure 3.2 : (a) Magnitude of the output signal after high-pass filtering. First, electrode pair 1 was selected and NTS flowing through the channel constriction (denoted by the arrow in a-1) induced a transient peak in the trace. Once all the NTS have passed, electrode pair 2 was switched on, and the movement of the NTS between the electrodes (a-2) caused fluctuations in the recorded signal. After
addition of DMSO, the NTS died and stopped moving (a-3), and a flat line was recorded. (b) Signal recordings from three different analysis units in parallel. Analysis units 1 and 3 were loaded with NTS, while only medium was present in analysis unit 2. No crosstalk was observed between the analysis units. (c) Signal power in a 1-3 Hz bandwidth as a function of the number of NTS between the electrodes. The x-axis displays number of larvae used in each measurement. To compare the measurements, the signal power was normalized to the respective number of NTS in the analysis unit. The dotted line indicates the mean value of the signal power for alive (green) and dead (red) NTS. The shaded areas show the standard deviation. The signal power for live and dead NTS falls within narrow ranges and were significantly different (at least 4 times SD) from each other.

To validate the ability of the platform to detect the status of the NTS in the chip, we measured the viability of DMSO-treated and untreated NTS. As described above, DMSO affects the viability of the NTS if used at high concentrations. We analyzed two conditions: (i) NTS incubated for 30 minutes in standard medium with 30% DMSO; (ii) NTS incubated in standard conditions (untreated). Replicate measurements of the NTS for the two test conditions were acquired simultaneously in the platform. The impedance signal at lower frequencies (< 1 MHz) can be used to extract information on the size of the particles passing over the electrodes. Here, we used the peak height recorded at 48 kHz to estimate the size of the NTS under different conditions (Figure 3a). No significant size difference was observed for treated and untreated NTS, which was confirmed visually. However, the NTS exhibited a large difference in motility under the two conditions (Figure 3b). The movement of the larvae between electrode pair 2 in the analysis unit results in fluctuations of the impedance signal, which were used to measure the larvae motility. DMSO treatment killed the NTS, which therefore did not move anymore so that no signal fluctuations were observed. The DMSO treatment resulted in a significant decrease in signal power in comparison to the untreated NTS, which confirms the validity of signal power as indicator of NTS motility and, consequently, viability.
Figure 3.3: (a) Normalized absolute amplitude of the peaks, generated by the passage of the NTS through the funnel constriction, of untreated and DMSO-treated NTS. The number of NTS was $n_{\text{untreated}} = 35$ in the untreated and $n_{\text{treated}} = 13$ in the DMSO-treated case. The peaks were measured with a 48 kHz carrier signal. No significant increase in size was detected by the EIS measurements, as has been confirmed by the micrographs displayed in (c). Scale bar 50 µm. (b) Normalized signal power, measured at 333 kHz for untreated and DMSO-treated NTS. Three replicates were acquired for each condition. Each circle represents a measurement of an analysis unit, and the mean is represented by the black line. (c) Micrographs of untreated and DMSO-treated NTSs

3.5.2 Impedance–based readouts for drug dose-response assay

We measured the variations in the viability of NTS that were exposed to different concentrations of a test compound using our impedance-based readout of size and motility, and compared the results to the standard visual-evaluation method. Although praziquantel is the most widely administered drug, it is known for moderate efficacy against NTS in vitro\textsuperscript{40}. Therefore, mefloquine was selected as a test compound, as this drug has shown potent antischistosomal properties, and is a good benchmark to test new screening methods owing to its fast activity and good dose-response effect in vitro\textsuperscript{41}. As a control, we measured also the
viability of untreated NTS and NTS incubated with the highest concentration of the drug vehicle, 0.5% DMSO (Figure S3.4).

Figure 3.4 shows the relative size of the NTS, as extracted from the impedance measurements, and representative microscopic images for comparison after 24 hours and 72 hours of incubation with mefloquine. After 24 hours incubation in the presence of mefloquine, NTS size, as well as the spread of the NTS size distribution increased, with increasing compound concentrations. However, after 72 hours, the NTS showed a similar size for both the lowest and the highest drug concentration tested, which indicates a subsequent shrinking of the NTS at high drug concentrations. A significant swelling of the NTS was detected for 3.3 and 6.25 µM mefloquine concentrations after 72 hours as compared to that of a 24 hour incubation. The impedance-based size readout correlated well with visual observation, shown by the micrographs recorded at 24 and 72 hours (Figure 3.4c and 3.4d, respectively), which proves that impedance measurements can be used to provide a qualitative phenotypical evaluation.

We measured the motility of NTS after incubation with mefloquine to detect NTS viability at different time points and as a function of drug concentration (Figure 3.5a). After 24 hours, no signal fluctuations could be detected for the NTS incubated with the highest drug concentration (25 µM), indicating that the NTS were no more motile. A high variability in signal power was observed between different replicates for 12.5 µM, which indicated that NTS viability was reduced and that not all NTS were still alive. The large variability is an effect of the low number of NTS in the analysis unit, so that the signal is highly dependent on the individual condition of the loaded NTS. After 72 hours of incubation, a drop in motility was observed for all drug concentrations. NTS incubated with 12.5 µM and 25 µM showed a very low signal power, which indicates loss of viability. A large reduction (~60%) in motility was also detected for the 6.25 µM mefloquine concentration, while, for lower concentrations, the signal power indicated still relatively motile NTS.
The current gold standard for assessing drug efficacy is based on visual evaluation of NTS’s morphology and motility. The visual scores at 24 and 72 hours are plotted in Figure 3.5b. The visual score was recorded with a higher number of NTS cultured under the same conditions. The trends of impedance–based motility scores and visual scores show good correlation for both time points.

We used the motility detection values to extract an IC₅₀ value of mefloquine for the two time points. IC₅₀ corresponds to the drug concentration at which 50% of the larvae were no more viable. A sigmoid fit (shown in black in Figure 3.5) was used to calculate the IC₅₀ both by using the impedance-based evaluations and the visual scores. The IC₅₀ value obtained from impedance motility recordings after 24 hours of incubation with the drug was 12.5 ± 0.01 µM, and decreased to 5.75 ± 0.07 µM after 72 hours. The IC₅₀ values obtained from the visual evaluation were 3.46 ± 0.07 µM after 24 hours and 1.94 ± 0.05 µM after 72 hours, respectively. Differences in IC₅₀ values obtained by the two methods were expected, as the differences in the readout methods are considerable. Visual scoring also includes phenotypical evaluation of the larvae, such as morphology, tegument appearance, and opacity of the larvae, as key parameters for the evaluation, so that lower visual viability scores can be expected for sub-lethal drug concentrations in comparison to an impedance-based evaluation, which is solely based on motility. Moreover, the morphology of the NTSs changes at higher drug concentration, and it is difficult to detect subtle movements of the larvae by eye, which, however, are detectable through an impedance. Upon comparing the motility values, obtained from the impedance chip to those obtained through visual scoring (Figure S3.5), we observed that for a number of drug concentrations (12.5 µM, 6.25 µM at 24h and 6.25 µM and 3.3 µM at 72 h post drug exposure), we were able to detect fluctuations and motility of the NTS, which were missed by the classical visual evaluation method. In all these cases, the evaluators gave an unjustified low viability score. Nevertheless, the IC₅₀ values obtained with the two different methods fall within the same order of magnitude, and only a slight increase in IC₅₀ values was obtained by using the motility information alone.
Figure 3.4: (a) EIS-size measurements of NTS, incubated with six different concentrations of mefloquine \( (n_{untreated} = 26 , n_{DMSO\ control} = 37 , n_{0.78} = 22 , n_{1.56} = 32 , n_{3.3} = 47 , n_{6.25} = 23 , n_{12.5} = 15 , n_{25} = 21 ) \) after 24 h. The size increased with increasing drug concentration. (b) Impedance-based size detection of NTS \( (n_{untreated} = 42 , n_{DMSO\ control} = 29 , n_{0.78} = 25 , n_{1.56} = 23 , n_{3.3} = 22 , n_{6.25} = 20 , n_{12.5} = 11 , n_{25} = 8 ) \) after 72 h of incubation. A drastic increase in NTS size for 6.25 µM of mefloquine was detected. As controls, also NTS incubated in pure medium and 0.5% DMSO were measured. (c),(d) – Micrographs of NTS after 24 h and 72 h of incubation with different concentrations of mefloquine and the DMSO control. Scale bar 50 µm.
Figure 3.5: (a) Signal power recorded from NTS incubated with different concentrations of mefloquine (0.78, 1.56, 3.3, 6.25, 12.5, 25 µM), for 24 h and 72 hours. Each circle represents a measurement of an analysis unit. The total numbers of samples that have been used for each concentration are given in the caption of figure 3.4. (b). Visual score evaluation of NTS (n = 200), incubated with mefloquine for 24 hours and 72 hours. A sigmoidal fit (in black) was used to calculate the IC₅₀ values for the impedance-based evaluation and for the standard evaluation of NTS viability.
3.6 Conclusion
Objective and quantitative methods for monitoring of NTS–stage schistosomes are needed to move towards high-throughput approaches for antischistosomal drug screening. In this paper, we have shown an integrated microfluidic platform that can be used to assess the viability of NTS using an impedance-based analysis method. The use of a microfluidic approach reduces the amount of NTS needed for the analysis, as compared to viability detection based on fluorescence/luminescence image detection or the standard visual evaluation method. The device features simple operation for loading the NTS and to detect their viability. Operation of the device is based on a pump-free microfluidic approach, so that the platform can be used with standard laboratory equipment. The impedance-based readout enables both an automated counting of loaded parasites, for comparing measurements between different analysis units, and subsequent NTS size and motility detection. Drug-dose responses of the larvae to different concentrations of mefloquine obtained via impedance detection showed good agreement with those obtained by standard visual evaluation of NTS, demonstrating that NTS motility could be used as a reliable estimator of viability.

Finally, we showed that impedance-based detection can be easily parallelized to increase throughput. The multiplexing capability of EIS recordings and the simplicity of the experimental setup enable further up-scaling. A higher throughput can be achieved either by increasing the number of analysis units on the platform or by stacking multiple platforms, which is possible due to the fact that optical access is not required for viability evaluation. Moreover, the use of EIS measurements can be extended to continuous monitoring of NTS viability, which could provide more insights into the dynamics of action of the drugs under evaluation, to allow the selection of fast-acting compounds. The presented platform paves the way towards the use of impedance-based detection for objective and automated screening of antischistosomal drug candidates.
3.7 Acknowledgements

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3.8 References


3.9 Supplementary Information

3.9.1 Device fabrication
The device consists of two parts: a poly(dimethylsiloxane) (PDMS) layer containing the microfluidic structures, and a glass slide with a patterned metal layer. The PDMS layer was cast from a mold, fabricated by a SU-8 photolithography process on a 4-inch silicon wafer (Figure S 3.1a). SU-8 3050 (MicroChem Co., Westborough, USA) was spin-coated to obtain a layer of 100 µm thickness and was then exposed to UV light through transparency masks for cross-linking. The mold was silanized for 3h with trichloro(1H,1H,2H,2H-perfluoro-octyl)silane (Sigma-Aldrich, Buchs, Switzerland) in a vacuum desiccator. A mixture of silicone and curing agent (10:1 w/w, Sylgard® 184, Dow Corning, Germany) was poured on the SU-8 mold and cured for 2 h at 80°C. The PDMS was then peeled off the mold and cut into single chips. Holes were punched for the device inlets and reservoirs.

The electrodes were fabricated on 4-inch 500-µm-thick glass wafers using a lift-off process (Figure S 1b). The wafer was spin-coated with lift-off resist (LOR3B, Microchem Corp., Newton, USA), followed by a positive photoresist (S1813, Rohm-Haas, Schwalbach, Germany) and patterned using photolithography. A sputtering process was applied to deposit a platinum film of 200 nm thickness on top of a 20 nm-thick W/Ti adhesion layer. Metal lift-off was carried out using mr-Rem 400 remover (Micro Resist Technology GmbH, Berlin Germany). After fabrication, the glass wafer was diced into individual glass slides. The PDMS device and glass accommodating the electrodes were aligned with each other using a custom-made alignment tool and bonded together using oxygen-plasma bonding.
Figure S 3.1: Fabrication steps of the device. (a) Fabrication steps of the PDMS layer using SU-8 photolithography and PDMS molding. SU-8 is spin-coated on a silicon wafers and patterned using a photolithographic process. After development of the SU-8, the three-layered SU-8 mold is silanized. Regular PDMS casting is done to realize the analysis unit and microfluidic channels. (b) Fabrication steps for patterning of the electrodes on glass using a lift-off process. The glass is spin-coated with lift-off resist (orange), followed by deposition of a positive photoresist (red). After developing of the resists and the subsequent metal deposition, a lift-off process is used to remove the remaining photoresist and leave the metal layer in the required areas. The wafer is then diced to obtain individual glass slides.

3.9.2 Effect of tilting angle on size and motility measurements

We performed an experiment with the same NTS sample in triplicates and measured NTS size and motility at three different tilting angles, 10°, 15°, and 20° (Figure S 3.2a). A larger angle will generate a higher flow rate, thus allowing more NTS to move from the loading to the sensing region. Nevertheless, the impedance-based counting of NTS in the chip provides
the exact number of larvae that enter the sensing region, which obviates any potential issues in comparing measurements at different angles or flow rates. No significant differences in size measurement values were observed for the three different tilting angles. The angle also had no effect on the values of the motility recordings (Figure S 3.2b). The motility of the NTS was recorded, when the larvae had passed through the funnel and were retained by the pillar structures. A tilting angle of ~15° was then later used for all experiments.

![Figure S 3.2: (a) Size measurements of live NTS of the same loaded sample, measured at different tilting angles of 10°, 15°, and 20°. A sample with a defined number of worms was loaded into the device, but not all loaded NTS arrived in the sensing region for every experiment or for every angle (n₁₀ = 18, n₁₅ = 25, n₂₀ = 21). (b) The signal power of live NTS of the same sample measured at different tilting angles in triplicates.](image)

3.9.3 Experimental setup
The device is placed in a custom-made PCB and holder, which allows for visual access to the chip via a microscope. The PCB consists of an Arduino to control the selection of the active pair of electrodes. A custom-made software, written in Python, is used to communicate with the Arduino to program the switching electrode pairs and the recording time of each analysis unit. An AC voltage of 300 mV at 48 kHz and 333 kHz is applied to the stimulating electrode, and the resulting current is measured. The current is then converted to voltage by
the trans-impedance amplifier and recorded by the impedance spectroscope (HF2-LI, Zurich Instrument, Switzerland). The recorded data are analyzed using custom-written scripts in MATLAB (The Mathwork Inc., Natick, United States).

![Figure S 3.3: (a) Schematic of the setup. Picture of the device in the holder on the microscope stage. The device is connected to a custom-designed PCB, which is used for automated switching between the “NTS counting” and “NTS viability” electrodes of the different analysis units. The PCB is used to route the selected electrodes to the impedance spectroscope for measurements. A custom-made software is used to communicate the switching protocol and recording duration of the electrodes to the PCB. This information is entered by the user into a custom-made software, and the analysis is then carried out automatically. An opening at the back side of the holder enables visual access to the analysis units.](image)
3.9.4 Control measurements for the drug-dosing experiment

![Graphs showing signal power and visual scores for different drug concentrations.](image)

Figure S 3.4: (a) Signal power plotted for untreated NTS (incubated only with medium), NTS with vehicle (0.5% DMSO in medium) and NTS treated with 25 µM mefloquine after 24 and 72 h. (b) Visual score values obtained for controls (untreated NTS and NTS with vehicle) and NTS with 25 µM mefloquine after 24 and 72 h, respectively. Error bars represent the mean error.

3.9.5 Comparing signal power and visual scores for different drug concentrations

![Graphs showing power of signal versus visual scores.](image)

Figure S 3.5: Power of the impedance signal plotted versus visual scores for different concentrations of mefloquine at 24 h (a) and 72 h (b). For certain drug concentrations, such as 12.5 µM and 6.25 µM at 24 h, and 6.25 µM and 3.3 µM at 72 h, a considerable NTS motility was obtained by using the impedance-based detection method, which was missed by classical visual evaluation. The respective visual scores were lower than 1, which is indicative of a severe loss of motility, which turned out to be unjustified low values, as the larvae were still alive.
4 CONCLUSION AND OUTLOOK

4.1 Conclusion

The application of electrical impedance spectroscopy as complementary readout or as alternative readout method to microscopy was described in the thesis. This thesis presents the design and development of microfluidic platforms that include automated and parallelized impedance-based readouts for (1) growth rate monitoring of yeast cells and (2) viability detection of parasitic worms.

The first half of the thesis focuses on the creation of a platform for long-term culturing and high-resolution imaging of yeast cells, while the growth rates of populations of cells could be assessed in parallel and in a label-free way by means of impedance measurements. The platform allows for simultaneous culturing of up to three strains under different microfluidic-flow and medium conditions. The use of flow resistors ensures controlled, unidirectional and parallel flow in the culturing chambers. Each chamber consists of five sets of analysis units for parallelized in situ impedance analysis of the growth rate. The design of the clamping pads and hexagonal structures was modeled using COMSOL and the design was optimized so as to ensure that the outgrowing cells passed over the electrodes for impedance measurements. The hexagonal structures also helped to provide support to the culturing chamber ceiling and to prevent collapsing. The spacing between the analysis units was also
modeled in COMSOL to avoid exchange of liquids and cross contamination. The diamond-like shape of the pads entailed a sufficient number of clamped cells and minimized the area of stagnant flow around the pads. The distance between hexagonal structures was chosen so that the cells could pass over the electrodes owing to the laminar flow within the device. Further, the hexagonal structures also helped to minimize the detection volume for impedance measurements so that passing cells could be detected with sufficient signal-to-noise ratio. The flow rate of 10 µl/min in the device was chosen to achieve fast replenishing of media in the device and to have enough media for the duration of experiments. The frequency of the applied AC signal that proved most sensitive to the passage of the cells over the electrode was chosen for the experiments. The availability of multiple analysis units enables the user to conduct experiments in the same platform in parallel in several replications, while having the capability to perform optical imaging. Importantly, the use of non-permanent vacuum-based bonding between the microfluidic structure and the glass slide hosting the electrodes allows for simple sample loading into the device and multiple usage of the glass slides.

The platform was validated by continuously monitoring the changes in the growth rates of differently engineered yeast strains, loaded in separate culturing compartments of the device, upon changing media conditions by using the impedance readout. The observed growth rates and their changes coincided with those recorded by using standard methods. The quality of the optical imaging was assessed by using a set of endogenously tagged proteins that are frequently used in yeast research. This experiment showed that continuous bright-field and fluorescence imaging of the cells at sub-cellular resolution are possible with our platform.

In the second half of the thesis, a platform for objective and quantitative drug screening for larval-stage parasitic worms, *S. Mansoni*, is described. The integrated microfluidic platform relies on an impedance – based readout for viability detection of the worms. The use of a microfluidic approach reduces the amount of NTS that are needed for the analysis as opposed to the classical method. The device is simple to operate and allows for the measurement of replicates in parallel. Operation of the device is based on a pump-free microfluidic approach, so that the platform can be used with standard laboratory equipment. The impedance-based
readout enables both, an automated counting of loaded parasites for comparing measurements between different analysis units, and subsequent NTS size and motility detection. The channel was designed with pillar structures on either side to prevent the NTS from moving into the reservoirs. The distance between the pillars (~15 µm) was designed to be smaller than the width of the NTS (~40 µm) to efficiently hold them back. The tapered shape of the channel from the inlet to the funnel constriction allowed to focus and direct the loaded NTS towards the sensing region. It also helped to spatially separate the worms so that single NTS passed through the funnel constriction. The width of the funnel constriction was designed, tested and adapted to 50 µm so that single NTS could pass without clogging the constriction. The distance between the electrodes was chosen to be 150 µm, which is similar to dimensions of the NTS, which ensures that enough conductive fluid between the electrodes is displaced upon passing of the NTS. A low frequency signal (48 kHz) was chosen to measure the NTS size, and a signal at a frequency of 333 kHz was chosen for the motility measurements, as this frequency range proved to be most sensitive to the movement of the NTS. Drug-dose responses of the larvae to different concentrations of mefloquine obtained via impedance detection showed good agreement with those obtained by standard visual evaluation of NTS, which demonstrates that NTS motility could be used as reliable estimator of viability.

4.2 Outlook
Automation and higher throughput are some of the key requirements for drug screening processes. The current platform for antischistosomal drug screening is limited by the number of samples that can be measured in parallel. However, the multiplexing capability of EIS and the simple operation of the platform, shown in chapter 3, enable further up-scaling. This up-scaling can be achieved by incorporation of additional chips in the setup with higher numbers of analysis units per chip.

The impedance–based viability monitoring of the parasitic worms can be extended to continuously monitor their health conditions and state. The current chip design was used to
monitor the motility of NTS for a period 18 hours (Figure 4.1). Two different conditions with 2.5 µM, 25 µM of the drug compound and control samples without any drug dosage were tested. A higher motility value was observed within the first few hours of the recording followed by a gradual decrease in viability for the 25 µM drug concentration. 2.5 µM of the drug compound also showed an increased movement during the first 12 hours in comparison to the control, which could be attributed to the drug-induced spasms. Thus, increasing the temporal resolution of the viability measurements can provide more insights into the drug dynamics, which could be missed by an end point assay. A key challenge in long-term experiments is to ensure minimal liquid evaporation. Larger medium reservoirs were used for the experiments, which could hold ~150µL of medium. Several measures, such as the addition of water pools on the chips and the use of polyester films could help to improve humidity control.

![Figure 4.1: Viability measurements of NTS during 18 hrs. of recording for three different conditions – 2.5 µM of drug compound (mefloquine), 25 µM of drug compound and control samples without drug dosage (adapted from ref 1).](image)

Figure 4.1: Viability measurements of NTS during 18 hrs. of recording for three different conditions – 2.5 µM of drug compound (mefloquine), 25 µM of drug compound and control samples without drug dosage (adapted from ref 1).
Studies have shown that patients suffering from schistosomiasis have an increased risk of bladder cancer and hepatocellular carcinoma. A combination of multiple organs on a chip with the parasites would enable simultaneous analysis of drug toxicity, efficacy, as well as the interaction between the parasites and other organs. The addition of a compartment to coculture liver spheroids with NTS to the current platform would be advantageous for studying (1) drug induced hepatotoxicity, or (2) the existence of pro-drugs, i.e., drugs that require biotransformation from an inactive into an active compound by liver enzymes. Studies have shown that eggs, laid by the adult worms, act as mechanical stimulants for bladder cancer.

The platform for the larval stage of the parasites could further be adapted for viability studies of adult-stage worms and their interaction with other cell types, such as uroepithelial cells, which are involved in squamous cell carcinoma of the bladder. Impedance could be used to read out the motility of the adult worms, while microscopy could be used as a readout for the cells.

PDMS is a widely used material for making microfluidic devices due to ease of fabrication of the devices along with other properties, such as optical transparency, biocompatibility, permeability to gases and capability of bonding it to glass. However, it has been shown that PDMS can absorb or adsorb small molecules and hydrophobic compounds over time, which renders PDMS unsuitable for drug screening applications. A few attempts have already been made to find better materials for lab-on-chip applications, such as thermoplastics, fluorine-based and thiol-based polymers. In order to create integrated microfluidic platforms, an alternative material to PDMS, which allows to pattern electrodes on it or to bond it to glass featuring electrode structures will be needed.
4.3 References


5  Toepke MW, Beebe DJ. PDMS absorption of small molecules and consequences in microfluidic applications. Lab Chip 2006; 6: 1484.

