

Single Cell Analytics for NanoBiology

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Abstract

Single cell analytics allows quantitative investigation of single biological cells from a structural, functional and proteomics point of view and opens possibilities to a novel unamplified cell analysis inherently insensitive to ensemble-averaging, cell-cycle or cell-population effects.

We report on three different experimental methods and their application to cellular systems with single molecule sensitivity at the single cell level. Firstly, atomic force microscopy (AFM) can be used to elucidate the surface structure of living bacteria down to the nanometer scale where identification of irregular surface areas and 2D-arrays of regular protein s-layers is possible. Secondly, single cell manipulation and probing experiments with optical tweezers (OT) force spectroscopy allows quantitative identification of individual recognition events of membrane bound receptors. And thirdly, a novel, single cell analysis for protein fingerprinting in structured microfluidic device format will allow a future (label-free) on-chip electrophoretical protein separation of single cells without preamplification.

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1. Introduction

In systems biology [1] a multitude of different disciplines from biology, chemistry, physics, material science, micro- and nanoengineering and (bio)informatics aim for linking molecular structural and functional (proteomic) information in a quantitative manner to the different genetically programmed and regulated networks in a living cellular organism. In contrast to optical microscopy where characterization of individual cells has been established for long, to date, proteoms are analyzed at the level of 10^5 - 10^6 cells accessing functional information only on the basis of that probed cellular ensemble. Therefore averaging effects from cell-cycle dependent states, the different and inhomogeneous cellular responses to external stimuli, or the introduction of genomic and proteomic variabilities during eucaryotic cell proliferation are completely neglected. The detection and analysis of single biomolecules, of smallest analyte quantities and the hunt for low abundant proteins at the single cell level, however, require new sensitive and efficient techniques. In systems nanobiology [2], microfabrication and nanotechnology offer novel tools to detect, image, measure, analyze, steer, and manipulate individual molecules and cells by AFM [3-6] and OT [7] and will allow more detailed insights into the physical mechanism of specific interaction, binding kinetics, and the interplay of genomic information and

functional peculiarity at the single molecule or single cellular level. Micro total analysis systems (μ TAS) or lab-on-a-chip systems [8,9] offer the possibility to handle minute volumes down to the pL and even fL range. In recent years, a number of new single cell manipulation and analysis technologies have emerged like dielectric field cages [10], electron tomography [11], microgenomics and single cell gene expression analysis [12], single cell MALDI [13], laser microdissection and single cell catapulting [14] and single cell protein profiling in capillary electrophoresis [15]. Some of these technologies rely on a subsequent amplification of preconcentration steps (like PCR), some of them allow a direct investigation of the cellular systems.

In this paper we present three techniques like atomic force microscopy, optical tweezers and a future single cell microfluidic device which ultimately exhibit single molecule sensitivity and can be applied to the analysis of single cells with respect of structural, functional and proteomic information.

2. Experimental

2.1 AFM-Experiments

We used a commercial AFM instrument (Bioscope, Veeco) with commercial standard Si-cantilevers (Pointprobe Plus, Nanosensors, Neuchatel) in tapping mode of operation. Surface topographs and phase signals were simultaneously

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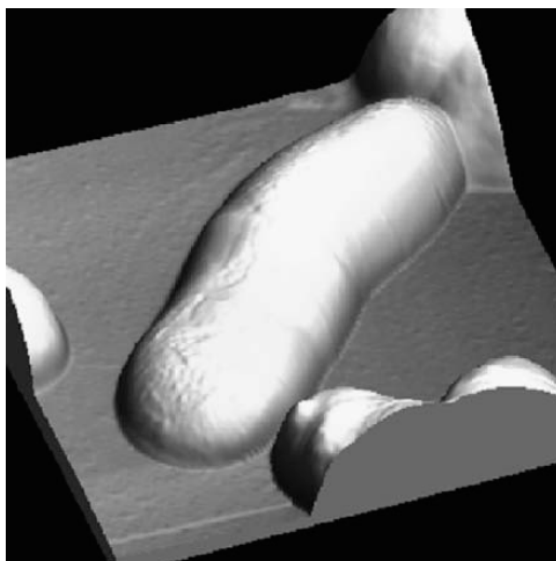


Fig. 1. AFM topography image of a living bacterium (5 μm scan).

recorded. Living bacterial cells *Corynebacterium glutamicum* (strain ATCC14067) were immobilized on amino-propyltriethoxysilane (APTES) functionalized mica or glass surfaces and kept wet and hydrated during the experiments. More detailed preparative instructions have been published recently [3].

2.2 Optical Tweezers Experiments

Our home-built single-beam OT setup is integrated into an inverted optical (bright-field & fluorescence) microscope (Axiovert 135, Carl-Zeiss AG), and is equipped with a Nd:YAG laser (1 W, 1064 nm). Manipulation and steering of micron-sized objects like beads, colloids and cells is possible with a spatial resolution of 1 nm, a force sensitivity of 0.1 pN and a maximum force of up to 300 pN. Instrumental details have been published recently [7]. Living chicken DT40 B-cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% heat-inactivated chicken serum, 10 units/ml penicillin, 10 $\mu\text{g}/\text{ml}$ streptomycin and 2 mM L-glutamine at 37°C and 5% CO_2 . Before starting the experiments, the B-cells were washed twice and suspended in PBS buffer (136 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4). In order to probe the membrane bound B-cell receptors (BCR) we used biotinylated mouse anti-chicken IgM antibodies (mIgM), recognizing the BCR (clone M-4; Soutern Biotech, Birmingham, AL) which were immobilized on streptavidin coated polystyrol beads (3180 nm diameter, binding capacity 60 pmol biotin / 1 mg particle; Spherotec, IL, USA).

2.3 Microfluidic Device for Single Cell Analysis

The poly(dimethylsiloxane) (PDMS) microchip device has a cross layout with microfluidic channels of dimensions 20 x 20 μm^2 (Fig. 6) and allows electrophoretical separation and detection of dye molecules, peptides and proteins with laser-induced fluorescence (LIF) in the visible [16] as well as in the UV spectral range [17]. Single cells were optically selected, trapped and injected into the microfluidic channel with OT. The manipulation of the cell and its transfer to the crossing of our microfluidic device (see Fig. 6) was realized with a dedicated x/y-stage. Cell lysis was performed by flushing 0.5% SDS in PBS by hydrostatic pressure from channel 4 to 2 or by applying an electric field pulse of 1250V/cm for 50 ms between channels 4 and 2. Subsequent electrophoretical protein separation and detection was performed in channel 2 by VIS-LIF detection (488 nm, 25 mW, Ar⁺). The separation buffer contains 100 mM Tris, 100 mM CHES, pullulan (4–8%) (pH 8.6). Sf9 insect cells (*Spodoptera frugiperda*) from Novagen (USA)



Fig. 2. AFM phase image of a living bacterium (5 μm scan).

were transfected with pIEx4-vector (Novagen, USA) containing the gene for the analyte fusion protein. The Sf9 cells expressed a 49.5 kDa GFP-labelled 'loss-of-function' mutant (T31N-GFP) of the cytoplasmic G-protein ArF1 of *Medicago truncatula*. A detailed description was recently published [17].

3. Results and Discussion

3.1 Measuring Cell Surface Structure by AFM

The surface structure of living cells like bacteria can be structurally investigated at the nm-scale by AFM. We chose a bacterial strain of *C. glutamicum* whose extracted and isolated s-layer cell wall already has been investigated in experiments with respect of their molecular structure and symmetry of the unit cell [3]. Under very humid and adequate imaging conditions these bacteria can be kept alive, remain in hydrated state, and allow investigation with AFM [20,21]. In Fig. 1 an AFM surface topograph of a bacterial cell in perspective representation is shown. It turned out that AFM imaging of these soft cellular systems is advantageous in tapping mode of AFM operation with simultaneous recording of topography and phase signal. Especially for resolving sub-cellular surface structure, AFM phase imaging renders a much more subtle nm-scale information. In Fig. 2 disordered surface structures of this bacterial cell, which currently undergoes a cell division process, is shown. A closer inspection (Fig. 3) reveals ordered structures which can be associated with the known 2D protein s-layer structure at a periodicity of ~ 18 nm [3].

3.2 Probing Cell Function With Photonic Forces

In order to probe individual cells in a functional way, single B-cells were optically trapped and transferred to a dedicated micropipette, where they were held by low suction pressure (Fig. 4a and b). A ms-IgM-functionalized microbead was trapped by the optical tweezers and carefully approached towards the B-cell with a constant velocity of 450 nm/s until a repulsive force of 5 pN was reached. Without delay the bead was retracted with a velocity of 1000 nm/s. In this so called force-distance curves the force interaction between the mIgM and the cell membrane bound BCR is monitored while retracting the bead, yielding an increasing attractive force signal until the bond dissociates. A typical force-distance curve is shown in Fig. 5a (1) with characteristic dissociation forces ranging from 10–40 pN. The loading rate of 45 \pm 5 pN/s was calculated from the retraction velocity and the slope of the force-distance curve just before the rupture. 30% of the force-distance curves show

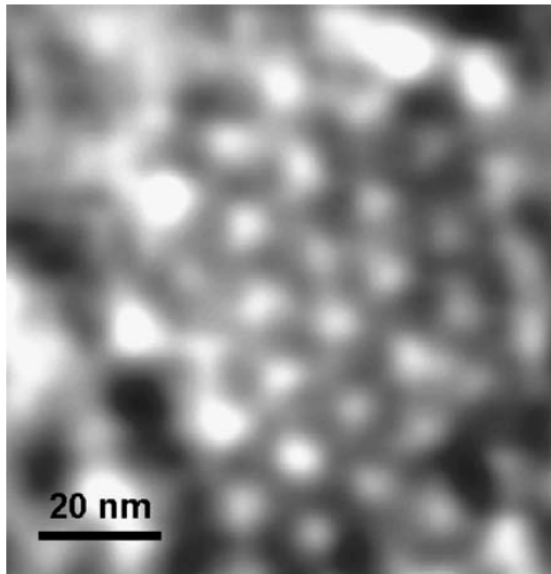


Fig. 3. High resolution AFM phase image of a living bacteria with 2D protein s-layer structure.

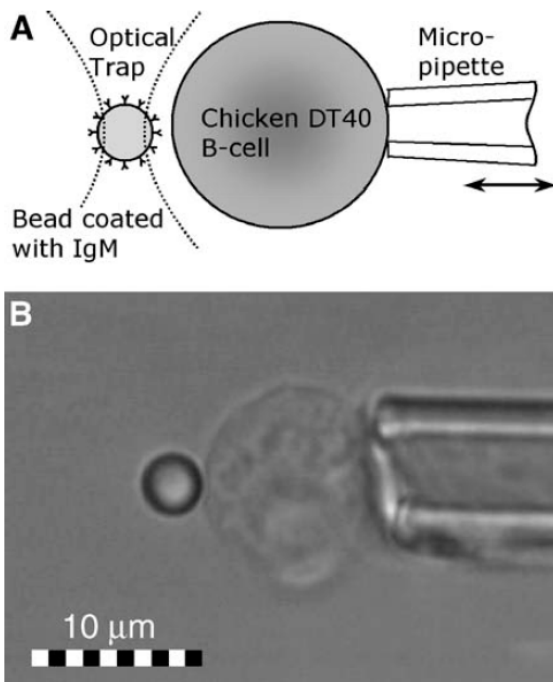


Fig. 4. Schematic view of single cell optical tweezer experiment (a). Microscopic top view image of the B-cell, held with the micropipette and the optically trapped bead (b). (From ref. 19.)

“cell tether” effects (Fig. 5a (2)) [18], where the mSIgM binds to the high viscous outer cell layer, causing an approximately constant force effect. These binding force results were excluded from further data analysis. Figure 5b shows the histogram derived from tether-unaffected rupture force measurements. In order to exclude non-specific background, only rupture events of more than 6 pN were considered. The specificity of the observed interaction was confirmed by two control experiments. 1) Competition with 15 pM free mSIgM exhibit a strongly reduced activity. 2) Using beads coated only with streptavidin instead of mSIgM show

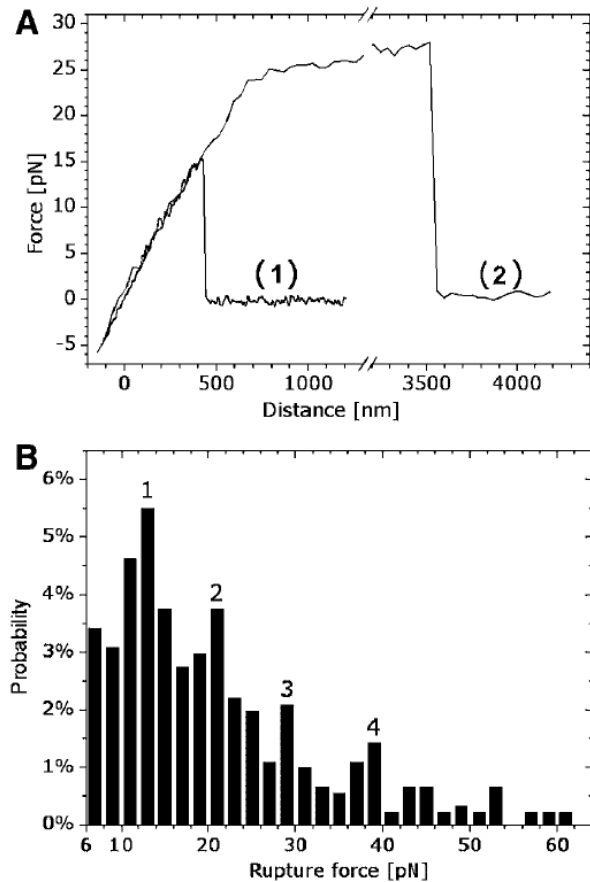


Fig. 5. (a) Typical force-distance curves obtained during retraction of the bead after forming a bond between the BCR and the mSIgM showing distinct curve breakups associated with single bond ruptures. (b) Histogram of the measured dissociation forces of the mSIgM – BCR binding at a loading rate of 45 ± 5 pN/s. Several peaks at 13 pN, 21 pN, 29 pN and 39 pN indicate an integer number of bindings forming between mSIgM and the membrane bound receptors (BCR). (From ref. 19.)

no evidence of specific binding. The force histogram in Fig. 5b exhibits individual peaks at ~ 13 pN, 21 pN, 29 pN and 39 pN, indicating an integer number of interactions between mSIgM and BCR. From a Gaussian distribution to the first peak a single bond binding force of 12.5 ± 0.8 pN for the given loading rate of 45 pN/s could be determined. The probability to observe single, double or multiple unbinding events is in good agreement with Poisson statistics and confirms the multiple rupturing characteristic of the investigated mSIgM-BCR bonds and reflects the membrane receptor density of the probed cell within the area of contact. This demonstrates the application of optical tweezers based force spectroscopy for the investigation of specific membrane bound receptors on living cells at the single molecule level. By using more appropriate immobilization schemes in the future, dynamic force spectroscopy experiments have the potential to measure the kinetics and thermodynamics of single membrane-bound receptors *in-vivo* and to investigate structurally hierarchic and more complex phenomena like receptor aggregation in biomembranes.

3.3 Towards Single Cell Protein Fingerprinting

In the third aspect of single cell analysis we aim for developing a novel microfluidic tool for single cell protein fingerprinting. In contrast to the two other surface sensitive techniques this analytical approach aims for surveying the protein expression level of individual cells by combining microfluidic on-chip protein electrophoresis with latest laser technology

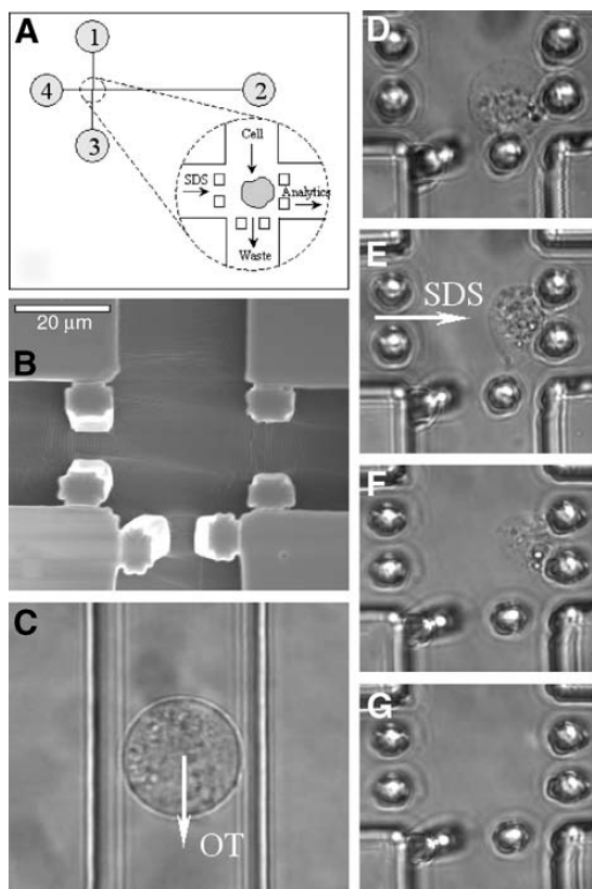


Fig. 6. Microfluidic device with cell trap composed of microstructured obstacles, (b) SEM micrograph of the cell trap, (c) single cell in a microchannel navigated by OT, (d–g) optical micrographs of a single cell at the injection position during SDS lysis (adapted from [17]).

for single cell manipulation and (label-free) protein detection. Namely, it is characterized by the following issues: 1) Single cells were trapped and navigated by optical tweezers (OT) in a PDMS microfluidic device, and lysed at a predefined position. 2) Separation and detection of proteins was achieved with protein electrophoresis and laser induced fluorescence (LIF) detection in the visible (488 nm). In Fig. 6 the experimental microfluidic setup with a series of optical micrographs of a controlled cell lysis is shown. An individual cell was optically selected, trapped and injected into the microfluidic channel with our optical tweezers setup. The individual cells were transferred to the crossing of our microfluidic device which is microstructured by vertical posts in order to act as a physical cell trap (Fig. 6b and d). Once the cell was navigated into this position the optical trap was switched off and the cell was allowed to adhere to the microchannel wall. Consecutively, cell lysis was performed by flushing a 0.5% SDS solution in PBS by hydrostatic pressure into the perpendicular channel (from channel 4 to 2). The cell lysis was visualized and controlled by optical bright-field microscopy. Figure 6d–6g demonstrate a sequence of snapshots from a single cell lysis at the injection point at the entrance of channel 2, in which subsequent analytics will be performed. Complete cell lysis was typically achieved within 6 seconds. After cell lysis, the proteom was electrophoretically driven (and separated) in the analytical channel (2) of the microchip. Figure 7 shows a single cell electropherogram recorded by VIS-LIF detection (488 nm, 2 mW, Ar⁺) of a GFP-transfected Sf9 insect cell. As expected from a single component analyte one distinct peak can be identified in the electropherograms which is attributed to the

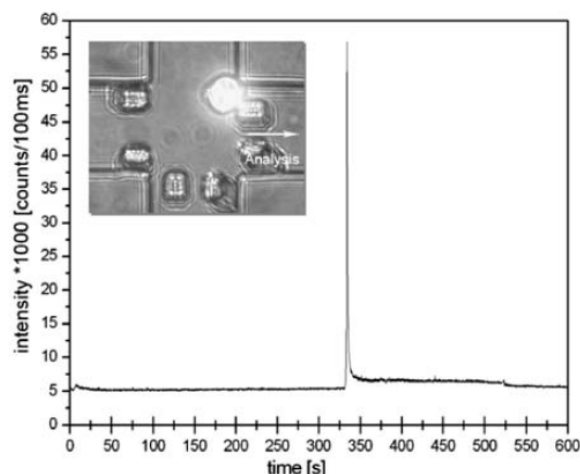


Fig. 7. Electropherogram of a single GFP-Sf9 insect cell with a distinct single component peak of the 49.5 kDa T31N-GFP variant. Inset: Fluorescence micrograph of a single GFP-Sf9 insect cell captured at the injection position (for more details see text and [17, 20]).

expressed GFP-construct protein [1]. In the future we will extend this method for label-free detection of proteins by monitoring the autofluorescence via UV-LIF [17,20].

4. Summary and Outlook

A brief overview of three different single cell analytical methods like atomic force microscopy, optical tweezers and future microchip-based protein fingerprinting device was given, which allow quantitative investigation of single biological cells from a structural, functional and proteomics point of view. This opens possibilities to a novel unamplified single cell analysis without being subjected to ensemble-averaging, cell-cycle or cell-population effects and applications in nanobiology.

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