Baktériumok optikai biochipeken: alapjelenségek és modell rendszerek (KH 126900)

FINAL REPORT

A kutatás eredményeinek rövid, tényszerű összefoglalása magyarul és angolul

A kutatási projekt célja élő sejtek adhéziós folyamatainak alapkutatása volt, melyet bioszenzorokkal, és egyedi sejtek mérésére alkalmas biofizikai módszerekkel valósítottunk meg. Előkísérleteket is végeztünk ezen módszerekkel (számítógép vezérelt mikropipetta, FluidFM, Epic Cardio bioszenzor), hogy még pontosabb eredményeket kaphassunk a további célzott méréseknél. A kutatás során modell felületeket is ki tudtunk alakítani, melyek baktériumokat és emlős sejteket taszító, illetve azokat specifikusan megkötő tulajdonságokkal bírnak. Méréseink során bakteriális flagellineket, illetve élő baktériumokat imitáló mikrogyöngyöket és nanorészecskéket is alkalmaztunk a folyamatok egyszerű modellezéséhez és megértéséhez. A projekt eredményeképp önszerveződő flagellin monorétegeket alakítottunk ki hidrofób felületeken. Molekuláris szintű interakciókat figyeltün meg, és az adhéziós erő mérését végeztük el mikrogyöngyök, mint modell rendszer, segítségével. Bakteriális sejtek emlős sejtekbe hatolását nanorészecskékkel imitáltuk, és valós időben monitoroztuk, így kinetikai információkat kaptunk a végbemenő folyamatokról.

The aim of the project was to perform basic research of living cell adhesion processes, which was carried out with biosensors and biophysical methods suitable for measuring individual cells. Preliminary experiments were also performed with these methods (computer-controlled micropipette, FluidFM, Epic Cardio biosensor), in order to obtain even more accurate results for further targeted measurements. In the course of the research, we were also able to design model surfaces that have repellent and binding properties on bacteria and mammalian cells. In our measurements, we applied bacterial flagellins, microbeads and functionalized nanoparticles to mimic living bacterial cells for simple modeling and understanding of the relevant biophysical processes. As a result of the project, we formed self-assembled flagellin monolayers on hydrophobic surfaces. Molecular-level interactions were observed and surface adhesion forcees were measured using microbeads as a model system. The penetration of bacterial cells into mammalian cells was mimicked with nanoparticles and monitored in real-time to obtain kinetic information about the processes.

Milestones and main results

1 Creating model surfaces using OWLS, QCM and EPIC BT biosensors

Hydration, viscoelastic properties and dominant structure of thin polymer layers on the surface of waveguide material were evaluated using optical waveguide lightmode spectroscopy (OWLS) and quartz crystal microbalance (QCM) methods. The fundamentally different principles of the two applied label-free biosensors enable to examine analyte layers from complementary aspects, e.g. to determine the amount of bound water in hydrated layers. In our

study *(Saftics et al. In situ viscoelastic properties and chain conformations of heavily hydrated carboxymethyl dextran layers: a comparative study using OWLS and QCM-I chips coated with waveguide material. Scientific reports, 2018)*, a new QCM instrument with impedance measurement (QCM-I) was introduced (Fig.1). Its specially designed sensor chips, covered by thin film of waveguide material (SiO2-TiO2), supply identical surface as used in OWLS sensors, thus enabling to perform parallel measurements on the same type of surface. Viscoelastic analysis of the measured data was performed by our evaluation code developed in MATLAB environment, using the Voinova's Voigt-based model. In situ deposition experiments on the ultrathin films of poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) were conducted for instrumental and code validation. Additionally, a novel OWLS-QCM data evaluation methodology has been developed based on the concept of combining hydration and viscoelastic data with optical anisotropy results from OWLS measurements. This methodology provided insight into the time-dependent chain conformation of heavily hydrated nano-scaled layers, resulting in unprecedented structural, hydration and viscoelastic information on covalently grafted ultrathin carboxymethyl dextran (CMD) films, which are basically antifouling coatings that can be specifically conjugated by adhesive motifs in order to arrange cells and bacteria on biosensor surfaces. The measured mass values as well as hydration and viscoelastic properties were compared with the characteristics of PLL-g-PEG layers.

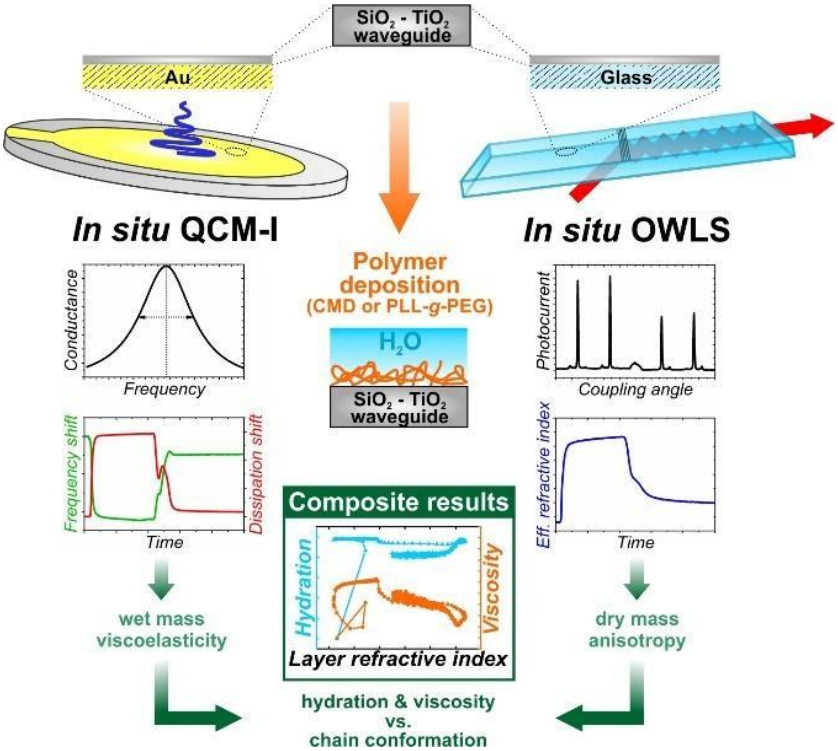

Figure 1 *Schematic representation of the measurement and data evaluation methodology developed and applied in this work to test and develop model surfaces for cell adhesion.*

In our other publication *(Peter et al. Horvath. High-Resolution Adhesion Kinetics of EGCG-Exposed Tumor Cells on Biomimetic Interfaces: Comparative Monitoring of Cell Viability Using Label-Free Biosensor and Classic End-Point Assays. ACS Omega, 2018)* a high-throughput label-free resonant waveguide grating biosensor, the Epic BenchTop was utilized to in situ monitor the adhesion process of cancer cells on Arg-Gly-Asp tripeptide (RGD) displaying biomimetic polymer surfaces. Using highly adherent human cervical adenocarcinoma (HeLa) cells as a model system, cell adhesion kinetic data with outstanding temporal resolution were obtained. We found that pre-exposing the cells to various

concentrations of the main extract of green tea, the epigallocatechin gallate (EGCG), largely affected the temporal evolution of the adhesion process. For unexposed and low dosed cells, sigmoid shaped spreading kinetics was recorded. Higher dose of EGCG resulted in a complete absence of the sigmoidal character, and displayed adsorption-like kinetics (Fig.2). By using the first derivatives of the kinetic curves, a simple model was developed to quantify the sigmoidal character and the transition from sigmoidal to adsorption-like kinetics. The calculations showed that the transition happened at EGCG concentration of around 60 µg/mL. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide end-point assay, we concluded that EGCG is cytostatic but not cytotoxic. The effect of EGCG was also characterized by flow cytometry. We concluded that, using the introduced label-free methodology, the shape of the cell adhesion kinetic curves can be used to quantify in vitro cell viability in a fast, cost-effective, and highly sensitive manner. This method probably can be used with bacterial cells as well to demonstate the antibacterial effects of certain further natural compounds.

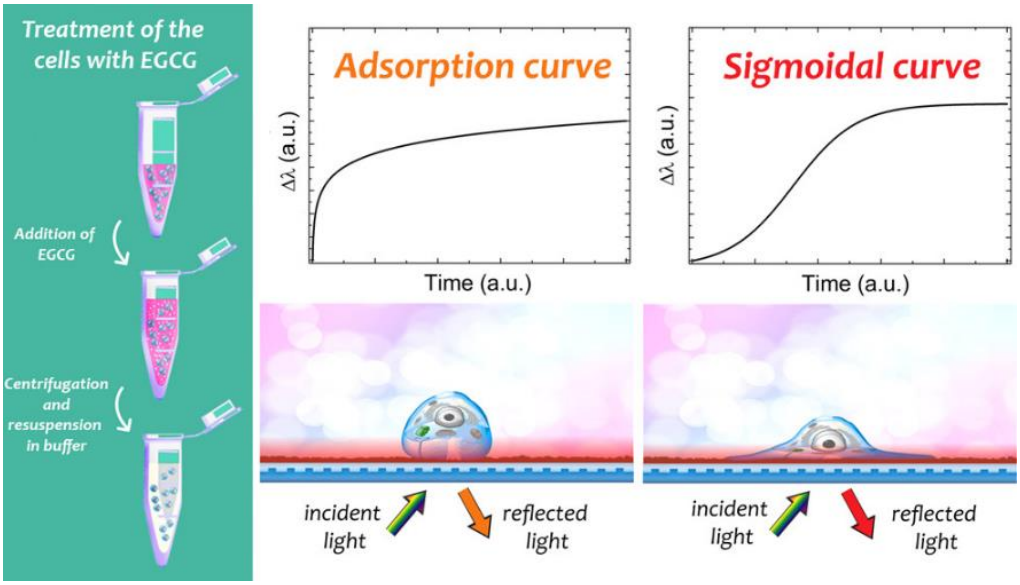

Figure 2 *Schematic illustration of the working principle of the Epic BT biosensor and the steps of the measurement (left). In case of adsorption kinetic curve, the cell attaches but does not adhere onto the biosensor surface. This phenomenon indicates a nonliving, "dead" process. Note, it also occurs when proteins adsorb onto the surface. In case of sigmoidal kinetic curve, the cells adhere onto the biosensor surface. This phenomenon indicates a "living" process.*

2 Immobilization of peptides and bacterial motifs on sensor surface and glass or latex beads

Flagellins (building blocks of bacterial flagellar filaments) does not preferentially adsorb on hydrophilic substrates, but very rapidly forms an oriented, dense and stable monolayer on hydrophobic surfaces, where the hypervariable D3 domain (one of the four flagellin domains) is oriented toward the solution. It presents a repellent surface coating to bacterial or cancer cells, thus this property can be utilized in biosensors and biofunctionalized surfaces. In our work *(Kovacs et al. Kinetics and Structure of Self-Assembled Flagellin Monolayers on Hydrophobic Surfaces in the Presence of Hofmeister Salts: Experimental Measurement of the Protein Interfacial Tension at the Nanometer Scale. J. Phys. Chem. C, 2018),* we monitored the adsorption–desorption kinetics and adsorbed layer structure of the bacterial protein flagellin in the presence of Hofmeister salts by a surface sensitive label-free optical biosensor (optical

waveguide lightmode spectroscopy, OWLS). The recorded OWLS data were analyzed by a computer code using a set of coupled differential equations modeling the adsorption–desorption process. By supposing reversibly and irreversibly adsorbed protein states with different adsorption footprints, the kinetic data could be perfectly fitted. We revealed that the proteins adsorbing in the presence of kosmotropic salts had smaller footprints, leading to a more oriented and densely packed layer (Fig. 3). Kosmotropic salts increased both the adsorption rate constant and the transition rate constants from the reversibly to the irreversibly adsorbed state. In contrast, chaotropic salts increased the desorption rate constant and led to decreased adsorbed mass and a more loosely packed film. Neither circular dichroism spectroscopy in bulk solutions or Fourier transform infrared spectroscopy of surface-adsorbed flagellins could reveal significant structural changes due to the presence of the Hofmeister salts, and supported our conclusions about the adsorption mechanism. On the basis of the measured kinetic and structural data (footprints of adsorbed proteins), we developed a model to calculate the protein–water-substrate interfacial tension in the presence of Hofmeister salts, and compared the experimentally obtained values with related literature data. The calculated values are consistent with previously published data of surface tension changes, and—to the best of our knowledge—represent the first experimental results for this quantity.

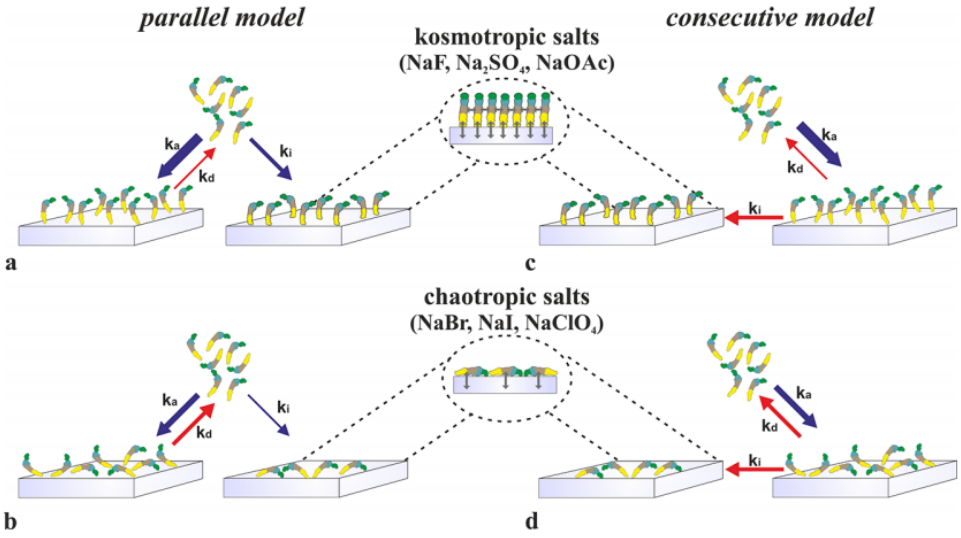

Figure 3 *Schematic illustration of the adsorption kinetics of flagellin proteins on a hydrophobic surface.*

Micropatterning of living cells or bacteria over millimeter-centimeter scale areas is of high demand in adhesion research. Micropatterning methodologies require both to have a biomimetic support that provides long-term cell or bacterium viability and high-contrast for printing as well as to have a printing technology offering reduced number of fabrication steps, high-throughput and a pattern resolution enabling the selective deposition of even single cells or bacteria. In our relevant study (*Saftics et al. Biomimetic Dextran-Based Hydrogel Layers for Cell Micropatterning over Large Areas Using the FluidFM BOT Technology. Langmuir, 2019*), we presented the micropatterning of living cells on carboxymethyl dextran (CMD) hydrogel layers using the FluidFM BOT technology (Fig.4). Micropatterning of living single cells and cell clusters over millimeter–centimeter scale areas is of high demand in the development of cell-based biosensors. Micropatterning methodologies require both a suitable biomimetic support and a printing technology. In this work, we present the micropatterning of living mammalian cells on carboxymethyl dextran (CMD) hydrogel layers using the FluidFM BOT technology. In contrast to the ultrathin (few nanometers thick in the dry state) CMD films

generally used in label-free biosensor applications, we developed CMD layers with thicknesses of several tens of nanometers in order to provide support for the controlled adhesion of living cells. The fabrication method and detailed characterization of the CMD layers are also described. The antifouling ability of the CMD surfaces is demonstrated by in situ optical waveguide lightmode spectroscopy measurements using serum modeling proteins with different electrostatic properties and molecular weights. Cell micropatterning on the CMD surface was obtained by printing cell adhesion mediating cRGDfK peptide molecules (cyclo(Arg-Gly-Asp-d-Phe-Lys)) directly from aqueous solution using microchanneled cantilevers with subsequent incubation of the printed surfaces in the living cell culture. Uniquely, we present cell patterns with different geometries (spot, line, and grid arrays) covering both micrometer and millimeter–centimeter scale areas. The adhered patterns were analyzed by phase contrast microscopy and the adhesion process on the patterns was real-time monitored by digital holographic microscopy, enabling to quantify the survival and migration of cells on the printed cRGDfK arrays. This technique is suitable for further micropatterning of bacteria repellent or adhesive surfaces as well.

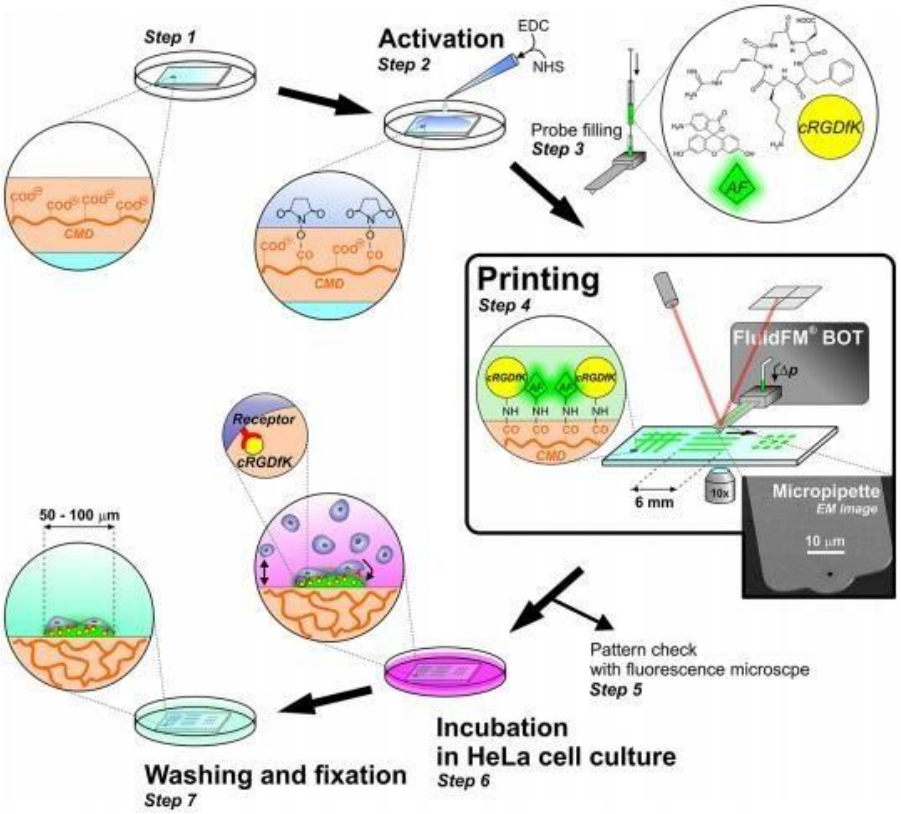

Figure 4 *Method used for cell adhesion experiments on the cRGDfK-printed CMD surface, where microprinting was performed with the FluidFM technique.*

3 Kinetic measurements with model systems

Functionalized nanoparticles (NPs) can penetrate into living cells and vesicles, opening up an extensive range of novel directions. For example, NPs are intensively employed in targeted drug delivery and biomedical imaging. However, the real-time kinetics and dynamics of NP−living cell interactions remained uncovered. In our study (*Peter et al. Interaction of*

Positively Charged Gold Nanoparticles with Cancer Cells Monitored by an in Situ Label-Free Optical Biosensor and Transmission Electron Microscopy. ACS Appl. Mater. Interfaces 2018), we in situ monitored the cellular uptake of gold NPs −functionalized with positively charged alkaline thiol− into surface-adhered cancer cells, by using a high-throughput label-free optical biosensor employing resonant waveguide gratings (Fig.5). The characteristic kinetic curves upon NP exposure of cell-coated biosensor surfaces were recorded and compared to the kinetics of NP adsorption onto bare sensor surfaces. We demonstrated that from the above kinetic information, one can conclude about the interactions between the living cells and the NPs. Real-time biosensor data suggested the cellular uptake of the functionalized NPs by an active process. It was found that positively charged particles penetrate into the cells more effectively than negatively charged control particles, and the optimal size for the cellular uptake of the positively charged particles is around 5 nm. These conclusions were obtained in a cost-effective, fast, and high-throughput manner. The fate of the NPs was further revealed by electron microscopy on NP-exposed and subsequently fixed cells, well confirming the results obtained by the biosensor. Moreover, an ultrastructural study demonstrated the involvement of the endosomal−lysosomal system in the uptake of functionalized NPs and suggested the type of the internalization pathway. NPs may act as model systems to imitate host cell-bacteria interactions and penetration in biosensor measurements. Furthermore, these measurement settings and results can be applied to study the uptake of NPs by bacteria as well.

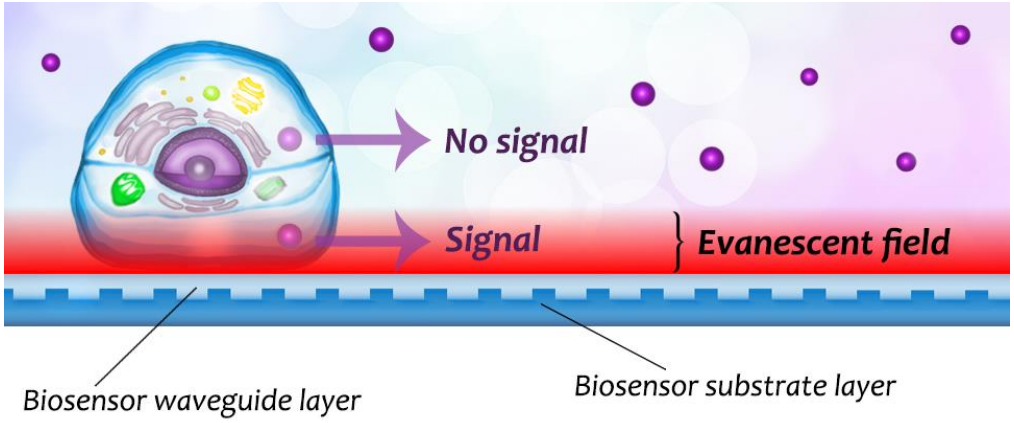

Figure 5 *Schematic illustration of the concept of evanescent labelfree biosensors in NP uptake detection.*

4 Experimental work on more complicated models using the combination of optical biosensors and computer controlled micropipette and FluidFM

Our aim was to perform biophysical measurements using optical biosensors, computer controlled micropipette and FliuidFM (and their combination) on more complex model systems.

We tend to measure cell adhesion and to print antibacterial coatings by fluidic force microscope (FluidFM). This technique can be considered as the nanofluidic extension of the atomic force microscope (AFM). It uses special probes with an integrated nanochannel inside the cantilevers supported by parallel rows of pillars. However, little is known about how the properties of these hollow cantilevers affect the most important parameters which directly scale the obtained spectroscopic data: the inverse optical lever sensitivity (InvOLS) and the spring constant (k). The precise determination of these parameters during calibration is essential in order to gain

reliable, comparable and consistent results. Thus the aim of our work was to investigate the calibration accuracy of these parameters and their dependence on: (1) the aperture size (2, 4 and 8 μm) of the hollow micropipette type cantilever; (2) the position of the laser spot on the back of the cantilever; (3) the substrate used for calibration (silicon or polystyrene). It was found that both the obtained InvOLS and spring constant values depend significantly on the position of the laser spot. Based on our results a calibration strategy is proposed and the optimal laser position which yields the most reliable spring constant values was determined and found to be on the first pair of pillars. Our method helps in reducing the error introduced via improper calibration and thus increases the reliability of subsequent cell adhesion force or elasticity measurements with FluidFM (Fig. 6). These results were published in Scientific Reports (*Nagy et al. Spring constant and sensitivity calibration of FluidFM micropipette cantilevers for force spectroscopy measurements, Scientific Reports, 2019*). These important findings were used in our other further measurements by applying FluidFM in this proposal.

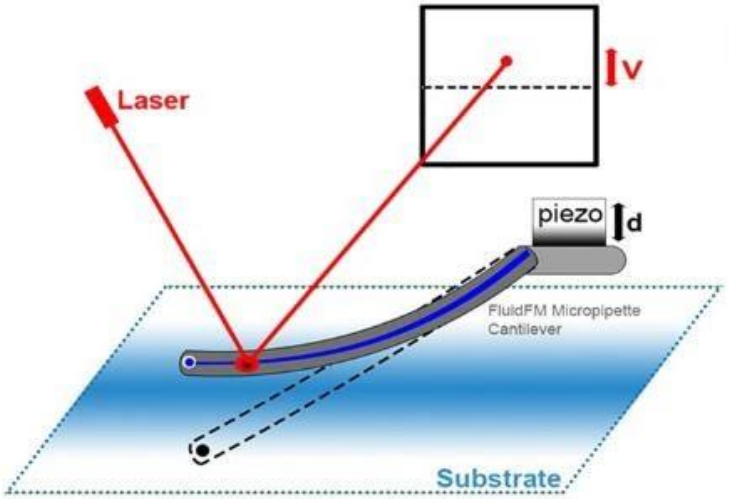

Figure 6 *Illustration of the laser beam reflection based optical setup of the FluidFM system.*

We used microbeads as simple models for bacteria for force measurements. Characterization of the binding of functionalized microparticles to surfaces with a specific chemistry sheds light on molecular scale interactions. Polymer or protein adsorption are often monitored by colloid particle deposition. Washing and centrifuge assays with (bio)chemically decorated microbeads provide better statistics, but only qualitative results without a calibrated binding force or energy value. In our publication (*Gerecsei et al. Adhesion force measurements on functionalized microbeads: An in-depth comparison of computer controlled micropipette and fluidic force microscopy. Journal of Colloid and Interface Science, 2019*) we demonstrated that a computer controlled micropipette (CCMP) is a straightforward and high-throughput alternative to quantify the surface adhesion of functionalized microparticles. However, being an indirect force measurement technique, its in-depth comparison with a direct force measurement is a prerequisite of applications requiring calibrated adhesion force values. To this end, we attached polystyrene microbeads to a solid support by the avidin-biotin linkage. We measured the adhesion strength of the microbeads with both a specialized robotic fluid force microscope (FluidFM BOT) and CCMP (Fig. 7).

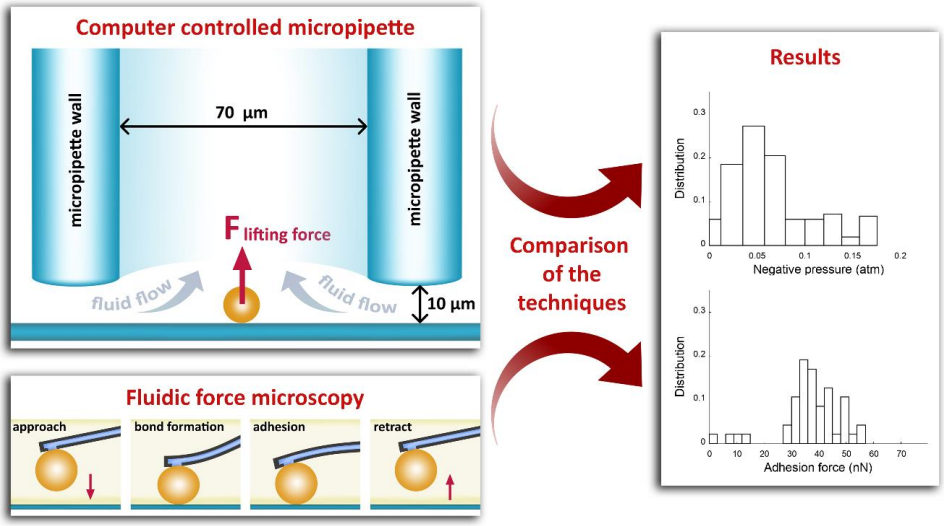

Figure 7 *Schematic illustration of the methods. Adhesion force measurements on microbeads were performed by using computer controlled micropipette (CCMP) and Fluidic force microscopy. The received results of the two techniques has been compared.*

We turned to single-cell measurements by using the combination of robotic FluidFM and Epic Cardio biosensor.

In this study a high spatial and temporal resolution resonant waveguide grating based label-free optical biosensor was combined with robotic fluidic force microscopy to monitor the adhesion of living cancer cells. In contrast to traditional fluidic force microscopy methods with a manipulation range in the order of 300–400 micrometers, the robotic device employed here can address single cells over mm-cm scale areas. This feature significantly increased measurement throughput, and opened the way to combine the technology with the employed microplate-based, large area biosensor (Fig.8). After calibrating the biosensor signals with the direct force measuring technology on 30 individual cells, the kinetic evaluation of the adhesion force and energy of large cell populations was performed for the first time. The present methodology opens the way for the quantitative assessment of the kinetics of single-cell adhesion force and energy even in case of bacteria as well with an unprecedented throughput and time resolution, in a completely non-invasive manner. We performed the measurements during the project period, however, these results were published in January 2020. (*Sztilkovics et al. Single-cell adhesion force kinetics of cell populations from combined label-free optical biosensor and robotic fluidic force microscopy. Scientific Reports, 2020*).

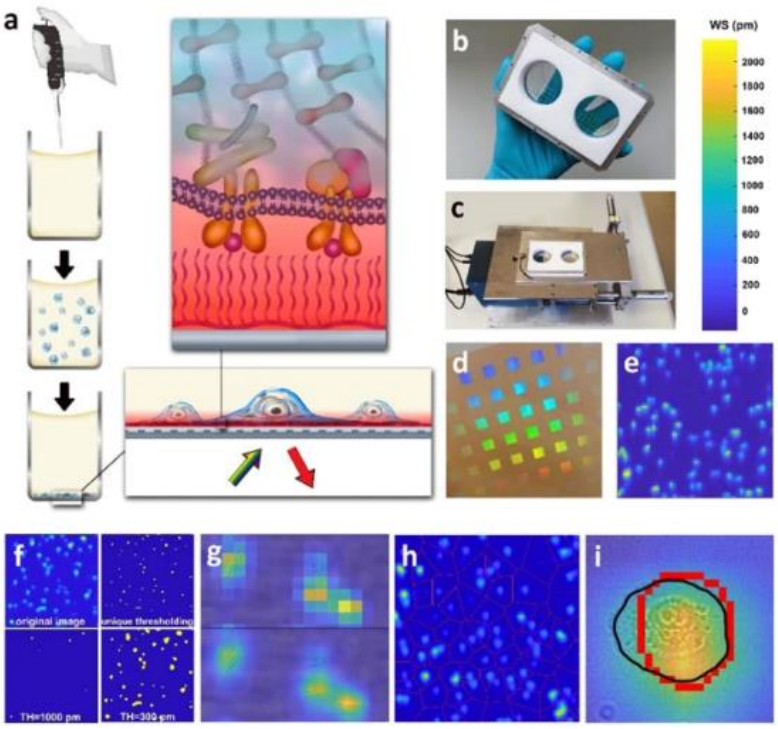

Figure 8 *The optical biosensor measurement workflow and results. (a) Schematic of the measurement workflow. (b,c) Photographs showing the custom-made biosensor insert holder (in a hand, and placed into the Epic Cardio device) with two circular wells optimized for subsequent FluidFM BOT measurements. (d) Photograph of the Epic Cardio biosensor insert. (e) Raw WS signal image of a single sensor area at t = 90 min. (f) Comparison of different thresholding strategies of recorded biosensor images. (g) Fused image of the biosensor signal and the brightfield picture, showing a clear correspondence between the two overlapping modalities. (h) The Voronoi tessellation of a sensor area. (i) Area matching segmentation: the combined optical biosensor and brightfield picture shows how the segmented cell perimeter (red) approximates the actual cell perimeter measured on the microscope image (black) after setting the optimal threshold.*

In this period, we wrote and published a review article as well (*Ungai-Salánki et al. A practical review on the measurement tools for cellular adhesion force. Advances in Colloid and Interface Science, 2019*).
This article provides a guide to choose the appropriate technique to answer a specific biological question or to complete a biomedical test by measuring cell adhesion (Fig. 9). In this work we dealt with mammalian cells, but we are working on an other review focusing on bacterial cells as well.

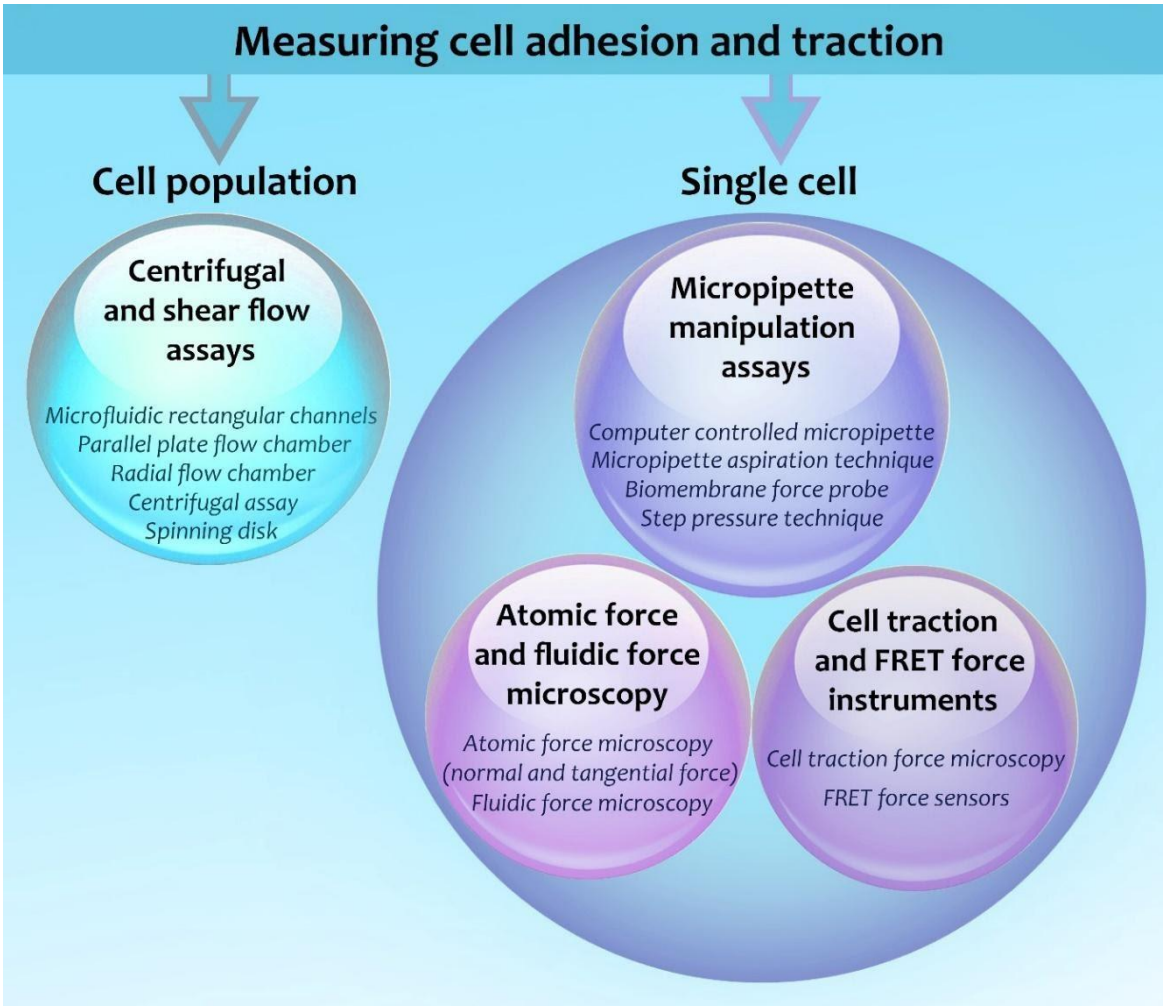

Figure 9 *Assays to measure cell adhesion and traction. These techniques are discussed in the review article.*

5 Setting up new biophysical models to explain the results

Our aim was to perform new biophysical models from kinetic data received by optical biosensors.

Optical Waveguide Lightmode Spectroscopy (OWLS) is widely applied to monitor protein adsorption, polymer self-assembly, and living cells on the surface of the sensor in a label-free manner. This method can be used for investigating bacterial cell adhesion as well. Typically, to determine the optogeometrical parameters of the analyte layer (adlayer), the homogeneous and isotropic thin adlayer model is used to analyze the recorded OWLS data. However, in most practical situations, the analyte layer is neither homogeneous nor isotropic. Therefore, the measurement with two waveguide modes and the applied model cannot supply enough information about the parameters of the possible adlayer inhomogeneity and anisotropy. Only the so-called quasihomogeneous adlayer refractive index, layer thickness, and surface mass can be determined. In our publication (*Kovács and Horvath. Modeling of label-free optical waveguide biosensors with surfaces covered partially by vertically homogeneous and inhomogeneous films. Journal of Sensors, 2019*), we constructed an inhomogeneous adlayer model (Fig.10). In our model, the adlayer covers the waveguide surface only partially and it has

a given refractive index profile perpendicular to the surface of the sensor. Using analytical and numerical model calculations, the step-index and exponential refractive index profiles are investigated with varying surface coverages from 0 to 100%.
Due to this new model, we created a software to fit kinetic data received by Epic BT resonant waveguide grating optical biosensor.

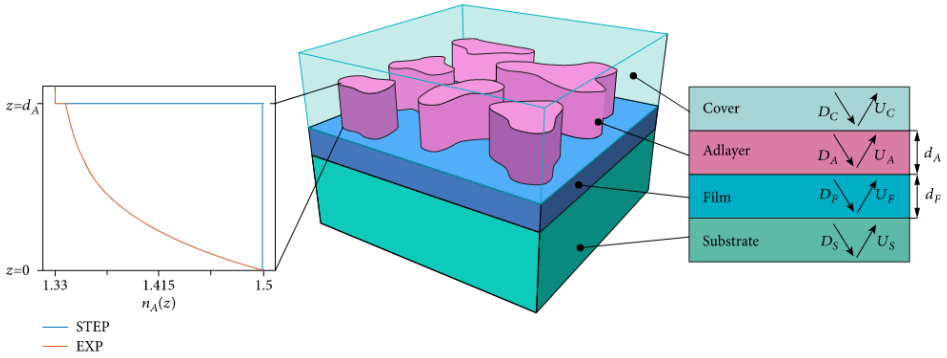

Figure 10 *The structure of the modeled OWLS waveguide chips with inhomogeneous adlayer. The modeled multilayered assembly consists of 4 layers: substrate, waveguide film, adlayer, and cover.*

Publication list

2018

Saftics et al. In situ viscoelastic properties and chain conformations of heavily hydrated carboxymethyl dextran layers: a comparative study using OWLS and QCM-I chips coated with waveguide material. Scientific reports, 2018

Peter et al. Horvath. High-Resolution Adhesion Kinetics of EGCG-Exposed Tumor Cells on Biomimetic Interfaces: Comparative Monitoring of Cell Viability Using Label-Free Biosensor and Classic End-Point Assays. ACS Omega, 2018

Kovacs et al. Kinetics and Structure of Self-Assembled Flagellin Monolayers on Hydrophobic Surfaces in the Presence of Hofmeister Salts: Experimental Measurement of the Protein Interfacial Tension at the Nanometer Scale. J. Phys. Chem. C, 2018

Peter et al. Interaction of Positively Charged Gold Nanoparticles with Cancer Cells Monitored by an in Situ Label-Free Optical Biosensor and Transmission Electron Microscopy. ACS Appl. Mater. Interfaces 2018

Szekacs et al. Integrin targeting of glyphosate and its cell adhesion modulation effects on osteoblastic MC3T3-E1 cells revealed by label-free optical biosensing.Scientific Reports, 2018

2019

Saftics et al. Biomimetic Dextran-Based Hydrogel Layers for Cell Micropatterning over Large Areas Using the FluidFM BOT Technology. Langmuir, 2019

Nagy et al. Spring constant and sensitivity calibration of FluidFM micropipette cantilevers for force spectroscopy measurements, Scientific Reports, 2019

Gerecsei et al. Adhesion force measurements on functionalized microbeads: An in-depth comparison of computer controlled micropipette and fluidic force microscopy. Journal of Colloid and Interface Science, 2019

Ungai-Salánki et al. A practical review on the measurement tools for cellular adhesion force. Advances in Colloid and Interface Science, 2019

Kovács and Horvath. Modeling of label-free optical waveguide biosensors with surfaces covered partially by vertically homogeneous and inhomogeneous films. Journal of Sensors, 2019

Visnovitz et al. An improved 96 well plate format lipid quantification assay for standardisation of experiments with extracellular vesicles.Journal of Extracellular Vesicles, 2019

Szekacs et al. In vitro SOD-like activity of mono- and di-copper complexes with a phosphonate substituted SALAN-type ligand. Chemico-Biological Interactions, 2019

2020

Sztilkovics et al. Single-cell adhesion force kinetics of cell populations from combined label-free optical biosensor and robotic fluidic force microscopy. Scientific Reports, 2020

Peter et al. Oxidization increases the binding of EGCG to serum albumin revealed by kinetic data from label-free optical biosensor with reference channel. Analyst, 2020

Franz et al. Subnanoliter precision piezo pipette for single-cell isolation and droplet printing. Microfluidics and Nanofluidics, 2020

Debreczeni et al. Human primary endothelial label-free biochip assay reveals unpredicted functions of plasma serine proteases. Scientific Reports
2020

Saftics et al. Dextran-based hydrogel layers for biosensors. Nanobiomaterial Engineering, 2020