

FINAL REPORT

1 General Information

DFG reference number: SCHU 3512/2-1

Project number: 493847477

Project title: Einflüsse und Resistenzentwicklung von Mikroorganismen auf niedrige Konzentrationen von Nanomaterialien in geometrisch definierten Umgebungen

Name(s) of the applicant(s): Dr. Julian Schütt (on 01.01.2023, the project coordination was shifted to Dr. Denys Makarov after Dr. Schütt took another position and left the host institution)

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Name(s) of the co-applicants: not relevant

Name(s) of the cooperation partners: not relevant

Reporting period (entire funding period): 01.04.2022 – 31.03.2025

2 Summary

English: This project focused on the development of an effective methodology for detecting and analyzing the emergence of microbial resistance to low concentrations of nanomaterials in environmental samples. To enable rapid screening and precise analyses, we tested various experimental approaches, all of which leveraged droplet-based microfluidics. This technology proved crucial for accurately controlling and analyzing individual liquid droplets, a key requirement for studying subtle effects at low nanomaterial concentrations. We explored physical detection techniques for directly sensing nanomaterials within droplets, including both magnetic and electromagnetic methods. For instance, we employed planar Hall effect sensors of magnetic fields to identify the presence of nanoparticles by measuring their magnetic influence within droplets. Similarly, we explored impedance measurements to detect changes in the electromagnetic properties of the liquid that nanomaterials induced. However, these direct physical methods lacked the necessary sensitivity for reliably detecting the extremely low and environmentally relevant concentrations of nanomaterials that could potentially induce microbial resistance. Consequently, we adopted a biological approach as our primary detection strategy. In this method, we exposed various bacterial strains to defined, low concentrations of nanomaterials and monitored their viability and resistance development over time. The microorganisms' biological response thus served as an indirect, yet highly

sensitive, indicator for the presence and biological efficacy of the nanomaterials, even at concentrations that we could not measure directly through physical means. The integration of precise microfluidic technology with this sensitive biological detection strategy led to the development of a measurement platform for follow-up research activities and technology transfer. In particular, the developed fluidic platform enables us to accurately and reliably analyze the effects of antibiotics and nanomaterials on microorganisms and their potential for resistance formation in complex environmental samples, significantly advancing our understanding of nanomaterials' environmental impact. Furthermore, the gained knowhow on specific processes for the fabrication of fluidic circuits, realization of fluidic components and detection modules is transferred to HZDR Innovation GmbH via appropriate licensing contracts.

German: Dieses Projekt konzentrierte sich auf die Entwicklung einer effektiven Methode zur Erkennung und Analyse der Entstehung mikrobieller Resistenzen gegen niedrige Konzentrationen von Nanomaterialien (NM) in Umweltproben. Um ein schnelles Screening und präzise Analysen zu ermöglichen, testeten wir verschiedene experimentelle Ansätze, die alle auf tröpfchenbasierter Millifluidik basierten. Diese Technologie erwies sich als entscheidend für die präzise Steuerung und Analyse einzelner Flüssigkeitströpfchen – eine wichtige Voraussetzung für die Untersuchung subtiler Effekte bei niedrigen NM-Konzentrationen. Wir erforschten physikalische Detektionstechniken zur direkten Erfassung von Nanomaterialien in Tröpfchen, darunter magnetische und elektromagnetische Methoden. Beispielsweise setzten wir planare Hall-Effekt-Sensoren für Magnetfelder ein, um das Vorhandensein von Nanopartikeln durch Messung ihres magnetischen Einflusses in Tröpfchen zu identifizieren. Ebenso untersuchten wir Impedanzmessungen, um Veränderungen der elektromagnetischen Eigenschaften der Flüssigkeit durch Nanomaterialien zu erfassen. Diese direkten physikalischen Methoden mangelten jedoch an der erforderlichen Sensitivität, um die extrem niedrigen und umweltrelevanten Konzentrationen von Nanomaterialien, die potenziell mikrobielle Resistenzen auslösen könnten, zuverlässig zu erfassen. Daher wählten wir einen biologischen Ansatz als primäre Detektionsstrategie. Bei dieser Methode setzten wir verschiedene Bakterienstämme definierten, niedrigen Konzentrationen von Nanomaterialien aus und beobachteten deren Lebensfähigkeit und Resistenzentwicklung im Zeitverlauf. Die biologische Reaktion der Mikroorganismen diente somit als indirekter, aber hochempfindlicher Indikator für das Vorhandensein und die biologische Wirksamkeit der Nanomaterialien, selbst bei Konzentrationen, die wir physikalisch nicht direkt messen konnten. Die Integration präziser Mikrofluidik-Technologie mit dieser sensitiven biologischen Detektionsstrategie führte zur Entwicklung einer vielversprechenden Plattform für weiterführende Forschungsaktivitäten und Technologietransfer. Insbesondere ermöglicht uns die entwickelte Fluidik-Plattform, die

Auswirkungen von Nanomaterialien auf Mikroorganismen und ihr Resistenzpotenzial in komplexen Umweltproben präzise und zuverlässig zu analysieren und so unser Verständnis der Umweltauswirkungen von Nanomaterialien deutlich zu verbessern. Darüber hinaus wird das gewonnene Know-how über spezifische Prozesse zur Herstellung von Fluidkreisläufen, zur Realisierung von Fluidkomponenten und Detektionsmodulen über entsprechende Lizenzverträge an die HZDR Innovation GmbH übertragen.

3 Progress Report

In the following, we present key achievements acquired during the project implementation:

Large libraries of unique codes in droplet fluidics: Droplet fluidics [1] is applied in medicine and biology in lab-on-a-chip (LOC) or micro total analysis systems (μ TAS). An easy and reliable tracking method is crucial for any running experiment since each droplet needs to be clearly identified and tracked during assays, which can last from minutes up to days and weeks. The major challenge is to realize large libraries of unique codes, which is required for current biotechnological and chemical state-of-the-art processes, i.e. up to several million samples in high-throughput screening (HTS) assays in drug discovery.

In the scope of this project, we proposed a new concept of multiparametric coding in droplet-based fluidics where we rely on the use of different sensing units (in our case: Planar Hall Effect (PHE) magnetic field sensor and impedimetric sensors) and targeted tailoring of the fluidic circuit and its complexification [2]. For instance, using a droplet code of 2 droplets of the same volume with 3 different concentrations of magnetic content in droplets, it is possible to fabricate 9 codes. In addition to the magnetic content, also the volume of droplets in the code can be varied leading to the multiparametric coding in droplet-based fluidics. This variation of droplet volumes of individual droplets within the code increased capacity of the code library, i.e. increasing the number of combinations from 9 to 36 codes in a two-droplet code. We demonstrate experimentally that by modular extension of the fluidic circuit to incorporate a third droplet with three different volumes for all droplets in the code, increases the coding capacity to 729 ($= 3^{(3+3)}$). Moreover, we demonstrate that the concept can be easily extended to other detection means as exemplarily demonstrated with impedance-based detection. With the current setup we demonstrate the possibility to prepare up to 3 millions unique codes. In a similar spirit, more detection parameters (physical or chemical) can be involved leading to practically unlimited code libraries.

Our concept offers practical way towards coding in functional fluidics, which outperforms the current state of the art methods by orders of magnitude. Therefore, the developed scalable approach towards coding in droplet fluidics can find a variety of practical applications, e.g. in pharmaceutical and biotechnological assays. Furthermore, we indicate that these libraries

will require advanced algorithms for code identification, e.g. supervised discriminant analysis or unsupervised k-means clustering. This will open a new page in the topic of functional fluidics for high-throughput screening assays in drug discovery.

Microfluidic droplet reader (MRD): We explored an alternative detection methodology, based on a millifluidic setup combined with a resonance detector [3], Figure 1. Its primary objective was to detect subtle changes in samples that exhibit magnetic, metallic, and/or ionic properties, whether these exist in liquids. To achieve this, we threaded a millifluidic tubing through a customized coil. This coil formed an integral part of an LC-tank (an inductor-capacitor resonant circuit) and electronically interfaced with a readout system. We then applied an alternating current (AC) to the LC-tank, establishing a resonant frequency. The design ensured that any material passing through the tubing and the coil, possessing different electromagnetic properties than the surrounding medium, would inherently alter the resonance frequency of the LC-tank.

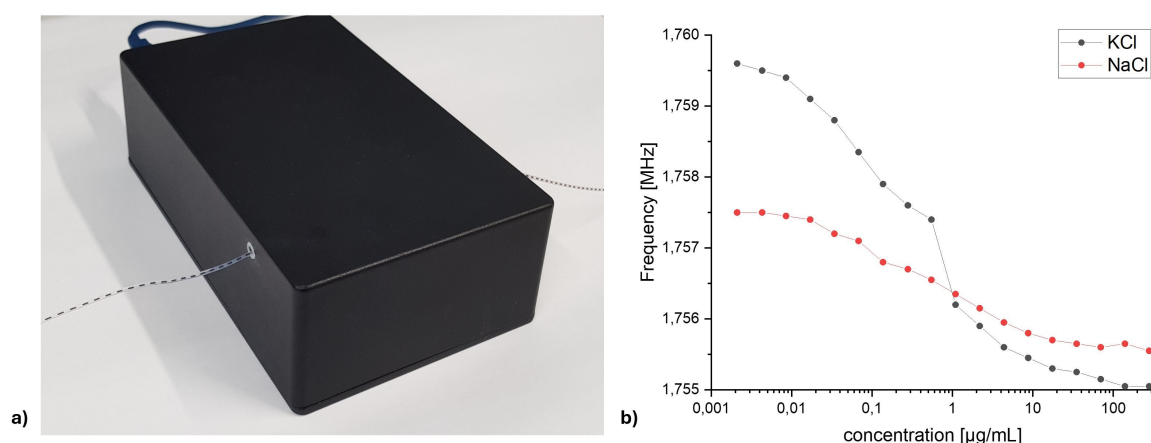


Figure 1: a) This image displays a custom-made MRD (Microfluidic Droplet Reader), where droplets in tubing pass through for analysis. b) The accompanying graph presents measurement data showing the response to varying concentrations of KCl and NaCl.

This shift in frequency, indicative of the sample's presence and characteristics, we could then read out using an oscilloscope. This approach offers a simple yet powerful means to sense changes in the sample's composition. Our tests using common ionic compounds, specifically Sodium Chloride (NaCl) and Potassium Chloride (KCl), yielded highly promising results. We determined the detection limit of this MRD setup for both NaCl and KCl to be 10 µg/mL. Notably, for these ionic salts, the MRD setup demonstrated a similar detection limit to that achieved for the nanoparticles in the previously discussed PHE setup. However, a significant distinction emerged: due to the inherently ionic nature of the salts, the resulting MRD signal

was substantially stronger compared to the signal from nanoparticles, which, in contrast, this particular MRD configuration could not detect. This highlights the MRD's particular strengths in detecting ionic or conductive species, while indicating specific applications where the PHE might be more suitable for magnetic nanoparticles without sensitivity to ionic contribution.

Millifluidic droplet analysis (MDA) setup: We realized a millifluidic setup as the core of this project. We designed this setup for the generation and subsequent analysis of millifluidic droplet sequences, each filled with bacterial suspensions. This setup drew inspiration from [4,5] adapting its principles to our specific research needs. In our experiments, we utilized fluorescent bacteria in conjunction with antibiotics and nanoparticles (NPs), which served as a stress factor. To achieve this, we established a millifluidic network (Figure 2). This network consistently generated over 400 individual droplets, each possessing a precise volume of approximately 100 nanoliters (nL). Following their generation, these droplets underwent continuous, real-time analysis. A finely tuned system accomplished this by shuttling each droplet back and forth through a dedicated detection area. The detection area itself received precise illumination from an optical fiber, which we directly coupled to a high-power LED light source. To ensure optimal excitation of fluorescent markers, we integrated an emission filter into the optical pathway. Complementing this, we focused a photomultiplier tube (PMT), equipped with a fluorescence filter, onto the detection area. As each of the 400 droplets traversed the detection area, the excitation light intermittently illuminated it. If appropriate fluorescent light was present within a droplet, the PMT accurately detected it, providing data for our analysis.

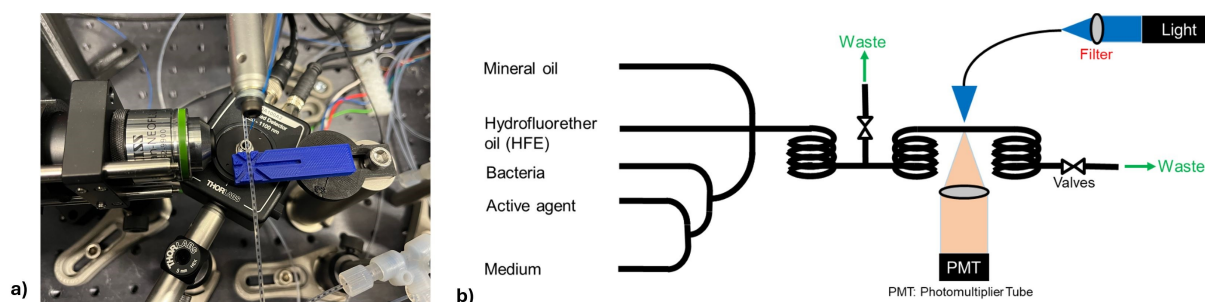


Figure 2: a) This figure depicts the Millifluidic Droplet Analysis (MDA) system. An objective lens focuses the light that the droplets emit onto the detector, which is a Photomultiplier Tube (PMT). b) This figure displays the fluidic network, including all tubing (represented by black lines) and the optical components for screening bacteria. All liquids enter the system on the left, and droplets form at the cross-junction before they move to the optical detection area.

We employed various fluorescent dyes as samples. These played a critical role in testing and calibrating the entire setup, ensuring its accuracy and reliability. Subsequently, we shifted our

focus to using fluorescent bacteria. We investigated these in combination with a range of different stress factors, including both antibiotics and silver nanoparticles (AgNPs), allowing for a comprehensive study of their effects on bacterial viability and response.

Testing with *Pseudomonas fluorescens*: During the initial phase of the project, we conducted tests using *Pseudomonas fluorescens*. These bacteria are naturally fluorescent, which made them an ideal and unrestricted choice for our early experiments. We cultured *P. fluorescens* cells in both their optimal nutrient broth and M9 media to assess their behavior under different nutritional conditions. We carried out all work involving *P. fluorescens* at their preferred temperature of 30°C. As a preparatory step, we counted cells to establish a correlation between cell concentration and a specific optical density (OD). Subsequently, we analyzed the cells' absorption and emission spectra using a commercial microplate reader. These data allowed us to equip our MDA setup with the appropriate optical filters to selectively detect the *P. fluorescens* fluorescence. However, during our experiments with *P. fluorescens* in the MDA system, we observed a significant challenge: their emission spectrum overlapped considerably with the emission of silver nanoparticles (AgNPs). This spectral overlap presented a hurdle for clear and distinct detection.

Testing with *Escherichia coli*: We experimented with *K12 E. coli*, which were modified to express a yellow fluorescent protein (YFP). The YFP exhibited an emission maximum at 535 nm, which is significantly distinct from the AgNP emission, typically around 400 nm. This substantial difference in emission spectra allowed us to achieve excellent optical filtering, ensuring that only the fluorescence that the *E. coli* YFP emitted was detected, minimizing interference from AgNPs. With these optimized optics, we generated droplets containing a low

concentration of *E. coli* YFP, specifically with an optical density at 600 nm of OD = 0.01. We continuously measured the droplets at a controlled temperature of 37°C. The entire droplet sequence took several minutes to pass through the detection area and be drawn back again, resulting in the measurement of each droplet every few minutes. Depending on the specific conditions, *E. coli* can have a doubling time ranging from 30 minutes to 1 hour. The initial

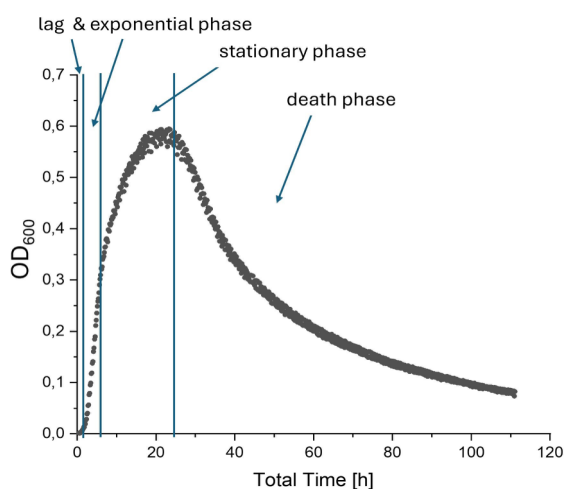


Figure 3: Exemplary measurement of one droplet out of 400 droplets measured for several days.

concentration of OD = 0.01 fell below the detection limit of our MDA setup, meaning the bacteria required a few hours to grow sufficiently and become detectable. The collected data showed a growth curve for *E. coli* YFP, illustrating the characteristic lag, exponential, stationary, and death phases of the bacterial growth. Figure 3 shows the signal of one droplet out of 400 filled with *E. coli* over the course of several days, highlighting stability of the developed fluidic setup.

Testing with antibiotics: We started our work program with exposing bacteria to antibiotics, which is a well-understood stress factor. This allowed us to evaluate and validate the functionality of the MDA setup. We tested several common antibiotics, including Cefotaxime (CTX), ampicillin, and streptomycin. A significant advantage of the MDA setup became apparent here: we could generate numerous droplets, each containing the same concentration of bacteria but with varying concentrations of antibiotics. This approach enabled us to create a precise gradient of stress conditions across a single droplet sequence. For example, Figure 4 illustrates an experiment with approximately 400 droplets containing CTX. The initial droplets in the sequence were free of CTX, and then we gradually increased the antibiotic concentration in a sigmoid-like manner, eventually reaching a maximum of 2 $\mu\text{g}/\text{ml}$. We developed a method for precisely determining the exact concentration of the stress agent within each droplet during our experiments.

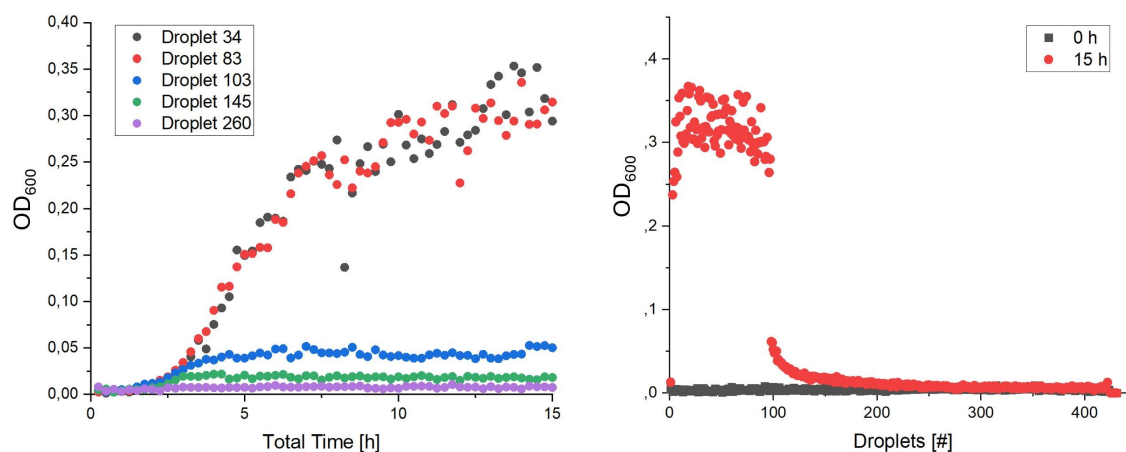


Figure 4: Example measurement with *E. coli* YFP and CTX.

Silver nanoparticle (AgNP) and silver nitrate (AgNO₃) experiments: One strategy for identifying an appropriate concentration range was based on dividing the entire droplet sequence into four to five distinct sections, with each section containing a different but fixed concentration of AgNPs. This allowed us to rapidly assess various AgNP dosages within a single experimental run. Subsequently, we adopted a gradient method, similar to that used

with antibiotics, for our AgNP experiments. This time, however, we combined the AgNPs with a fluorescent dye prior to droplet generation. Consequently, the very first scan of the droplets not only showed the expected low signal from the bacteria but also revealed a clear gradient signal originating from the dye and the AgNPs. Since this signal from the dye remained constant throughout the experiment, we could effectively subtract it from all subsequent data, isolating the bacterial fluorescence signal. The results of such an experiment are illustrated in Figure 5, left panel. After a 15-hour incubation period, only the first approximately 100 droplets showed a significant increase in the fluorescent signal, indicating bacterial growth. The remaining droplets, which contained higher concentrations of AgNPs, exhibited no signal increase, suggesting an inhibitory effect. By combining the information regarding the dye's distribution with the known AgNP concentrations, we were able to determine the minimal inhibitory concentration (MIC) of AgNPs for *E. coli* YFP in M9 media. In droplets with a higher concentration than the MIC, the bacteria no longer grew. We calculated the MIC value as 5.4 $\mu\text{g}/\text{mL}$.

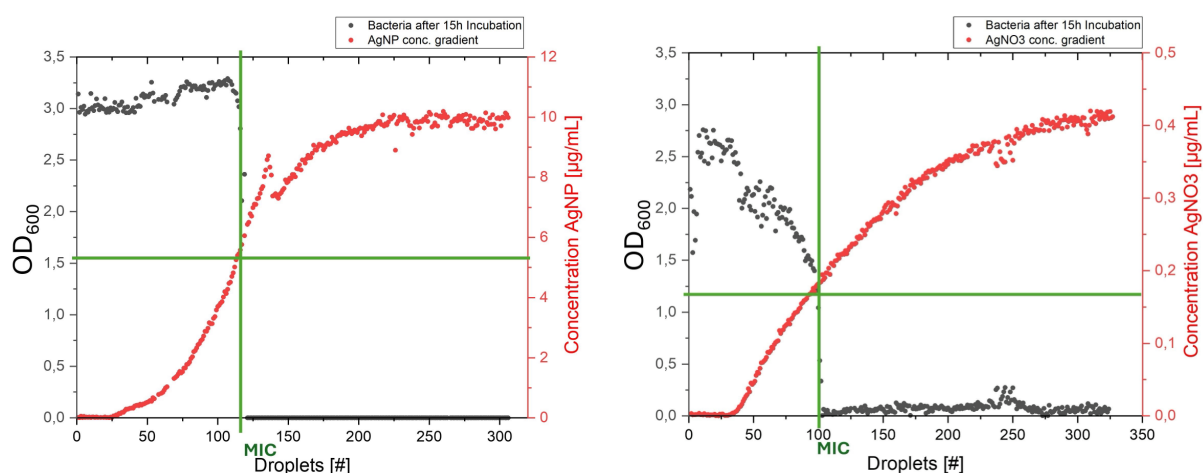


Figure 5: (left) An experiment to determine the MIC with *E. coli* YFP and AgNP in M9 medium. (right) An experiment to determine the MIC with *E. coli* YFP and AgNO_3 in M9 medium.

To gain a deeper understanding of silver's influence on *E. coli* and to provide a comparative baseline for our AgNP studies, we also utilized the well-known silver agent, silver nitrate (AgNO_3). When we add AgNO_3 to M9 medium, it readily dissolves, dissociating into nitrate (NO_3^-) and silver ions (Ag^+). The nitrate ions remain stable in the M9 medium and have only a negligible effect on bacteria. Silver ions (Ag^+), however, exert their well-documented toxic effect on bacteria. It is important to note that even though the silver ions are toxic, even when a significant portion of them will bind with the chloride ions present in the M9 medium, forming silver chloride (AgCl), which is less soluble. We performed a MIC experiment, similar to

the one performed with AgNPs, to directly compare their effects. Figure 5 (right panel) presents the results of this specific experiment.

References

- [1] T. S. Kaminski et al. *Droplet microfluidics for microbiology: techniques, applications and challenges*. Lab Chip 16, 2168 (2016). doi: 10.1039/C6LC00367B
- [2] J. Schütt et al., *Modular droplet-based fluidics for large volume libraries of individual multiparametric codes in lab-on-chip systems*. Adv. Sens. Res. 2, 2300101 (2023). doi: 10.1002/adsr.202300101
- [3] D. Karnaushenko et al. *Monitoring microbial metabolites using an inductively coupled resonance circuit*. Scientific reports 5, 12878 (2015). doi: 10.1038/srep12878
- [4] L. Baraban et al. *Millifluidic droplet analyser for microbiology*. Lab Chip 11, 4057 (2011). doi: 10.1039/C1LC20545E
- [5] X. Zhao et al. *Coexistence of fluorescent escherichia coli strains in millifluidic droplet reactors*. Lab Chip 21, 1492 (2021). doi: 10.1039/D0LC01204A

4 Published Project Results

4.1 Category A – Articles in peer-reviewed journals, contributions to peer-reviewed conferences or to anthology volumes, and book publications

1/ publication

[4.1.1.A] (open access publication) J. Schütt, H. Nhalil, J. Fassbender, L. Klein, A. Grosz, D. Makarov, “Modular droplet-based fluidics for large volume libraries of individual multiparametric codes in lab-on-chip systems”, Advanced Sensor Research 2, 2300101 (2023). DOI: 10.1002/adsr.202300101

2/ peer reviewed conferences

[4.1.2.A] (contributed talk) S. Schuba et al., “Evaluation of nanoparticle influence on living microorganisms”, DPG-Frühjahrstagung der Sektion Kondensierte Materie, 18.-19.03.2024, Berlin, Germany

[4.1.2.B] (poster presentation) S. Schuba et al., “Evaluation of nanoparticle resistance development of microorganisms”, DPG-Frühjahrstagung der Sektion Kondensierte Materie, 26.-31.03.2023, Dresden, Germany

[4.1.2.C] (invited talk) D. Makarov et al., “Flexible and printed electronics: from interactive on-skin devices to bio/medical applications”, Joint European Magnetic Symposia (JEMS), 24.-29.07.2022, Warsaw, Poland

4.2 Category B – Any other form of published results

The project results were communicated to the public in the frame of the following events:

1/ workshops and summer schools

[4.2.1.A] (poster presentation) S. Schuba et al., “Evaluation of nanoparticle influence on living microorganisms”, Seminar of the Institute of Ion Beam Physics and Materials Research, Schmochtitz 2024, 25.-26.06.2024, Schmochtitz, Bautzen, Germany

[4.2.1.B] (invited talk) S. Schuba et al., “Antibacterial effect of nanoparticles”, Summer school “Smart Devices and Their Applications” organized in the frame of the EU project BioNanoSens. Dresden, Germany. June 5-7, 2023.

[4.2.1.C] (contributed talk) S. Schuba et al., “Optical analytics of nanoparticles in liquids”, Priority Training School, Recent Trends in Microplastic research, 24.05.2023, HZDR, Dresden, Germany

[4.2.1.D] (invited talk) D. Makarov et al., “Intelligent Materials and Systems”, Seminar at the Central European Institute of Technology (CEITEC), 03.11.2022, Brno, Czech Republic

[4.2.1.E] (poster presentation) S. Schuba et al., “Evaluation of nanoparticle resistance development of microorganisms”, HZDR DocSeminar 2022, Wrocław, Poland, 19.-21.10.2022, Wrocław, Poland

2/ public events

[4.2.2.A] Participation in the demo stand of the Helmholtz-Zentrum Dresden-Rossendorf e.V. highlighting the possibilities of fluidic technologies in biology and medicine. Dresden Science Night 2023. Dresden, Germany. June 30, 2023.

[4.2.2.B] Participation in the demo stand of the Helmholtz-Zentrum Dresden-Rossendorf e.V. highlighting the possibilities of fluidic technologies in biology and medicine. Dresden Science Night 2022. Dresden, Germany. July 8, 2022

4.3 Patents (applied for and granted): there are no patents or patent applications.