

Project title: **InfectoOptics – Combating infectious diseases with advanced optical methods**

Project number: W8 /2018

Executive Summary

The **Leibniz ScienceCampus (LSC) InfectoOptics – Combating infectious diseases with advanced optical methods** is a joint project of the Leibniz Institute for Natural Product Research and Infection Biology (Leibniz-HKI) and the Leibniz Institute of Photonic Technologies (Leibniz-IPHT), alongside the Friedrich Schiller University Jena (FSU), Jena University Hospital (JUH), and the Fraunhofer Institute for Applied Optics and Precision Engineering (IOF) combine their efforts in the investigation of infections and microbial pathogens. In its second funding period, the LSC **InfectoOptics** focused on high quality research in infection biology, optics, photonics, and microsystem technology. It bridged basic and applied research, supporting projects like DFG Collaborative Research Centres, the BMBF-funded InfectoGnostics Research Campus, and the Leibniz Centre for Photonics in Infection Research. The research program included four main projects: **PNEUTHERA**, **VersaDrop**, **HoT-Aim 2.0**, and **iTag**, each with specific objectives and milestones. **IntraPerSpective** was also part of the LSC InfectoOptics without receiving funding.

PNEUTHERA used high-resolution optical technologies and quantitative image data analysis to investigate a complex lung cell culture model (Alveolus-on-Chip) for optimal therapy conditions for viral pneumonia with secondary bacterial infection. Milestones included optimizing infection experiments with influenza A virus and *Staphylococcus aureus*, enhancing automated image analysis pipelines, and developing advanced lung-on-a-chip models from induced pluripotent stem cells. PNEUTHERA also secured funding for research on SARS-CoV-2 and advanced microphysiological systems research.

VersaDrop developed a multiplexing platform for droplet generation and high-throughput optofluidic measurements. It resulted in new droplet deposition technology and a digital microfluidic platform, along with image-based fuzzy logic control and software for a hyperspectral snapshot camera, improving droplet-based analysis and microbial cultivation for antibiotic susceptibility testing.

HoT-Aim 2.0 combined (bio-)molecular methods with high-resolution microscopy to understand human pathogenic fungal infections. It made significant progress in fungal research, identifying candidalysin as a hemolytic factor in *Candida albicans* and visualizing non-mating receptors in *Schizophyllum commune*. Advanced imaging techniques, including tip-enhanced Raman spectroscopy, were used to study virion and fungal membrane surfaces and nanomechanical properties, enhancing the understanding of fungal biology and microscopic interactions.

iTag implemented Raman tags for imaging virulence factors and antimicrobial agents and explored synthetic biology methods for non-invasive in vivo imaging. It developed multimodal nonlinear imaging platforms and achieved proof of concept for coherent anti-Stokes Raman scattering imaging, expanding tools for studying infections.

InfectoOptics ensured equal opportunities for all researchers by publicly advertising positions and fostering a culture of inclusivity. Collaborations were established across all projects, involving various research laboratories and institutions. Regular meetings and project-specific discussions facilitated the exchange of knowledge and the advancement of projects. The results were published as open access, with regular reporting and advisory discussions ensuring data integrity. Group members participated in annual seminars on good scientific practice.

In conclusion, the LSC **InfectoOptics** successfully advanced research at the intersection of infection research and optics, contributing innovative models, imaging techniques, and analytical platforms despite the challenges posed by the pandemic. The findings of the project were disseminated through 150 publications, the majority of which are open access and therefore accessible to the wider scientific community. Furthermore, the LSC **InfectoOptics** has established a robust foundation for future research endeavors and methodologies that hold significant promise.

1. Achievement of objectives and milestones

Significant progress was made in a number of areas of biomedical and optical research. Infection experiments involving the influenza A virus and *Staphylococcus aureus* were optimized, with advanced imaging techniques and automated analysis methods developed to visualize and quantify viral loads and track bacterial localization. Induced pluripotent stem cell (iPSC) cultures were differentiated into epithelial cells to support full viral replication cycles, thereby validating their use for antiviral drug testing and immune response studies. Furthermore, this research secured funding for further research on SARS-CoV-2 and chronic disease mechanisms.

Technologies for the generation and manipulation of droplets were refined, incorporating high-throughput optofluidic measurements and advanced microscopy modalities, thereby enabling precise analysis and imaging of droplets. Notable innovations included the integration of angle-resolved light scattering and the development of hyperspectral imaging software.

Research on *Candida albicans* identified the key factors involved in toxin secretion and employed advanced imaging techniques to study fungal interactions and membrane properties. The implementation of structured illumination microscopy and single-molecule localization techniques enabled a detailed cellular analysis to be conducted.

The development of Raman tags for imaging infection processes has resulted in significant advancements in multimodal nonlinear imaging and the introduction of new contrast mechanisms. Experiments conducted with ultrafast tunable lasers were employed to optimize CRS setups, and novel molecular markers were utilized to facilitate the study of microbial interactions and biological systems.

Research and the employment of students, PhD students and post-doctoral researchers were financed directly from the project budget. The financial and time plans were largely adhered to, with the exception of minor deviations. In addition, **InfectoOptics** was an important lever for research funding, and **InfectoOptics** was also able to attract further third-party funding (*see 3. Results and successes and attached Excel table, sheet 3.4 "results and successes"*).

2. Activities and obstacles

PNEUTHERA

The project's progress was impeded by restrictions related to the SARS-CoV-2 virus, resulting in delays in planned procedures. Despite these challenges, infection procedures, staining protocols for microscopy, and initial treatments with Tamiflu and clarithromycin were established and tested in single cell culture experiments. The original plan was to use primary cells for a lung-on-a-chip model. However, the cells could not be sufficiently expanded and lost their phenotype during the process. This led to a shift towards the use of induced pluripotent stem cells (iPSCs) due to their ability to expand indefinitely and differentiate into the required cell types, offering a personalised model without the risk of allogenic rejection. iPSCs sourced from D. Kotton's lab at Boston University improved the study of human immune responses to infections like IAV in a controlled in vitro setting. This shift in focus resulted in a delay in the establishment of the lung-on-a-chip model and the development of specific quantitative image analysis. The success of infection in the transwell system was primarily monitored via plaque assays, with some challenges in image quantification due to unspecific signals from Matrigel, which is necessary for cell cultivation. Conventional wide-field fluorescence microscopy was used to confirm infection of AT2 epithelial cells, but higher resolution microscopy and improved staining protocols are needed for future studies.

VersaDrop

At the Leibniz-HKI, fluorescence dyes have been utilized to color code experimental conditions within droplets. A machine learning approach has been successfully developed to identify different droplet populations analyzed using brightfield and fluorescence microscopy. The color-coded droplets have been successfully analyzed for high-throughput screening campaigns using an optofluidic droplet analysis platform. The platform was developed according to the specified requirements and the bottom glass substrate was coated with two silver electrodes (Ag) via inkjet printing, at the IOF. The microfluidic platform can be characterized as follows: The surface on which drops can be moved has a size of 44 x 44 mm and is illuminated by a conventional light projector. By applying an alternating voltage for moving individual droplets was achieved, best achieved when the non-illuminated spot matches the droplet's radius. The droplets can be moved in every direction on the x-y-plane and can be merged or split, creating numerous possibilities to generate different dilutions. The development has not reached the stage to conduct droplet experiments with the HKI droplet system, yet. Triggered data acquisition has been successfully implemented, and angle-resolved light scattering measurements have been obtained for defined low cell numbers in picoliter droplets during microfluidic flow. A robust and compact scattering sensor has been developed and combined with the microfluidic platform. A special chip for electromagnetic droplet fusion and fission, multimodal

nonlinear imaging, sorting, and dielectrophoresis has been designed for small-sized droplets. However, the realization of this chip with high-precision bonding of the wafers has not yet been successful. At current, the observation time of the droplets is limited to about 10 seconds due to fluctuations in the pressures. This can be changed only with constant feedback, which we actually implement.

HoT-Aim 2.0

With respect to the adaption of the initially planned experiments to target systems, namely *S. commune* and *C. albicans* we are naturally slightly behind schedule, as during the pandemic, the instrumentation had to be developed mainly using in-house model systems. Novel approaches like nanoIR, Spatial Light Modulators (SLM) and Fluidic force microscopy (FluidFM) were used to close this gap.

iTag

The iTag project was successful in evaluating Raman imaging to identify virulence factors containing polyunsaturated moieties and in preparing a range of natural product conjugates. The preparation process was more time-consuming than anticipated due to the limited availability of pure compounds and the challenging synthetic procedures. Physiological concentrations of active compounds often fell below Raman microscopy's detection limit. The heterologous production of polyalkynes was hindered by toxic side effects. Efficient marker development was found to be crucial for successful Raman spectroscopic visualization, but balancing scattering efficiency with biocompatibility proved difficult. Consequently, the development of switchable alkyne-modified biomarkers was postponed to first investigate the minimization of stress on biological systems and better understand the within iTag designed biomarkers. Superresolution CRS imaging was challenging due to the need for high signal markers, leading to a focus on SRS and SREF for improved contrast and sensitivity. Spontaneous Raman spectroscopy was more sensitive than CARS and SRS for detecting low concentrations of Raman tags, identifying low concentrations of active agents in cellular environments as a major challenge.

3. Results and successes

InfectoOptics was involved in 150 publications, comprising 117 journal articles, four conference transcript articles, 29 monographs, and one book chapter. (*see Annex, Excel table, sheet "results and successes", 3.1*). Furthermore, numerous scientific events and seminars (*see Appendix, Excel table, sheet "results and successes", 3.2*) were held, providing students and scientists with valuable opportunities for exchange. **InfectoOptics** also provided support for 34 theses, comprising 27 doctoral dissertations and seven bachelor's/master's theses. A total of 21 theses were financed directly from project funds. A total of 13 of the 34 projects were funded exclusively by other sources. (*see Appendix, Excel table, sheet "results and successes", 3.3*). Furthermore, there were numerous instances of scientific collaboration with other projects (*see Appendix, Excel table, sheet "results and successes", 3.5*). It is also noteworthy that three patents were submitted and a book chapter will be published (*see Appendix, Excel table, sheet "results and successes", 3.5*).

This shows that project funding plays an incredibly important role in initiating research; it acts as a lever for the realization of broad-based research.

It should also be emphasized that a total of € 30.684.261,00 in third-party funding was generated by other funding sources (*attached Excel table, sheet 3.4 "results and successes"*), which was raised in direct connection with the **InfectoOptics** project. This also shows that **InfectoOptics** funding ultimately benefits a broad field of research and makes new funding possible.

In addition, numerous scientific events took place, as listed in the attached Excel sheet (*3.2 "scientific events"*). These included colloquia, seminars, workshops and summer schools aimed at undergraduates, PhD students and postdocs. **InfectoOptics** has thus fulfilled its commitment to promoting young scientists. The general public was also reached through exhibitions and other events. For example, scientists from the InfectoOptics project were involved in events such as the MINT Festival, the Long Night of the Sciences and the "Forscher Schüler Tag".

PNEUTHERA

PNEUTHERA has achieved promising results with the use of induced pluripotent stem cells (iPSCs) to develop advanced lung-on-a-chip models for the study of influenza A virus (IAV) infection. Initial findings from transwell studies indicate the efficacy of iPSC-derived AT2 epithelial cells in facilitating the complete replication of IAV. These findings are being compiled into a manuscript that will detail the experimental procedures, outcomes, and the advantages of using iPSCs over primary cell materials. The manuscript will also highlight the shift towards a more scalable and stable cell source for in vitro models. In addition, our protocol for generating an iPSC-based lung-on-a-chip model has been accepted for publication as a book chapter in the second edition of 'Influenza Virus: Methods and Protocols' in the Methods in Molecular Biology series published by Springer titled 'Human induced pluripotent stem cell-based alveolus-on-chip model to study influenza virus A infection'. Furthermore, insights from applying this lung-on-a-chip model to study influenza virus infection are being prepared for another manuscript. This publication will discuss the model's

utility in analysing the human immune response to IAV infections, emphasizing the potential of iPSC-based models in personalized medicine and advanced virological studies.

VersaDrop

The developed multiplexing platform has been successfully employed in a demonstration microbial cultivation experiment for antibiotic susceptibility investigations. Optofluidic analysis of multiplexed droplets showed the successful identification of different droplet populations measured by in-flow laser-induced fluorescence, which was analyzed offline. The technology for controlled droplet deposition onto agar plates was published.

For the angle-resolved light scattering setup, the results prove a very high sensitivity for microfluidic droplet analysis by showing a detectability of less than five cells down to single cells in one droplet. Different droplet amounts can be analyzed regarding cell number and cell type. First application tests show early detectability of cell growth after only 2-3 hours as well as growth inhibition accordingly, such as caused by antibiotic activity.

An imaging platform for TPEF, CARS, SRS and FLIM has been combined with a pressure-based microfluidic chip platform and automated imaging with different modalities of 50 pre-sorted droplets with bacterial content was demonstrated.

HoT-Aim 2.0

In project 1 it was shown that voriconazole adaptation leading to a higher accumulation of lipid rafts at the hyphal tip, associated with hyperbranching and in directional hyphal growth a specific connection to surface attachment was detected. Then in project 2, it was shown that Ece1 precursor is not required to block premature pore-forming toxicity, but to prevent intracellular auto-aggregation of candidalysin sequences. Significant progress was made in project 3 by implementing a high-resolution microscopy prototype attached to a commercial microscope. F. Wechlser and R. Heintzmann developed Julia-based image processing software packages, which are open access (NDTools.jl, FourierTools.jl, PointSpread-Functions.jl). In Project 4 a novel method was developed to suppress protein degradation on plasmonic substrates, namely silver. This proved to be a crucial step towards TERS experiments on Candidalysin. Additionally, Force-distance experiments have been established for the fast evaluation of nanomechanical properties of fungal membranes.

iTag

The iTag project demonstrated that the addition of Raman tags can enhance detection limits, particularly when utilising alkyne tags. Furthermore, deuterated substances with numerous C-D bonds also yield high signals.

In collaboration with the Glorius group at the University of Münster, a series of alkyne-based tag structures were designed and evaluated for use with cholesterol mimetics. The most favourable outcomes were observed in structures comprising multiple alkyne units. Nevertheless, the selection of tags must take into account their size and chemical properties. Consequently, it is of the utmost importance to retain the biological and chemical properties of vibrational probes, which must be verified in biological assays or test experiments prior to their use.

Furthermore, natural products were isolated and several tagged derivatives were synthesised. In addition to imaging virulence factors, a noteworthy accomplishment was the visualization of a peptide in a model nematode through a collaborative project with Hertweck-Popp-Hoffmeister. This was achieved through the use of a biosynthetic approach for the implementation of tags, which ensured bioavailability. An imaging approach of FLIM and Raman spectroscopy was used.

All publications, events and transfer activities in connection to InfectoOptics are listed in the attached excel table, sheet "results and successes".

4. Equal opportunities, career development and internationalization

The LSC **InfectoOptics** strives to provide the best possible working conditions for all its members—regardless of nationality, gender, sexual orientation, age or health. All institutions participating in the LSC **InfectoOptics** employ an equal opportunities officer and adhere to the “Leibniz Guidelines on Career Development”, the “Research-Oriented Standards on Gender Equality” and the “Standards for the appointments to academic executive positions within the Leibniz Association”.

By organizing numerous events for young scientists, **InfectoOptics** fulfilled its commitment to promoting young scientists (*see also 3. Results and attached Excel table, sheet 3.2 "results and successes"*). Equal opportunities are a central part of the staff development strategy. In order to guarantee that all researchers have equal opportunities regardless of gender, origin, age or other individual characteristics, the relevant positions were publicly advertised in gender-neutral language. During the candidate selection process, care was taken to avoid unconscious biases and to recruit the best suited researchers. To increase the percentage of female scientists in higher positions, the LSC **InfectoOptics** offers mentoring and training to its talented

female scientists to encourage them to pursue an academic career. It further aims to increase the representation of women in appointment- and steering committees, discussion panels, as seminar speakers and conference contributors. In the second funding period, 59 % of all **InfectoOptics** researchers were female. In greater detail 66 % of all doctoral researchers, 36 % of all postdocs were female. Attention has been paid to creating a culture of equal opportunities to encourage the full potential of all participants and to improve the quality of research. The total number of foreign nationals among the scientists is 31%, which serves to illustrate the distinctive cultural characteristics of different nations.

All employees are listed in the attached Excel table, sheet 4. "Eq.opp.+career+internat."

5. Structures and collaboration

The projects described involve extensive collaborations and research efforts across various fields, including iPSC-derived lung cells, organ-on-a-chip models, hyperspectral imaging, microfluidic platforms, and inkjet printing technologies. Key contributions include the establishment of new partnerships, significant funding acquisitions, and successful implementation of advanced experimental methods. These collaborative efforts span multiple institutions within the University Jena and Heidelberg and with Leibniz-Institutes with various disciplines, with a focus on exploring mechanisms of diseases, developing new technologies, and enhancing experimental procedures. *(see also attached Excel table, sheet 5. "Structures & cooperation", 5.1 Close academic partners)* Additionally, we are pleased to report that, through the efforts of our esteemed cooperation partners, namely ChemBioSys, FungiNet 3D-Sensation, InfectoGnostics Research Campus, InfectoControl, Centre for Sepsis and Care (CSCC), and of course Leibniz IPHT. *(see also attached Excel table, sheet 5. "Structures & cooperation", 5.2 associated Cooperation's)*, we have achieved remarkable scientific advancements.

6. Quality assurance

Results are published as open access, when possible. All projects performed regular reports und advisory discussions. Data and results are frequently reviewed within the groups and stored securely. Whenever possible, the data is published as open access. We are especially pleased to say that 76% of this data is freely accessible to science and society. The results have also led to a book chapter and three patents. *(see also 3. Results and attached Excel table, sheet 3. "Results and successes", 3.1 Publications)*. A total of 34 theses are related to **InfectoOptics**, of which 7 master's/bachelor's theses and 17 doctoral theses have already been completed. 10 dissertations are still in progress. *(see also 3. Results attached Excel table, sheet 3. "Results and successes", 3.3 Theses and dissertations)*. Group members participate in annual good scientific practice seminars. In addition, numerous courses, conferences and colloquia have been supported and organised as part of **InfectoOptics**.

At the **InfectoOptics**, we are committed to the highest standards of animal care and ethical research. Animal testing has been conducted in accordance with the European and German animal welfare regulations. All experiments were approved by the Committee for Animal Research of the State of Thuringia.

7. Additional resources

Within **PNEUTHERA** biochemical consumables worth ≈8000 € were used in the Eggeling and Ehrhardt laboratories. In the Erhardt laboratory, the personnel costs for the technical assistant were supported with 10 % and in the Eggeling laboratory for a post-doc position with 5 %. For the **VersaDrop** the experimental infrastructure for performing the multimodal nonlinear imaging experiments has been provided by the IPHT. Two microfluidic pressure-based flow control systems (Fluigent, Biophysical tools) for setting up a simple microfluidic experiment and two hyperspectral cameras were used. Fabrication of microfluidic chips in the clean room of the Leibniz-IPHT (20 wafer pairs, non-scientific staff). A laser scanning microscopic platform (SP8 Falcon, Leica Microsystems GmbH) in combination with a custom ultrafast fiber laser for coherent Raman imaging (DeltaEmerald, APE GmbH). In the **HoT-Aim 2.0** there were no additional resources. In the **iTag** project the spectroscopic research was founded by other projects.

8. Outlook

The project is making noteworthy advancements in a number of scientific and technological domains. The project is dedicated to investigating viral and bacterial infections in lung models, with a particular focus on the use of antiviral drugs and microbial metabolites. Furthermore, the project is dedicated to enhancing lung-on-a-chip technology to more accurately simulate physiological conditions. Additionally, a new microfluidic platform for high-throughput screening of bacterial droplet cultivation experiments will be developed. The research has also been expanded into coherent Raman scattering (CRS) methods for large-area

and live imaging applications, building on previous studies with Raman micro spectroscopy. These initiatives are focused on enhancing our understanding of disease mechanisms and developing innovative therapeutic strategies.

Three patent applications have been submitted. The objective of these patents is to drive scientific advancement and promote broader utilisation. Once the **InfectoOptics** project has concluded, they should be used and spread to other research initiatives.