DELNAM
DELIVERY OF NUCLEIC ACID MIMICS

SUMMER SCHOOL 2

FISHING BACTERIA

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Book of Abstracts

Theoretical Sessions

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Invited Speakers

Jesper Wengel, University of Southern Denmark, Denmark

Nuno F. Azevedo, Faculty of Engineering of the University of Porto, Portugal

Govind Kaigala, IBM Research, Zurich, Switzerland

Zach Hensel, Instituto de Tecnologia Química e Biológica, Portugal

Paolo Bianchini, Istituto Italiano di Tecnologia, Italy

Jeffrey J. DeStefano, University of Maryland, USA

Alex Valm, State University of New York at Albany, USA

Hatice Ceylan Koydemir, University of California, Los Angeles, USA

Laura Cerqueira, LEPABE, formerly BIOMODE, Portugal

Ricardo Cunha, BlueClinical, Portugal

Chemically modified nucleic acid mimics (NAMS) for improved RNA targeting

Jesper Wengel
University of Southern Denmark, Denmark

LNA (locked nucleic acid) and the corresponding 2'-amino-LNA derivatives mediate strong RNA targeting and antisense potency. This will be highlighted together with developments like Lipo-LNA, i.e., novel lipophilic 2'-amino-LNA-based antisense compounds, and DNA-mimicking modifications for improved selectivity of RNA-targeting.

Nucleic acid mimic applications in fluorescence *in situ* hybridization

Nuno F. Azevedo
Faculty of Engineering of the University of Porto, Portugal

DNA probes have been the molecules of choice in fluorescence *in situ* hybridization (FISH) for the identification and spatial characterization of microbial communities. The introduction of nucleic acid mimics has improved the robustness of FISH in terms of sensitivity and specificity of the method. Several nucleic acid mimics have been used, of which the most relevant are peptide nucleic acid (PNA), locked nucleic acids (LNA) and 2'-O-methyl RNA (2'OMe). During this talk, I will address the advantages and limitations of using PNA and LNA/2'OMe probes both in: i) classical FISH applications such as pathogen detection in food matrices, spatial location of cells in biofilms and multiplex FISH and ii) novel FISH applications such as fluorescence *in vivo* hybridization or delivery of nucleic acids for therapeutic applications.

Micro fluorescence *in situ* hybridization (μFISH) using a microfluidic scanning probe

Govind Kaigala
IBM Research, Zurich, Switzerland

The widespread use of FISH for diagnostics is limited, mainly because the FISH probes are expensive and the assay is time-consuming. To this end, we developed a microfluidic FISH implementation for rapid cytogenetic analysis of cells and tumor sections while also being conservative of the cytological sample and reagents. We demonstrated spatially multiplex chromosomal enumeration in adherent cells. Further, we adapted this MFP-based FISH implementation for rapid detection of the HER2 gene, a diagnostic biomarker, in breast tumor sections. We also developed a new method for probing FISH hybridization kinetics of FISH signals in real-time. This method allows the effects of various reaction parameters in FISH reactions to be quantified, which is important for modelling intracellular hybridization and the development of new FISH assays.

Single-molecule mRNA FISH in bacteria

Zach Hensel
Instituto de Tecnologia Química e Biológica, Portugal

Single-molecule FISH (smFISH) makes it possible to identify, count and localize single mRNA molecules in fixed samples. In bacteria, small cell volumes and the cell wall introduce complications for smFISH sample preparation and quantification. In this talk, I will briefly discuss sample preparation and data analysis for smFISH, focusing on protocols in bacteria and new methods for signal amplification and multiplexing. I will show how we are using smFISH to characterize live-cell mRNA imaging methods to determine whether or not they impact mRNA localization or degradation.

Nanoscopy tools to image and dynamically interact with molecular cell processes

Paolo Bianchini
Istituto Italiano di Tecnologia, Genoa, Italy

The study of intracellular process requires a detailed exploration of all the cell components and activity, such as molecular interaction and dynamics, structural organization, and conformation. Optical nanoscopy has emerged as a fundamental tool to investigate the cell at nanoscale resolution.

Several optical diffraction-unlimited methods have been introduced in the past decade; however, this seminar will focus on some of them: image scanning microscopy (ISM), stimulated emission depletion (STED) Nanoscopy and expansion microscopy (ExM). We will discuss both theoretical and practical aspects of the methods. There will be the chance to have a close view of a bench-top custom-built ISM-STED nanoscope, providing the description and function of the main components constituting it.

Aptamers to target microorganisms

Jeffrey J. DeStefano
University of Maryland, College Park, MD, USA

Aptamers are small (~20-90 nucleotides) pieces of nucleic acid with high affinity to a target (often a protein). They are typically selected using a process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Originally aptamers were made of natural RNA and DNA but more recently, Xeno-Nucleic Acid (XNA) alternatives have become popular. XNAs are nucleic acid mimics with modifications in base, sugar, or phosphate moieties. The alterations can lead to greater resistance to degradation and potentially enhance binding to the target through further interactions with the modified moiety and the ability of XNA to explore unique structural space. In collaboration with Dr. Philip Holliger's lab (Cambridge, England), our groups were the first to select FluoroArabino Nucleic Acid (FANA) XNA aptamers to proteins. FANA aptamers to HIV reverse transcriptase (RT), and integrase (IN) bind in the pM range and the latter bind 1-2 orders of magnitude better than previous RNA and DNA aptamers. More recently we have prepared XNA aptamers to the SARS-2 Covid-19 receptor binding protein. The possible use of these aptamers for inhibiting HIV and SARS-2 will be discussed.

Practical and Theoretical Aspects of CLASI-FISH imaging applied to microbial communities

Alex M. Valm
State University of New York, Albany, USA

Although the number of species present in a microbial community may number in the hundreds or more, until recently, fluorescence *in situ* hybridization has been used to label, at most, only a handful of different taxa in a single sample. We recently developed a technique, CLASI-FISH for combinatorial labeling and spectral imaging - fluorescence *in situ* hybridization, to greatly expand the number of distinguishable taxa in a single FISH experiment. The CLASI technique involves labeling microbes of interest with combinations of probes coupled with cutting edge spectral imaging instrumentation and state-of-the-art spectral deconvolution algorithms to allow the use of fluorophores with highly overlapping excitation and emission spectra. In this talk, the basic principles and theory of CLASI-FISH along with some guidelines for performing CLASI-FISH experiments will be presented. Recent developments in highly multiplexed labeling and probe design will be presented as well as their application to multi-species oral biofilms

Mobile Microscopy

Hatice Ceylan Koydemir
University of California, Los Angeles, USA

Smartphones have been a part of our daily lives since they are not only devices to make calls but also equipped with several sensors (e.g., accelerometers, gyroscopes, and image sensors) and computation power. These devices with their advantages such as availability across the world and field portability offer a great promise as the next generation's biosensing and bioimaging tools at the point of care. In this talk, several field-portable, cost-effective, and computational mobile microscopes developed at the Bio- and Nanophotonics Laboratory at the University of California, Los Angeles will be discussed in detail. These mobile platforms were designed and developed using optical parts and 3D printed tools, and integrated with artificial intelligence for automated detection, classification, and enumeration of the biological analytes of interest for a variety of applications. The handling steps for sample preparation were minimized to simplify the measurement flow and reduce the time to result starting from the sample collection. The image quality and efficiency of the platforms were confirmed using high-end microscopes as our ground-truth. These mobile microscopes can be useful sensing and imaging tools for health, water, and food quality monitoring applications.

Taking FISH from lab to market

Laura Cerqueira

LEPABE, formerly BIOMODE, Portugal

Nowadays, scientific community is facing a paradigm change as great deal is given to highly focused technologies that seek to solve specific problems or provide innovative solutions issuing to benefit the society and drive the economy. Even though, for the researchers, inventors and entrepreneur's standpoint, there is a long way to go from developing scientific findings from the laboratory and introducing them onto the market. In here an overview on a Lab-to-Market path will be given, with a special emphasis on Biomode, a company founded by Laura Cerqueira (LEPABE), Nuno Azevedo (LEPABE) and Carina Almeida (INIAV) that was intended to develop and commercialize PNA-FISH kits for the detection of pathogens in the health and food safety sectors. A glance on the FISH technology transfer, and its winnings and pitfalls, towards the market, will be provided.

Drug Development – From bench to bedside

Ricardo Cunha

BlueClinical, Portugal

Drug discovery and development is a highly complex and time-consuming process. Although it is commonly characterized as a "pipeline", suggesting a consistent unidirectional flow of a candidate therapeutic in unchanged form from basic research discovery until its regulatory approval, this is in stark contrast with the reality of drug development, full of advances and setbacks.

The drug development process is comprised of a non-clinical development phase, where the drug will be evaluated in animals to assess its safety profile, prior to its first administration to Humans, and a clinical development phase. The clinical development phase is comprised of phase I, phase II and Phase III studies, prior to obtaining marketing authorization, and phase IV studies, when the drug is already available for widespread use.

Over the last decade, pharmaceutical companies are following through on their pledge to break down some of the high walls built around R&D as they go out and work more with academic investigators, biotech companies and other pharmaceutical outfits in the much heralded "open innovation" model that is being fostered worldwide.

Oral Communications from selected abstracts

Linda Mezule, Riga Technical University, Latvia

Viktorija Denisova, Riga Technical University, Latvia

Joana Castro, University of Minho, Portugal

Aliona Rosca, University of Minho, Portugal

Murad Ghanim, Volcani Center, Israel

Rapid fluorescence-based assays for *in situ* enumeration of microorganisms

L. Mezule

Water Research Laboratory, Faculty of Civil Engineering, Riga Technical University, Riga, Latvia

Many microorganisms in the environment and engineered systems are constantly fighting with various stress inducing factors. This further induces microbial starvation, dormancy or cell entrance into a viable but non-culturable (VBNC) state where they cannot be detected and enumerated with traditional culture-based assays that require microbial growth and subsequent formation of a colony. In many of the engineered systems, e.g., water supply, hospital appliances, these organisms can cause serious health hazards. Alternative methods are generally molecular based and aim to determine cellular genetic material or *in situ* analyses which include epifluorescence staining of the samples without prior cultivation. Despite wide scale availability of various fluorescent stains, these techniques are often inappropriate for the analyses of environmental and surface material samples due to high cell variability, presence of various inhibitors that can induce autofluorescence or incompatibility with sample pre-processing approaches, e.g., immobilization, fixation or permeabilization and sample type as such.

The general objective of the research is to design protocols for rapid and simple *in situ* enumeration of microorganisms in their natural and man-made environments.

Current research activities of RTU Water research laboratory have been linked with designing viability staining and fluorescent *in situ* hybridization (FISH) protocols or combination of both for enumeration of total and viable *Escherichia coli*, bacterial spores, *Clostridium*, methanogenic archaea and many more. FISH alone is rapid and robust enough to be employed on a routine basis, however, its wide scale application is usually based on the necessity to design a customized protocol for each specific microorganism (group) in a specific matrix. Introduction of viability staining allowed to discriminate among viable and non-viable cells, simultaneous use of multiple probes in FISH allowed to discriminate among various microbial groups in biogas reactors. Use of analytical software allowed to set certain fluorescence thresholds to minimize the impact of autofluorescence and nonspecific binding that seemed to be as one of the biggest drawbacks of FISH. Further research is dedicated to development of automated detecting and analysis systems to minimize manpower needed for the analysis.

In conclusion, the results showed that most of these protocols can be validated in environmental systems and yield as a good indicator for overall population quality.

Acknowledgements

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Disinfection of non-cultivable microorganisms in water supply systems

V. Denisova

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The presence of pathogenic microorganisms in drinking water causes waterborne diseases such as diarrhoea, dysentery, cholera, typhoid and polio. According to World Health Organization's 2018 report data, globally around of 2.1 billion people do not have access to microbiologically safe drinking water, and about 500 000 people die of diarrhoea each year [1]. The majority of these deaths are in developing areas where water sources are not adequately protected, and typical centralized water treatment plants are not applicable because of prohibitively expensive costs for their building and maintenance [2]. Therefore, the development of point-of-use (POU) water treatment systems at home play an important role in decentralised drinking water safety.

Traditionally, the microbiological quality of drinking water is based on heterotrophic plate count (HPC) method [3]. The method is based on microbial ability to form colonies on the artificial media, which represent viable microorganisms. However, this method has two disadvantages: 1) long cultivation time – from 24 hours up to 7 days to obtain the results; 2) not all microorganisms could be detected by this method. Moreover, many microorganisms, when subjected to stress may be present in the drinking water in a viable but nonculturable (VBNC) state. It is assumed that bacteria in the VBNC state fail to grow on routine bacteriological media, on which they would normally grow and develop into colonies but are alive and capable of renewed metabolic activity. Nevertheless, it has been shown that in this state, microorganisms are able to retain their pathogenicity and even resuscitate. As a result, drinking water supply systems are not completely protected, and that in turn can create a new risk for waterborne diseases caused by viable but nonculturable microorganisms.

The serious danger of microbial contamination is its rapid spread in drinking water supply systems, which can effect large number of people in a short period of time. This is particularly noticeable in the villages and small towns due to the relatively small number of people and rapid spread of pollution. Therefore, the aim of this study is to develop effective and affordable drinking water treatment technologies for rural communities (villages) in order to decrease the number of viable but nonculturable (VBNC) microorganisms in water storage and supply systems.

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PNA-FISH approach in Bacterial Vaginosis: from diagnosis to unveiling etiology

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Bacterial vaginosis (BV) is a common vaginal infection occurring in women of reproductive age, but its etiology is not yet fully understood. What is widely accepted is that BV is often characterized by a shift in the composition of the normal vaginal microbiota, from a *Lactobacillus* species dominated microbiota to a mixture of anaerobic and facultative anaerobic bacteria. During BV, a polymicrobial biofilm develops in the vaginal microenvironment, being mainly composed of *Gardnerella* species. The interactions between vaginal microorganisms are thought to play a pivotal role in the shift from health to disease and might also increase the risk of sexually transmitted infections acquisition.

In this last decade, our research group developed peptide nucleic acid probes (PNA) specific for the identification of vaginal bacteria to be used in fluorescence *in situ* hybridization (FISH) method. This approach combines the simplicity of traditional staining procedures with the unique performance of PNA probes to provide a rapid and accurate diagnosis of BV. As such, our novel multiplex PNA FISH method is based on the specificity of PNA probes for *Lactobacillus* spp. and *Gardnerella* spp. visualization, allowing a trustful evaluation of the bacteria present in vaginal microbiota and avoiding the occurrence of misleading diagnostics [1].

Aside from to be a valuable alternative method for BV diagnosis, PNA-FISH has been also emerging as a crucial tool for better understanding BV progress, through the study of ecological interactions between (i) endogenous bacteria adhered to vaginal epithelial cells and BV-associated bacteria [2], and (ii) between BV-associated bacterial species in a multispecies biofilm [3]. Of note that the combination of this method with confocal laser scanning microscopy allowed the study of spatial organization and changes of specific members of complex microbial populations without disturbing the biofilm structure.

In conclusion, PNA-FISH methodology represents a valuable alternative in relation to conventional methods for BV diagnosis and presents a unique contribution to gain insights into the phenomena responsible for the events that elicit BV.

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Acknowledgments

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Characterization of bacterial vaginosis-associated multi-species communities by multiplex PNA-FISH

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Bacterial vaginosis (BV) is one of the most common bacterial vaginal infections among women of reproductive age, characterized by a dramatic shift in the vaginal microbiota from the beneficial lactobacilli to a polymicrobial community, consisting of strictly and facultatively anaerobic bacteria [1]. It is noteworthy that a hallmark of BV is the presence of a highly structured polymicrobial biofilm on the vaginal epithelium, presumably initiated by a facultative anaerobe, *Gardnerella spp*., which then become a scaffold for other species to adhere [2]. While not much is known about multi-species interactions within BV biofilms, *Atopobium vaginae* is often associated with *Gardnerella spp*. biofilms and is rarely detected without *Gardnerella spp.* [3].

In the present study, we aimed to assess the interactions between *Gardnerella spp*. and *A. vaginae*, analysing both mono- and dual-species cultures. Firstly, we evaluated the impact of *A. vaginae* on a pre-established *Gardnerella spp*. biofilm, by determining the total biofilm biomass using the crystal violet method. Furthermore, bacterial distribution and biofilm structure were evaluated by Peptide Nucleic Acid Fluorescence *in situ* Hybridization (PNA-FISH) with specific PNA probes for *Gardnerella spp*. and *A. vaginae* using confocal laser scanning microscopy. Afterward, quantification of viable bacteria within pure or dual-species planktonic cultures was performed by also using PNA-FISH method.

We observed that in vitro, *A. vaginae* was not able to establish a single-species biofilm but easily incorporated a pre-formed *Gardnerella spp*. biofilm. Interestingly, *A. vaginae* lost viability after 48 hours of single-species planktonic growth but was able to maintain viability when co-cultured with *Gardnerella spp*. This demonstrated that in vitro *A. vaginae* is dependent on *Gardnerella spp*. to survive, providing an explanation of the co-occurrence of these two species in vivo. Overall, this study underlined the importance of the ecological interactions between these BV-associated species, which might delineate the development of BV.

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Acknowledgments

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Localization of plant pathogenic viruses and bacteria, and endosymbiotic bacteria in plants and insect vectors using FISH methods

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Transmission of persistent plant pathogenic viruses and bacteria by insect vectors is a complex process that involves proteins and mechanisms of both the pathogen and the insect vector, which are essential to ensure successful transmission [1]. Spatial localization and visualization of viruses and bacteria during the infection process is an essential step that complements expression profiling experiments in response to different stimuli. Studying the spatiotemporal localization of these microorganisms is essential for complementing biological, physiological and molecular experiments. Several techniques for visualizing viruses and bacteria such as reporter gene systems or immunohistochemical methods are time-consuming, and some are limited to model organisms, and involve complex methodologies. FISH that targets RNA or DNA species in the cell is a relatively easy and fast method for studying spatiotemporal localization of genes and for diagnostic purposes. This method can be robust and relatively easy to implement when the protocols employ short hybridizing, commercially available inexpensive probes. This is particularly robust when sample preparation, fixation, hybridization, and microscopic visualization do not involve complex steps. We have developed several protocols for localizing plant pathogenic bacteria and viruses, insect bacterial endosymbionts and mRNA transcripts in several plant pathogen-vector systems for studying their complex interactions and revealed new results at the cellular, organ and organism levels. Those methods, the studied systems and representative results will be presented and discussed.

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