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Quantitative Analysis of Spatial Association between Discrete Events Represented by Small Foci in Multicolor 3D Confocal Microscopy

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KEY WORDS: image analysis, correlation, multicolor confocal microscopy, replication foci, DNA damage

Background: Laser scanningcytometry (LSC) studies of cell populations has demonstrated that the level of DNA damage induced by topoisomerase I inhibitor camptothecin, or oxidative damage induced by H_2O_2 is higher in cells replicating DNA than in nonreplicating G1 and G2 cells. In order to establish a potential interdependence between replication and damage, it was necessary to analyse quantitatively the distances between replication and DNA damage foci in 3D space.

Goal: The goal of this research was to construct a method of quantitative analysis of spatial association between small foci, representing discrete subnuclear processes.

Methods: Sites of DNA replication were labeled using click-it chemistry, whereas the foci of phosphorylation of histone H2AX (a marker of double strand breaks) were labeled by immunofluorescence. 3D confocal microscopy images of A549 nuclei of human lung carcinoma cells were deconvolved and subjected to image analysis.

Results: We developed an image segmentation routine in order to isolate the point-like fluorescence signals corresponding to DNA damage and replication and determine their spatial positions within cell nuclei. The relationship between the spatial distributions of these signals was analyzed using nearest-neighbour (nn) distance to isolate the populations of replication-dependent and replication-independent damage signals. This separation was performed with simple thresholding of the nn distances or with distributions un-mixing where the set of nn distances between replication signals was taken as the reference. The nn distance calculation was supplemented with analysis of cumulative distribution of all possible distances (Ripley's K functions) between signals of the same and different kinds. Using the K functions specific association between damage and replication signals can be distinguished from their local grouping produced by inhomogeneity in nuclear distribution of chromatin or differences in the number (density) of replication and damage signals.

Conclusions: We found an expected, statistically significant spatial correlation between replication and damage induced with topoisomerase I inhibitor, but only a negligible correlation in cells subjected to oxidative damage. This approach analysis of spatial association of two nuclear events is expected to be suitable for analysis of a relationship between any other type of cellular events represented by small foci in multicolor patterns found in 3D confocal images.

Key Words: image analysis, correlation, multicolor confocal microscopy, replication foci, DNA damage

Microbiology and Aquatic Sciences (B211 – B223)

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Community Flow Cytometry – An Efficient Tool to Monitor Microbial Population Dynamics in a Biogas Reactor

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The use of biomass as a renewable energy resource is becoming increasingly important in the substitution of fossil fuels. Over 5000 biogas plants of different scales are already installed in Germany and cover already about 1.5 % of our total energy demand. An upward trend is found worldwide.

Biogas reactors contain a highly divers microbial community comprising of Bacteria and Archaea. They are able to degrade complex substrates, ranging from energy crops to organic wastes and distillers grains, to biogas mainly composed of methane and carbon dioxide. Substrate choice, temperature, retention time, pH and the presence of trace metals or noxiouscompounds are just some factors influencing the community composition and the total reactor performance. So far, most studies only focused on the microbial composition at certain time points using fingerprinting techniques. Differences were found but distinct correlations to total reactor performance could hardly be made.

At the moment only little is known about the behaviour of the biogas microorganisms in dependence on the bioreactor scheme. Flow cytometry offers a tool to follow the dynamics of these complex microbial communities as was already shown for wastewater systems (Guenther et al. 2011). The combination with cell sorting of interesting subcommunities (either very stable or very fluctuating) and their molecular characterisation can help to identify the key organisms behind a stable or unstable process performance.

The microbial community in a biogas reactor, run at the German Biomass Research Centre (DBfZ), was investigated over a period of 9 months and first results on the question "How stable is stable – community dynamics in a biogas reactor" will be presented with the aim of understanding the dynamics behind functional stability.

Guenther et al. 2011: Correlation of Community Dynamics and Process Parameters As a Tool for the Prediction of the Stability of Wastewater Treatment. *Environmental Science & Technology***2012***46* (1), 84-92

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Characterization of *E. coli* Morphology from Light Scattering

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Background: Characterization of bacteria cells by their static and dynamical characteristics has direct medical applications, e.g., strain identification and antibiotic sensitivity tests. Current implementations of the latter take a lot of time, up to several days. Accurate measurement of changes of morphological characteristics of bacteria over time can significantly decrease this time, making it smaller than one cell cycle. In this work we studied *E. Coli*, as a common model for rod-shaped bacteria.

Methods: We used scanning flow cytometry, allowing the measurement of angle-resolved intensity light scattering patterns (LSPs) of individual particles, and solution of the inverse light-scattering (ILS) problem. We measured LSPs with the Scanning Flow Cytometer fabricated by CytoNova Ltd. (Novosibirsk, Russia, http://cyto.kinetics.nsc.ru/). The solution of ILS problem is based on fitting an experimental LSP by theoretical ones, calculated from the modeling *E. Coli* cell as a cylinder capped with hemispheres. To accelerate the fit a precalculated database of 600 000 LSPs of individual bacteria is used. This solution allows determination of length, diameter, and refractive index of individual bacterium including errors of these estimates.

Results: Developed method was tested on two strains of *E. coli* bacteria. Obtained length and diameter distributions showed a good agreement with both literature data on these strains and microscopic measurements of the same samples.

Conclusions: We present a new approach for accurate and statistically reliable measurement of *E. Coli* morphology, including length and diameter, using light-scattering flow cytometry. The presented method allows characterization of population of any rod-shaped bacteria cells by their length and diameter distributions. It can be used for determination of dynamic characteristics of bacteria, such as growth rate via length enlargement. This approach can be also used for kinetic studies of cell cycle.

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Storage Methods for Flow Cytometric Analysis of Bacterial Cells

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Flow cytometry (FCM) is a powerful high-throughput method for evaluating microbial cell samples. The experimental design of studies with cell sampling at different time points often requires the storage of these cells for further analysis. Here, bacterial cells of the standard laboratory species Pseudomonas putida KT2440 and Escherichia coli K12 were fixed and stored using three different methods: sodium-azide fixation, deep-freezing and vacuum-drying.

The cells were stored up to four weeks and the cell's morphology, scatter characteristics and DNA content were determined at different time points (fresh, 2d, 28d storage) using flow cytometry. Furthermore, the impact of each fixation method and storage time on the proteome profile was evaluated using tandem mass spectrometry (LC-MS-MS) with label free quantification of identified proteins. The effect of sodium-azide fixation/storage on scatter parameters and DNA content of bacterial cells was strong, whereas deep-frozen and vacuum-dried samples showed high similarity with the respective fresh samples. The proteome analysis revealed that each storage method has its characteristic effect on the proteomic profile. It is therefore advisable to stick to one particular method during one series of experiments. In conclusion, it could be shown that simple vacuum-drying of bacterial cells is a convenient and effective storage method with similarly small impact on cell morphology as deep-freezing.

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Microfluidic Cytometer for Measurement of Photosynthetic Characteristics and Lipid Accumulation of Individual Algal Cells

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Background: Significant attention is focused on the potential use of microalgae-derived lipids for the production of transportation fuels. Research and development is still needed at all stages of the production process, including characterization of algal strains, genetic engineering for higher productivity, and optimization of growth conditions. To this end, we are developing flow-cytometry methods for screening of algal photosynthetic efficiency and lipid content.

Photosynthetic efficiency may be determined from chlorophyll (Chl) fluorescence induction measurements at an excitation intensity that saturates photosystem II (PS2), by closing the reaction centers in $<10^{-4}$ sec, prior to electron transfer. When a rectangular light pulse is applied to each cell, the Chl fluorescence intensity (F_0) is low when the PS2 reaction centers are "open" or capable of accepting excitation energy. In the light-saturated state, r[1]eaction centers are "closed" and fluorescence yield (F_m) is maximal. The normalized variable fluorescence, $F_v/F_m = (F_m - F_0)/F_m$ represents the quantum yield of excitation trapping by the PS2 reaction center, which is thus the ceiling for the quantum yield of photosynthesis. It is a well-established measure of the solar energy conversion efficiency. Its value ranges from 0 for dead cells to ~0.8 for healthy cells.[1]

We built a microfluidic flow cytometer that measures F_v/F_m, forward scatter, and fluorescence from a lipophilic stain (Nile Red; NR) from each cell at a rate of ~10^3 cells/sec, and used it to characterize nutrient-replete and stressed (nutrient-limited) cultures of the marine diatom *Phaeodactyl tricornutum*.

Methods: Cells flowing at 1 cm/sec in a fused-silica 2D hydrofocusing microfluidic chip (channel cross-section of 80×100 µm) were illuminated by a sub-µsec pulsed 470 nm LED for exciting Chl and NR, and a 785 nm diode laser (which does not excite Chl or NR), for forward light scattering. An objective lens collected fluorescence, which is split into three channels by dichroic mirrors and detected by separate PMTs for Chl and NR emission. An AC-coupled photodiode detects forward-scattered light. Data collection, analysis of fluorescence transients, and instrument control was performed with home-built circuitry and software.

Results: Cytometry measurements on light-adapted *P. tricornutum*cells show significant differences between stressed and unstressed cells, *e.g.* higher Fv/Fm for unstressed (0.763±0.152) *vs.* stressed (0.459±0.108) cells and higher lipid content (a 77% increase in NR fluorescence). We compared the average value of Fv/Fm for these two samples with bulk measurements of Fv/Fm on the same cultures obtained on a commercial fluorometer. Differences between cytometry and bulk values (unstressed: 0.771±0.003; stressed: 0.370±0.014) are more significant for the stressed cells. The values differ due to the difference in the spectral ranges of the two instruments. The cytometer Chl channel selectively detects fluorescence from PS2, whereas the fluorometer detects both PS1 and PS2. Since PS1 is known to have a lower quantum efficiency, it is to be expected that selective measurements of PS2 will observe higher values of Fv/Fm.

Conclusions: These results are consistent with bulk observations that lipid production in algae is triggered by stressed conditions, and also indicate that the stress results in larger decrease in the quantum yield of PS2 relative to the quantum yield of PS1.

[1] Olson *et al.* (1999) *Cytometry***37**, 1-13

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Immunodetection of Bacterial Surface Structures by ELISA and Flow Cytometry

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Although enzyme-linked immunoassay (ELISA) has been used effectively for antibody titration and quantifying the amounts of specific antigens for decades, flow cytometry (FCM) is becoming the technique of choice for measuring complex antigen-antibody interactions such as those occurring at the bacterial surface. Here we investigated the suitability of these approaches to measure changes at the surface of lactobacilli species using polyclonal antibodies directed against a specific cell surface protein, mucus-binding (MUB) protein from *Lactobacillus reuteri* ATCC 53608