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Meeting report

The 6th International Conference on Analysis of Microbial Cells at the Single Cell Level, Retz, Austria, 19–22 July 2015

ABSTRACT

The 6th International Conference on Analysis of Microbial Cells at the Single Cell Level, held in Retz, Austria from 19 to 22 July 2015, brought together experts from different areas working with bacterial, yeast and mammalian cell systems. The conference highlighted the importance of dissecting cell behaviour down to the single cell level, as analysis of mixed populations can obscure crucial cell-to-cell variations. The sessions covered advances in the fields of image analysis and microscopy, flow cytometry and cell sorting as well as bioinformatics, including recent developments and new applications of existing tools. In addition, a high speed poster slam session contributed to the lively discussions and exchange of expertise among academic and industrial researchers.

Introduction

This paper summarizes and highlights the contents of the 6th International Conference on Analysis of Microbial Cells at the Single Cell Level held in Retz, Austria from 19 to 22 July 2015. The conference was organized by Nicole Borth, Michael Sauer, Diethard Mattanovich and Monika Debreczeny on behalf of the European Federation of Biotechnology 'Microbial Physiology' Section. The aim was to enable researchers from various disciplines of biotechnology to share state-of-the-art technologies associated with single cell analysis.

A total of 22 talks (5 keynotes, 17 selected from abstracts), a poster slam session including 8 short talks as well as a total of 16 posters spanning the diverse areas of single cell analysis (bacterial, fungal, mammalian) yielded a very comprehensive overview of the field. Supported by exceptionally hot weather due to the consequences of climate change, the conference on the whole, but especially the poster sessions, turned into a place of not only vivid but also extraordinarily 'hot' discussions. The social programme included typical Austrian food and a guided wine tasting in the underground labyrinth of wine cellars beneath Retz.

Image analysis and microscopy

The first session of the conference was opened by Pekka Hänninen (Faculty of Medicine at University of Turku, Finland), who focused on novel applications of super-resolution microscopy at the cellular and molecular level. Hänninen gave a concise introduction and overview of current technologies and software solutions that his group is developing including super-resolution compatible nanoprobes, super-resolution compatible *in vitro* models, and Stimulated Emission Depletion microscopy. This groundbreaking imaging technology enables researchers to resolve structures at a much higher resolution than conventional confocal microscopy due to the application of laser bleaching of a given sample area to minimize the illuminated area and thus overcome the diffraction limit of light microscopy. Furthermore, label free technologies such as photoacoustic microscopy methods, second harmonic generation microscopy, stimulated RAMAN and CARS microscopy were discussed in detail.

Verena Puxbaum (Austrian Centre of Industrial Biotechnology, Vienna) presented recent data on a study that aimed at tracing two recombinant model proteins, human serum albumin (HSA) and a single chain Fv-Fc fusion antibody, on their way through the secretory pathway of the yeast *Pichia pastoris* and Chinese Hamster Ovary cells (CHO). Using a live-cell microscopy approach it could be shown that HSA in *P. pastoris* is mainly localized in the ER as expected, while the more complex antibody localized to the vacuole. In CHO cells, HSA is found in the ER and Golgi, while the antibody is predominantly found in the ER. Remarkably, in *P. pastoris* the secretion of HSA is directed through the bud of the maturing yeast cell.

The last presentation of the session by Matthias Steiger (Austrian Centre of Industrial Biotechnology, Vienna) described the impact of zinc on glutathione production in *Saccharomyces cerevisiae*. Two yeast strains, which were engineered for increased production of glutathione either on high zinc levels or on acrolein, were used for the study [1]. Transcriptomic data of the strain that was selected at high zinc levels revealed that zinc finger-containing transcription factors were significantly upregulated. Low zinc levels of this strain induced glutathione formation even

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further. Single cell analysis revealed that in the cells with high glutathione accumulation, the intracellular distribution of zinc was disturbed. These results are the basis for subsequent evaluation of the formulation of current buffer systems for glutathione producing yeast strains.

Flow cytometry and cell sorting

This session was opened by Johan Rockberg (KTH-Royal Institute of Technology, Stockholm), who presented a novel high throughput screening system for single cell characterization and sorting of CHO producer cells using droplet microfluidics and split-GFP (green fluorescent protein) complementation. A short tag, comprising the 11-strand of GFP, was genetically fused to either the monoclonal antibody Herceptin or erythropoietin and expressed as a fusion protein in CHO cells without affecting production or function of the reporter protein. Upon addition of GFP_{1-10} to the GFP_{11}-tagged fusion protein the chromophore is matured, yielding fluorescent GFP (see Fig. 1) [2]. It could be demonstrated that split-GFP signals correlated with the secreted amount of protein. A high-producing clone was selected via droplet screening, whereby single CHO cells are encapsulated and cultivated in monodisperse droplets in a microfluidic device, allowing for high throughput droplet sorting based on protein secretion. By adding GFP_{1-10} to the droplets, secretion of the tagged protein could be monitored upon complementation. To speed up detectability, complementation with matured GFP_{1-10} can be performed, where the rearrangement of the chromophore is captured in its active form [3]. After isolation of a production clone, the spit-GFP-11 tag is removed by insertion of a stop-codon upstream of the tag via CRISPR/Cas9 genome editing. Better screening of libraries for the identification of CHO cells with improved antibody secretion is anticipated using this high throughput system.

Reliable gene expression was also the topic of Michael Jahn (Helmholtz Centre for Environmental Research, Leipzig), working with the model strain *Pseudomonas putida*. Jahn analysed plasmid copy number (PCN) fluctuation, which can compromise the yield of biotech processes, by cell sorting via flow cytometry and droplet digital PCR [4]. After optimization of the PCR conditions, cell disruption and number of sorted cells, precise determination of PCN was possible [5]. The established workflow (see Fig. 2), using duplex detection of genomic and plasmid DNA of only 1000 cells, revealed remarkable heterogeneity of the plasmid copy number in a population of *P. putida* KT2440. Furthermore it was shown that although plasmid loss is a function of copy number, increasing antibiotic concentrations did not boost the PCN.

New developments

In this session new perspectives for the analyses of individual cells, covering yeast, bacteria and mammalian cells, were highlighted. The keynote lecture was given by Anna-Karin Gustavsson (University of Gothenburg), who summarized her work on glycolytic oscillations in individual yeast cells. Glycolytic oscillations in dense synchronized yeast populations have been reported since the 1950s, but these studies have been limited to observations of average oscillatory behaviour [6]. Several attempts to determine possible heterogenous behaviour at the single cell level have so far been inconclusive [7–9]. Gustavsson presented a novel approach for single cell analyses combining optical tweezers for cell positioning [10], microfluidics for environmental control [11,12] and fluorescence microscopy for detection. Using this experimental approach, sustained glycolytic oscillations in isolated yeast cells could be demonstrated for the first time by exposing the cells to glucose and cyanide [13] and measuring the NADH autofluorescence signal from individual cells. The heterogeneous cell responses from individual cells were categorized and successfully described with a detailed kinetic model [12]. It could further be confirmed that the oscillatory behaviour is determined by allosteric regulation of phosphofructokinase, as suggested previously [14]. Subsequent experiments investigating the entrainment of glycolytic oscillations by periodic external perturbations showed that the mechanism behind the synchronization of individual oscillatory yeast cells is phase synchronization, rather than frequency or amplitude modulation. This robust mechanism is both insensitive to cell heterogeneity and universal in that it is similar for different types of external perturbation [15]. These studies led to a deeper understanding of complex reactions in energy metabolism, cell-cell interactions and synchronization. Pursuing these studies will hopefully provide further information about oscillations and communication in other biological systems, such as pulsatile insulin secretion from islets of β-cells, which would make it very interesting in the understanding of diabetes.

Another hot topic discussed in this session was RAMAN microspectroscopy for label-free single cell imaging. An introduction into this emerging technology was given by Heidi Kremling (Cell Tool GmbH, Bernried). RAMAN microspectroscopy is based on the detection of light inelastically scattered by molecules. It is increasingly used for biomedical applications, providing vast amounts of biochemical data from living cells with high spatial and temporal resolution. Thus, it can reveal rich information on the metabolomic state of cells and tissues like a photonic fingerprint

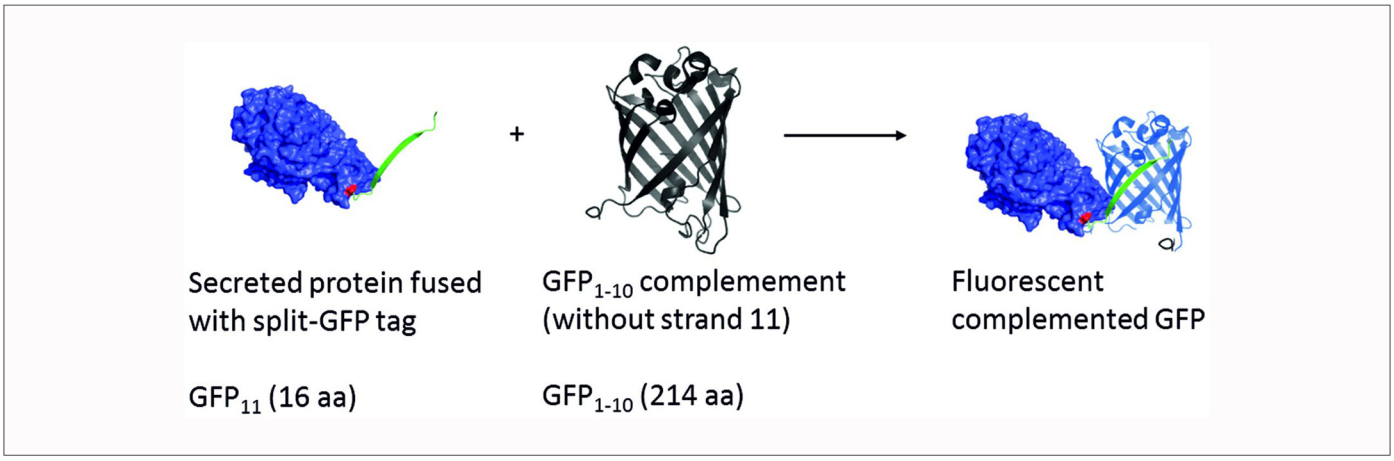

Figure 1. Split-GFP complementation mechanism. Reassembly of the GFP-11 tag (comprising the 16 amino acids long 11th strand of GFP) genetically fused to the secreted protein, with GFP_{1-10} (the first 214 amino acids of GFP, including residues 65–67 that become the chromophore). The chromophore is not formed in GFP_{1-10}, but matures upon addition of GFP_{11} [2].

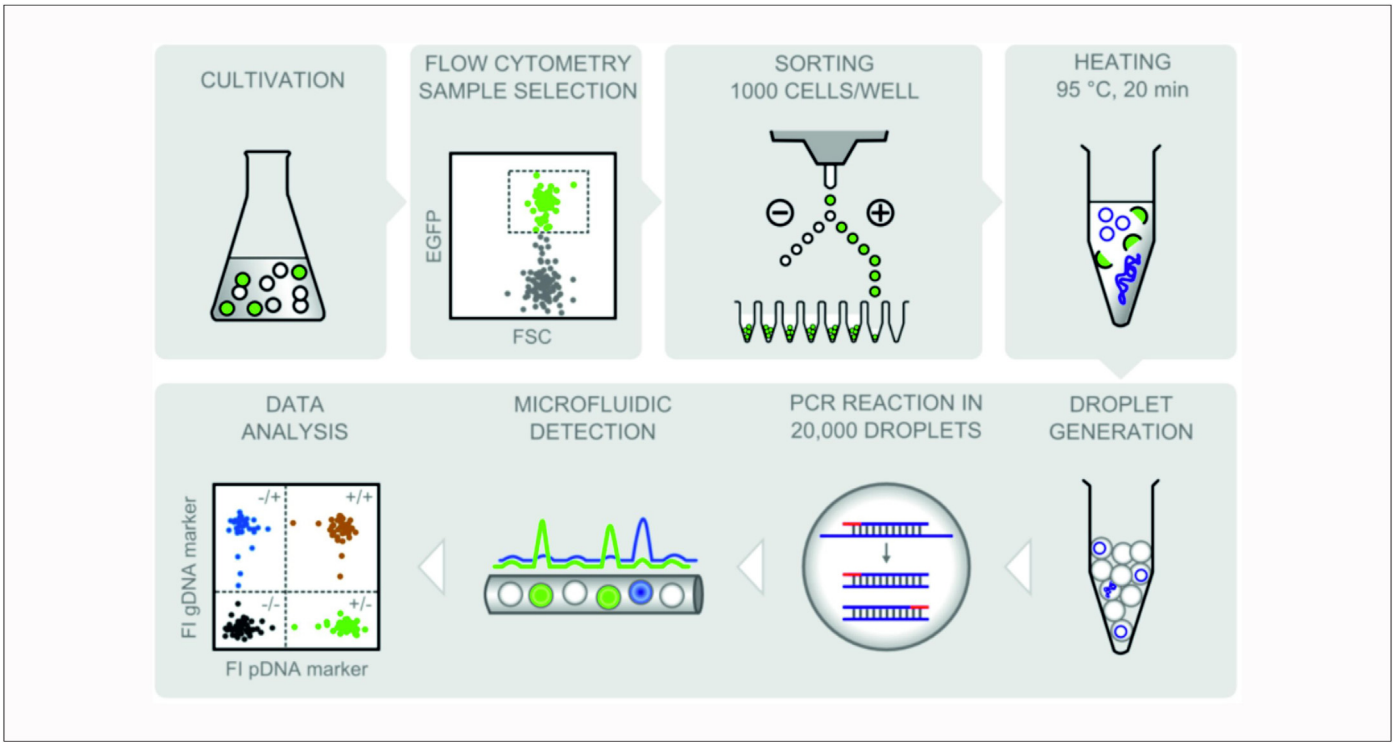

Figure 2. Workflow for determination of plasmid copy number (PCN) combining cell sorting and digital PCR. (Reprinted with permission from [5]. Copyright (2014) American Chemical Society.) Cells were cultivated, analyzed via flow cytometry and a small and defined number of cells were deposited in micro wells via cell sorting (i.e. 1000 cells/well). The cells were then disrupted by heating at 95°C for up to 20 min, followed by addition of PCR master mix and droplet generation. Duplex PCR was performed in every droplet individually, and the mixture of positive and negative droplets was analyzed via a microfluidic analyzer (genomic DNA marker in blue, plasmid DNA marker in green, double positive in brown). Absolute copy number was calculated using Poisson statistics [4].

[16]. Heidi Kremling described the application of RAMAN microspectroscopy to distinguish between various types of airborne microorganisms and patient derived bacteria. She showed evidence that it is not only possible to discriminate between different types of bacteria, but also between different strains of one species. In human blood monocytes, RAMAN imaging showed different fingerprints for non-infected cells and cells infected with *Chlamydia pneumoniae*, mainly due to changes in lipid/fatty acid content [17].

Katherine A. Hollywood (University of Manchester, UK) described the applicability of RAMAN microspectroscopy for the analysis of living mammalian cells via the incorporation of an online incubator within the microscope chamber to allow control of temperature, humidity and atmospheric conditions. She demonstrated the detection of the small molecule drug dithranol within human keratinocyte cells, where changes in cellular distribution and localization over time could be assessed [18]. It was shown that this technique can offer continuous assessment of a process rather than capturing snapshots and that it is adaptable to multiple adherent cell lines, provided that some pre-knowledge about the specific cell system is available.

Eva Harreither (BOKU, Vienna) demonstrated that RAMAN imaging of CHO cells enables clear discrimination between host and producer cell lines. Cluster analysis and Principle Component Analysis also showed that cell lines producing different proteins give distinct signals that can be detected. Moreover, the cells could be clustered according to the protein content of different areas within the cells. Vertex Component Analysis further enabled cellular imaging of key cellular components, that is, the lipid plasma membrane, the cytoplasmic protein as well as DNA/RNA, without the presence of a label. Future work will focus on the applicability to identify high producing cells in a high throughput screening set up.

Summarizing the presentations, RAMAN imaging has proven to be a very useful label-free tool due to high spatial resolution, speed of acquisition and non-invasive nature.

Bioinformatics for single cell analysis

The bioinformatics session focused on the analysis of data obtained by flow cytometry and the challenges associated with the evaluation of data from high throughput applications including multiple parameters. Technical advances have allowed state of the art flow cytometers to acquire 30+ parameters per single cell, but for quite some time computational methods for analysis have been lagging behind. Ryan Brinkman (British Columbia Cancer Agency, Vancouver) promoted the implementation of a more widespread use of automated tools to fully extract the information contained in such extensive data sets. Robust computational algorithms are often superior to conventional analysis based on manual gating and the number of available tools as well as their quality and reliability is increasing. In this context the work of FlowCAP (Critical Assessment of Population Identification Methods) was highlighted. Furthermore, examples of the successful establishment of pipelines for the automated identification of biomarkers in clinical research were presented.

The study of microbial community dynamics in natural systems was the topic of the talk of Susann Müller (Helmholtz-Centre for Environmental Research GmbH – UFZ, Leipzig). Microbial flow cytometry in combination with DNA staining provides a method for rapid cell analysis to determine quick changes in the structure of a community. A pipeline for data evaluation with the newly developed bioinformatic tools flowCyBar and flowCHIP was established. In addition, separately determined abiotic parameters were included for complete data interpretation [19]. Ruben Props (Ghent University) presented

recent work using flow cytometry data for microbial community fingerprinting to monitor the dynamic changes of the microbiome of a water cooling system during the different operational phases of a nuclear test reactor.

Applications

As keynote speaker, Hsiang-Yu Wang (National Tsing Hua University, Taiwan) opened the session and provided insight into the use of microfluidic techniques for the optimization of microbial fuel cells (MFC). MFCs are used for the generation of energy. The range of possible substrates is very diverse with one major application being wastewater treatment. In order to facilitate the investigation of different microorganism cultures and carbon sources, a laminar flow based microfluidic MFC (LF-μMFC) was developed. This novel set-up allows a rapid detection of microorganism electroactivity and reduction of sample size, without the need of a proton exchange membrane. The separation of anode and cathode in the reaction chamber with a width of 1 mm is achieved by two streams of laminar flow. Influences of different parameters can be determined in just one hour and conclusions obtained with this system can be transferred to the properties of larger MFCs [20]. Furthermore, the development of an air-cathode μMFC was introduced, which can be helpful in evaluating strategies to improve the performance of MFCs in terms of charge-transfer resistance and power output. One major issue for MFCs is the formation of biofilms, which can hinder the effective transfer of electrons. Wang showed results of recent work in which the application of polyelectrolyte polymers was studied. For this, microorganisms were encapsulated in conductive polymer material forming microparticles, which when attached to the anode provide a means to retain the microbes and to expand the surface area for electron transfer [21]. It was shown that the use of polyelectrolyte polymer particles gave rise to a 56% reduction in charge-transfer resistance, resulting in a doubling of power-output.

Ivana Marova (Brno University of Technology) described methods to investigate the performance of carotenogenic yeasts at a high throughput level. Production of carotenoids has been mainly focused on chemical synthesis, but as the market for carotenoids and their applications in pharmaceutical, cosmetic, food and feed industries are growing, there is also an increased interest in implementing biotechnological production processes [22]. Carotenoids in yeasts serve as membrane-protective antioxidants. Their formation increases as part of a stress response to a variety of triggers [23]. This study showed how a Bioscreen C system for high throughput microcultivation coupled to online FTIR analysis can be used to test the effects of various C/N ratios and carbon source compositions on the formation of lipids and pigments in three different yeast genera. Several additional analyses, including RAMAN microspectroscopy, fluorescence correlation spectroscopy (intracellular location of lipids and pigments) and electron microscopy (changes in morphology), provided insights into the intracellular metabolic processes.

Another product with increasing industrial relevance is poly-3-hydroxybutyrate (PHB), a carbon storage polymer formed by many species of bacteria. Tim Overton (University of Birmingham, UK) presented how flow cytometry can be applied for fermentation optimization in *Cupriavidus necator* and as a tool to observe the effectiveness of novel isolation strategies. The production of polyhydroxyalkanoate (PHA), which has superior material properties compared to PHB, was monitored by staining with lipophilic dyes in parallel with viability assessment using propidium iodide. Here, lactone was added to the feed during fed batch cultivation, as it can be converted to PHA by the endogenous set of enzymes. One restriction for the broader use of polymers of this class is the need to use halogenated solvents in the extraction process, which is also a time-consuming multistep protocol. The less hazardous dimethylsulphoxide was introduced as an alternative to chloroform for the isolation of PHA [24].

Christian Dusny (Helmholtz-Centre for Environmental Research GmbH–UFZ, Leipzig) showed very promising advances in the field of microfluidic single-cell analysis. An Envirostat microbioreactor allows the contactless trapping of a single cell and its observation under defined environmental conditions, where its metabolic activity is independent from that of other population members. Among other studies, the individual growth rates of single cells of *Hansenula polymorpha*, *P. pastoris* and *Corynebacterium glutamicum* were determined, which increased by up to 120% compared to the average population value under similar conditions [25]. Overall the presented work could clearly show that the Envirostat is a promising tool for the investigation of various aspects of microbial physiology.

For the final talk of this diversified conference, Christopher Love (Koch Institute at MIT, Cambridge, Mass.) focused on the perspective of using yeast production systems for the manufacture of monoclonal antibodies and presented a method based on microengraving for high throughput screening of the secretory capacities of *Pichia pastoris*. Per array, $\sim 10^4$ individual cells can be investigated, with the size of the wells being in the subnanoliter range [26]. Based on in-well imaging cytometry, it was concluded that although most clonal yeast cells produced properly folded protein, only a subset secreted recombinant protein efficiently. Furthermore the capacity of the cells to transport the processed protein from the ER to the Golgi and the cell surface was identified as a limiting process, with transcription and translation as less substantial determinants for the resulting degree of secretion [27]. Interestingly the balance of protein reduction by ER-associated degradation (ERAD) and protein secretion dictates the secretory potential of a cell. Genome-wide transcriptional analysis of cells with increased secretory capacity identified the upregulation of genes involved in ER-resident glycosylation, ERAD and proteasome activity. The increased expression of these genes is explained as part of a stress response, which is necessary to cope with the burden of heterologous protein production, as it supports the prevention of excessive protein accumulation in the ER and the transition into an 'off' state in terms of secretion.

Concluding remarks

In summary, the individual sessions highlighted the value of emerging technologies in single cell analysis and gave insights into upcoming applications. The main attraction of this conference series is that it explores new methods across different types of cells and applications, thus generating stimulating cross-fertilization between different areas of expertise. In addition, this international conference was very successful in bringing together academic as well as industrial researchers from different areas of biotechnology. Not only did it attract experts in the field from Europe, but also scientists from other parts of the world including the USA, Canada, Brazil and Taiwan. Furthermore, a well-balanced mix of young and experienced researchers resulted in a vibrant and productive exchange of thoughts and ideas.

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