Label-free Identification of Microorganisms using a Contact-less Dielectric Microsensor

AUSTRIAN RESEARCH CENTERS

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Abstract

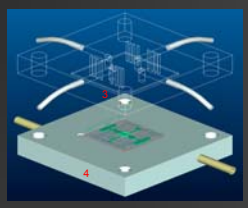
Microfabricated biochips are developed to continuously monitor cellular phenotype dynamics in a non-invasive manner. In the presented work we describe the novel combination of contact-less micro-dielectric sensors and microfluidics that promote biofilm formation for quantitative cell analysis. The cell chip consists of a polymeric fluidic (PDMS) system bonded to a glass wafer containing the electrodes, while temperature and fluid flow are controlled by external heating and pumping stations. The high-density interdigitated capacitors are isolated by a 550 nm multi-passivation layer of defined dielectric property that provides stable, robust and non-drifting measurement conditions. The performance of this detector is evaluated using various bacterial and yeast strains. The high sensitivity of the developed micro-dielectric sensors allows direct identification of microbial strains based on morphological differences. The novel biofilm analysis platform is used to continuously monitor dynamic responses of *C. albicans* and *P. pastoris* biofilms to increased shear stress, temperature and antimicrobial agent concentration. While the presence of shear stress triggers significant changes in yeast growth profiles, the addition of 0.5 µg/mL amphotericin B revealed two distinct behaviors of *C. albicans* biofilm. In contrast to visual results, impedance spectra initially increased linearly at 30 Ohm/h followed by 10 Ohm/h (at 50 kHz) rate over 10 hours. These results demonstrate the ability to directly measure sub-cellular components with high sensitivity within a living cell population.

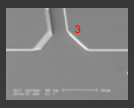
Introduction

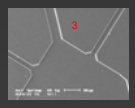
Microbial biofilms exist in virtually all nutrient-sufficient ecosystems and are phenotypically distinct from their planktonic or free floating counterparts. Biofilms are structured microbial communities attached to surfaces, sometimes embedded within a matrix of extracellular polymers, and are significantly less susceptible to antimicrobial agents. It is also recognized that a substantial proportion of human infections involve biofilms found on surfaces of implants or medical devices, such as catheters, joint replacements, prosthetic heart valves and others. The interrelated and complex nature of cellular dynamics within biofilms require the creation of an analysis technology capable of continuously monitoring phenotypic changes such as adaptation, aging and etc. throughout the entire life cycle. We have therefore developed a microfluidic biochip capable of detecting phenotypic changes continuously in a non-invasive manner while maintaining physiological conditions throughout the experiment.

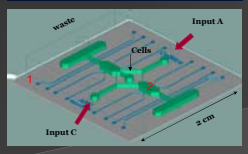
Microfluidic Biochip Design

1. Micro-electromechanical systems (MEMS) Technology
   - Two-component chip using different materials (glass & polymer)
2. Multiparameter detection platform
   - High-density interdigitated capacitors (µIDC) and band electrodes
   - Integrated reference arm
3. Integrated microfluidic system
   - Three input reservoirs (flow rate & shear stress control)
4. Heating system
   - Maintaining constant temperature over long periods of time
5. Continuous studies of fungal biofilms









Microfluidic layout and chamber geometry is designed to promote biofilm formation

Results

Characterization of Cell Chip

The aim of this research is the development and characterization of technology capable of continuously monitoring cell growth, viability and morphology changes with high sensitivity. An important feature of the newly developed technology is the complete isolation of the µIDC that guarantee non-invasive monitoring conditions. The physical separation of micro-dielectric sensor from the liquid environment eliminates the interaction of electroactive ion species (e.g. electrode polarization) or bubble formation leading to stable non-drifting measurement conditions. Single, bi- and multi-layer passivation strategies using epoxy (350 nm), spin-on-glass (100 nm), silicon nitrite (100 nm), and combinations thereof are investigated.

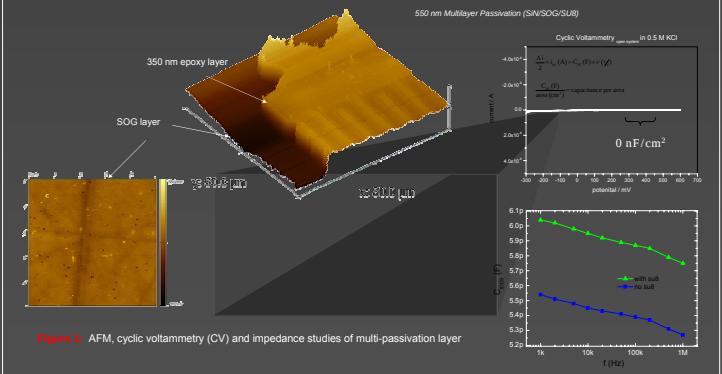


Figure 1: AFM, cyclic voltammetry (CV) and impedance studies of multi-passivation layer

Principle of Cellular Dielectric Spectroscopy using µIDC

Dielectric Spec. measures the response of biological material to an applied electric field and is described by its conductivity (let charge pass) and permittivity (store charge). Cellular dielectric spectroscopy provides information about cell morphologies such as cell wall, cytoplasm, and etc. Drift analysis using saline buffer yielded a relative standard deviation of 1.5% RSD over a period of 30 hours indicating that changes to the baseline greater than 3% RSD can be readily detected. The absence of charging currents or ohmic contributions provide stable and non-drifting measurement conditions

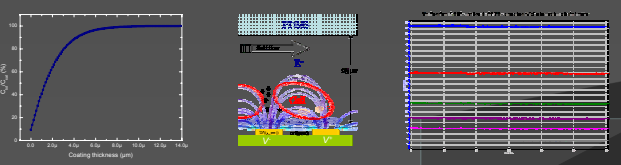


Figure 2: A) Calculation (conformal mapping technique) of electric field distribution using present sensor geometry 5 x 5 µm (finger width and gap). B) Schematic representation of dielectric spectroscopy. C) Stability of micro-dielectric sensor over 20 h measuremnet

Performance Characteristics of Contact-Less Micro-Dielectric Sensors

It is imperative for the development of a novel analysis technique to use well-known and established systems. The best way to initially investigate morphological and metabolic changes at the cellular level is to employ a simple model system because the biology can be easily standardized. In contrast to existing phenotype technologies that rely on an organism's ability to reproduce (depending on the organism, taking hours to days), the developed micro-dielectric sensor provides continuously quantitative results allowing the adaptive phenotypic changes to be monitored through the entire life cycle of a cell.

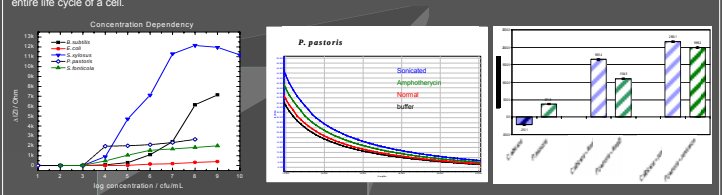


Figure 3: Performance characteristics of the contact-less micro-dielectric sensor in the presence of A) increasing concentrations of microorganisms, B) raw data of live, dead and disrupted yeast cells, and C) comparison between *C. albicans* and *P. pastoris* cell cultures

Label-Free Identification of Microorganisms

Next we investigated the ability of the developed detection methodology to directly distinguish and identify individual strains in a nanovolume setting in the absence of any labels or indicators. Initially we evaluated the influence of media components on dielectric spectra using chemometric data analysis. Principle component analysis (PCA) is applied to impedance and phase angle signals obtained from cell suspensions and compared to cell-free extracts of their respective growth media. Generated pattern recognition plots show distinct groupings of microorganisms while cell free media extract exhibits only random distribution as seen in Figure 4A. These results clearly indicate that the identification of organisms is not affected by media components or metabolites generated during microbial cultivation.

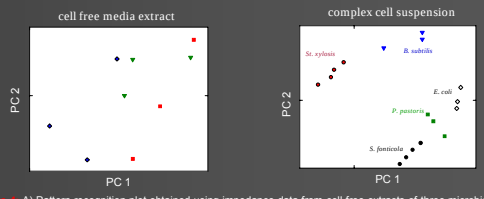


Figure 4: A) Pattern recognition plot obtained using impedance data from cell-free extracts of three microbial growth media. *E. coli* K12 (◊), *B. subtilis* (▼) and *P. pastoris* (■) were cultured under standard conditions, harvested in the exponential phase, and centrifuged. 1 µL aliquots of supernatant were injected into the biochip and allowed to settle for 30 min prior measurement. B) Pattern recognition plot using phase angle values obtained in the presence of *B. subtilis* (▼), *S. xylosis* (○), *E. coli* K12 (◊) , *P. pastoris* (■), and *S. fonicola* (●).

On-chip Monitoring of Biofilm Dynamics

Yeast growth profiles were initially determined by calculating area expansion rates of individual colonies over a 5 hour period using time-lapse microscopy. Figure 5 shows a comparison of calculated expansion rates of *P. pastoris* microcolonies in the absence and presence of fluid flow (0.12 µL/min). In the absence of continuous media supply, surface expansion rates followed known growth characteristics of liquid batch cultures. In the presence of fluid flow, however, cell growth was significantly influenced exhibiting linear growth rates (colony diameter in flow direction) of 8.3 and 12.5 µm/h, respectively.

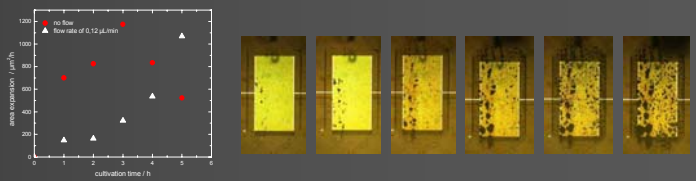


Figure 5: Growth curve of *P. pastoris* using calculated colony area expansion rates obtained by time-lapse microscopy in the absence (●) and presence (▲) of 0.12 µL/min fluid flow.

Fungal Biofilm Response to Shear Stress

Cellular dielectric spectroscopy was used to continuously monitor dynamic responses of yeast biofilms to shear stress. A flow rate increase (solid arrow) with *P. pastoris* from 0.12 µL/min to 0.5 µL/min maintained the cell population constant over a period of 6 hours by washing out newly formed cells, while a decrease of fluid flow (dashed arrow) induced rapid cell growth in the proliferation chamber. In turn, a flow rate increase from 0.12 to 0.5 µL/min with *C. albicans* caused an initial loss of cells followed by a continuous increase in cell number until complete coverage of the sensor was obtained.

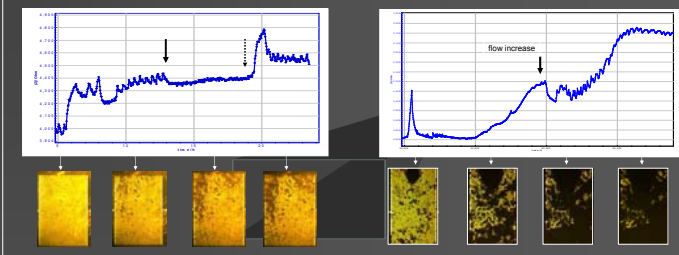


Figure 6: A) Growth profile of *P. pastoris* biofilm before and after shear stress increase (solid arrow) and subsequent decrease (dashed arrow) from 0.12 to 0.5 µL/min to 0.12 µL/min. B) Dynamic response of *C. albicans* biofilm in the presence of increased shear stress. Impedance data obtained for every minute at 50 kHz are plotted against time.

Dynamic Response of Candida Biofilm to Fungicide

Figure 7 shows the dynamic response of the *C. albicans* biofilm before and after the administration of 0.5 µg/mL amphotericin B (red arrow). Interestingly, impedance signals started to increase linearly at an initial rate of 30 Ohm/ h (first 2 hours) followed by 10 Ohm/h (at 50 kHz) over a period of 10 hours suggesting a decrease in cell population. However, corresponding pictures taken every 30 min during the same time period showed that the *C. albicans* biofilm actually continued to spread. These results indicate that the monitored signal changes are caused by morphology changes induced by the incorporation of amphotericin B into the plasma membranes. Although, the effects can be readily quantified, it is not clear whether observed rates are caused solely by the fungicide or by intrinsic morphology changes due to activation of resistance genes such as the expression of efflux pumps, change of cell wall composition and etc.

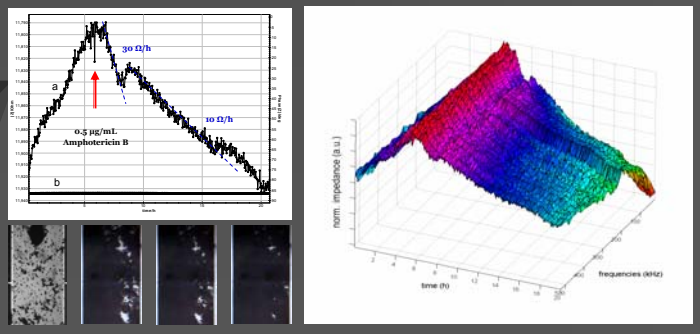


Figure 7: (A) *Candida* biofilm response to 0.5 µg/mL amphotericin B (solid arrow) where (a) impedance signals and (b) phase angle values obtained at 50 kHz are plotted against time. (B) Dielectric Spectra of *C. albicans* biofilm response to amphotericin B.

Conclusions

We have developed a novel microfabricated biofilm analysis platform that provides quantitative results to the continuous monitoring of the adaptive phenotypic changes of the entire life cycle of a cell population. The precise control of critical electrode characteristics, such as size, shape and passivation composition, as well as thickness, makes microfabrication an attractive tool for the development of contact-less dielectric sensors. The interdigitated capacitors are designed to monitor living cells in a space of approximately 20 nL volume and 5 µm thickness. An important feature of the sensor design is the application of electrical insulation making non-invasive, stable and robust measuring conditions possible. In this work we evaluated the multi-passivation layer and characterized the detector configuration utilizing various bacterial and yeast strains. We successfully demonstrated that microorganism can be readily identified and that any morphological changes induced by external stimuli can be continuously monitored using dielectric sensors. We have also shown that induced shear stress has significant effects on biofilm formation and that, by controlling fluid flow, growth rates can be adjusted and used for cell analysis. Using the developed technology we have monitored *C. albicans* biofilm dynamics and response to the antifungal agent amphotericin B. The ability to monitor sub-cellular components with high resolution within a cell population is expected to open new insights into cellular phenotype dynamics. Extension of this work to quantify biofilm dynamics and its application to a wide range of external stimuli is underway.