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Executive Summary

For the purpose of preparing the D2.1 -- Internal literature review report on SoA, the deliverable report was divided into the following sections: summary, introduction, methodology, topics, conclusion and references. The topics section was divided to the following subsections: basic principles of digital holographic microscopy (DHM), technology implementation: from prototype to industrial applications, CBRN standards and protocols, CBRN bioterrorism, application of machine learning methods in digital holographic microscopic image analysis, microorganism detection limits, cell identification by DHM, bacteria size and shapes, sampling methods, exploitation in medicine, supplementary modalities, microscopy contaminants, emerging technologies on bio detection, user needs and technology implementation. In order to prepare a comprehensive analysis, the involved consortium members collected the relevant literature positions concerning each of the above mentioned topic sections and placed the link in the Zotero platform. While preparing the comprehensive analysis of each topic, the following needed to be avoided: wrong study design, the study not relevant to the scope of the deliverable, not original study, not available in English, insufficient data in the study, limited data, no full text available, the scope of performed study not clearly described. On the basis of the literature inclusion criteria, the relevant literature positions were chosen in order to prepare the topic reviews.

Introduction

The HoloZcan project brings a new tool for security actors (police, relief workers, disaster managers, crisis managers, stakeholders responsible for public safety, critical infrastructure, and service providers) notably in the fields of autonomous detection and response capabilities.

The project increases (environmental and exhaled) bio-aerosol sensing/measurement capability of CBRN practitioners by developing a high resolution, large throughput, automatic and highly portable detection system for making automatic classification of pathogens and particles. HoloZcan develops of a novel holographic microscopy and imaging technology for rapid and cost-efficient screening of potential biological threats and unknown, potentially dangerous substances, combined with methods of artificial intelligence and machine learning. It establishes a framework of a dynamic feature selection and validation algorithm to support the continuous innovation capability of the system in the field of adaptive learning and database optimization for specific bioinformatics applications. The project also develops comprehensive and innovative means of respiratory, ventilation and environmental biological data sampling that can be used in a real-time, standoff or in mobile bio-detection context.

Airborne diseases can be transmitted by a wide variety of infectious pathogens, such as variola virus, measles virus, influenza A viruses, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae* or *Bordetella pertussis* [1]. Some other pathogens that can be transmitted by this route are not very efficient in human-to-human transmissibility, but present high concerns because of their potential as bioterrorism agents, as *Francisella tularensis* or *Coxiella burnetii* (CDC, USA, 2021; [1], or to produce outbreaks due to inhalation of contaminated aerosols, such as *Legionella pneumophila* (WHO, 2021). Bioaerosols can be also comprised by fungal spores and fungal hyphae, pollen grains and other biological material [2]. The emergence of coronaviruses able to cause severe respiratory diseases, as SARS-CoV and MERS-CoV, and the most recent pandemic SARS-CoV2 identified in December 2019 [3], placed the airborne diseases at the centre of the attention.

The D2.1 -- Internal literature review report on SoA is a deliverable prepared by 2.1 Task Leader (University of Lodz) with the following consortium members participation (Sioux, DMI, Ideas Science, Institut Pasteur, Zug Med, Datasenselabs, Polimi) under the supervision of WP2 (Biohazard classification system development) Leader (Sioux). The deliverable objectives were aimed to describe the work done in the previous 4 months in order to provide an essential and extensive literature review. The aim of D 2.1 -- internal literature review report on SoA is to provide comprehensive information on the base of literature review in the field of the state of the art in methods of biohazard classification and system development. To provide a full picture of the literature review, the analysis was divided into subsections, where dedicated literature positions were placed. The following subsections (Table 1) were created:

I. Basic principles of Digital Holographic Microscopy (DHM)

II. Technology Implementation: from Prototype to Industrial Applications

III. CBRN standards and protocols

IV. CBRN Bioterrorism

V. Application of Machine Learning Methods in Digital Holographic Microscopic Image Analysis

VI. Microorganism Detection Limits

VII. Cell Identification by DHM

VIII. Bacteria Size and Shape

IX. Sampling Methods

X. Exploitation in Medicine

XI. Supplementary Modalities

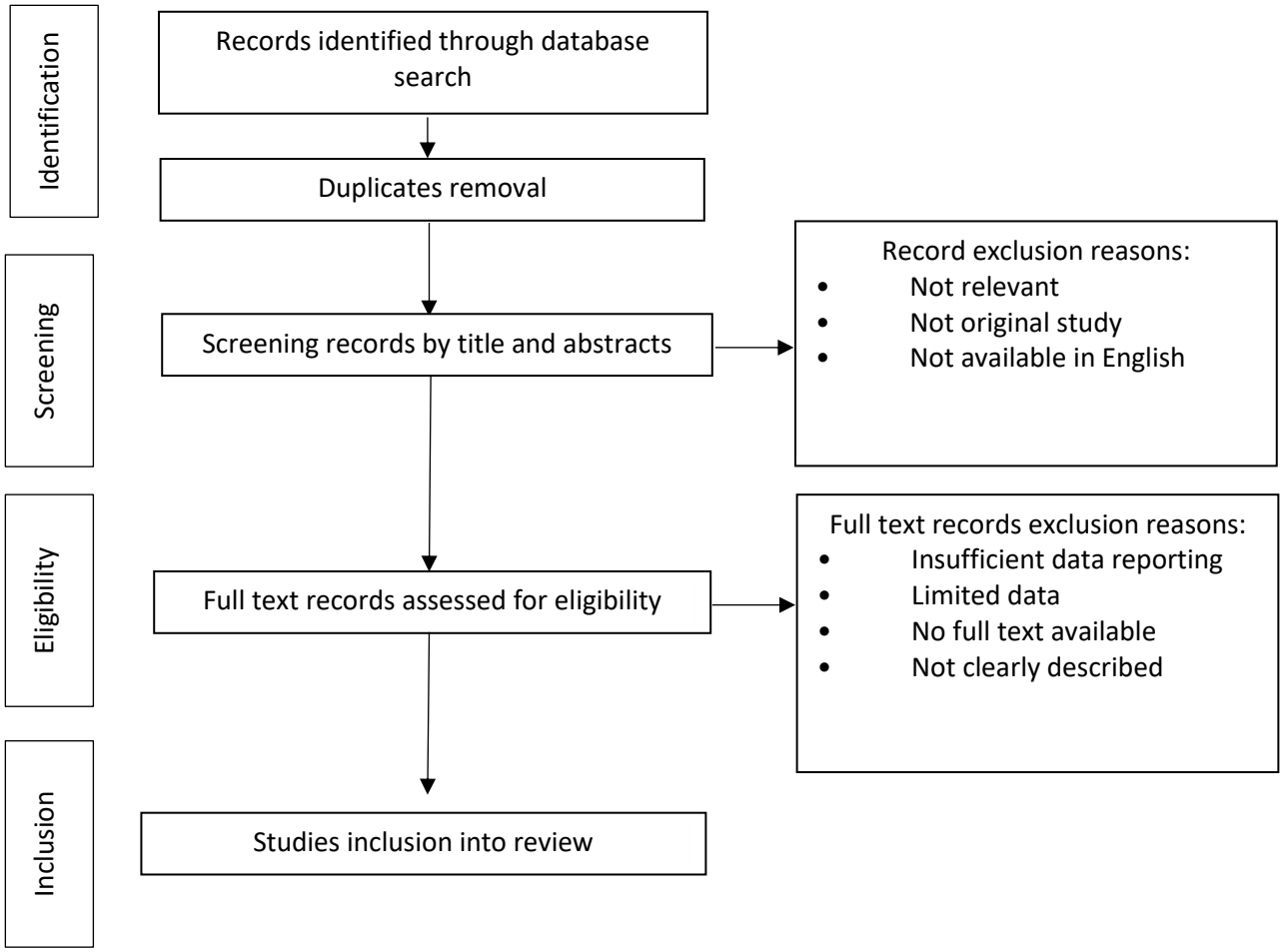
XII. Microscopy Contaminants

XIII. Emerging Technologies on Biodetection

XIV. Users' Needs and Technology Implementation

Methodology

For the purpose of this survey, 961 literature positions were chosen and placed in the Zotero platform (https://www.zotero.org). The Zotero platform was used as a tool that allowed the gathering and pairing of links to the discovered literature positions and was used for creating the D 2.1 deliverable list of references. Before final inclusion as reference, the following scheme was used. Using the above-mentioned and below illustrated scheme, the final list of references consisting of 177 literature positions was used for preparing the comprehensive literature analysis.



Topics

I. Basic principles of Digital Holographic Microscopy (DHM)

Digital holographic microscopy (DHM) is one of the comparatively new technologies in the field of high-resolution microscopy. Substitution of conventional holography photochemical procedures with electronic imaging creates the opportunity to utilize microscope visualization in a wide range of possible applications from other fields e.g. microbiology, medicine. The holography concept is not new and has been known for decades, however has not been used due to mainly technological imperfections. It is built on the holography concept, which was invented in the 1940s. In 1948, the technology inventor Denis Gabor received a Nobel Prize for his work [4] His primary effort was to improve the electron microscopy resolution due to the technical difficulty in the correction of electron lenses. During his work, he dispensed the electron imaging lens and figured out that the electron pattern diffraction beam possesses complete information concerning the amplitude and phase of the electron wave. As a result of this, the electron wave diffraction record can be applied to optically synthetize the observed object. Nowadays, the progress in the field of processing and acquisition of digital images enables the advancement of DHM in comparison to other techniques in image acquisition. Furthermore, the increased capability of current computers in numerical processing provided the DHM superiority in regard to robustness, measurement rates and ease of use. As the principles, in DHM, the pattern of holographic interference is generated optically by a reference beam and object superpositioning and then digitally sampled by charged coupled camera (CCD) and retrieved in a computer as array numbers – numerical reconstruction of analyzed image. DHM possesses two separate modes, which allow to analyze the sample in reflection or transmission mode. The transmission mode is particularly useful in biological samples analysis and has no analogy in another interference microscopy. The basic setup of DHM consists of 4 main elements: Illumination source, digitalizing camera, interferometer, and a computer equipped with dedicated software. As for the illumination source, currently the laser (helium–neon, diode, diode pumped, argon, neodymium-doped yttrium aluminium garnet, tunable dye, sapphire femtosecond lasers) can be used [5]. In order to obtain multiwavelength, the multiple-laser-light should be applied. As for the interferometer, two main types can be used in the DHM technique. The first one is the Michelson type, with reflecting objects and Mach-Zehnder type for transmissive objects. In both cases, the object is illuminated by plane wave and the reference is placed in CCD. The Mach-Zehnder interferometer needs more elements but offers more alignment flexibility. Both interferometers can be equipped with attenuators and optics, which can be managed through the reference and ration of object intensity and with polarization optics, which allow image birefringence. Modulators can also be part of the set. Modulators such as: electro-optic, acousto-optic, piezo-mounted optics, liquid crystal phase allow to modulate the received signal. As an open set, the DHM can be equipped as needed with a lensless Fourier kit useful in object magnification, however, obtainable magnification is limited and relay on magnification is achieved by microscope lenses [6]. The set can also be equipped with other lenses, which can be applied in matching the object curvature and in wavefronts reference. The CCD and recently introduced Complementary Metal Oxide Semiconductor sensor (CMOS) camera are part of the DHM set. Both are used to capture the obtained interference pattern and digitize them. Usually, the device pixel size range is several microns and counts to 10002 [7]. These parameters are the main limitation in DHM resolution, however the technological progress in the field of camera technology will definitively broaden the range of DHM applications and improve their performance. The digitized interference

pattern is transferred to a computer as a 2D array of integers with grayscale resolution. The computer performed numerical diffraction results in a holographic image of 2D numbers. Further computing tasks are: the pre- and post-processing of the images, the rendering and storing of the images. The common characteristic of DHM is the 3D content of the image. In this technique in order to provide 3D content, single hologram is used to recreate the optical field at all distances from hologram. In simple terms, the image is calculated at certain distances and then congregated into one clip as a result of computing images with adjusting the distances from the particular parts of an object. In comparison to conventional microscopy where the focus is placed on the object surface, which is fixed and further recorded, however the information concerning the observed object, which is placed apart from the focus area will be totally lost. When comparing DHM with interference microscopy, the major differences are as follows: The surface topography in DHM is obtained by single acquisition, mechanical or spectral scan is not required, in comparison to coherence scanning interferometry (CSI), where the sample vertical scan is needed or to phase-shifting interferometry (PSI), where certain image acquisition process of sample placed in a different position is required [8]. The fact that in the case of DHM the scan is not needed , is a vast superiority in the field of robustness against vibration. Furthermore, the image acquisition is performed very rapidly and is only dependent on the CCD camera refreshing rate. This allows to capture the hologram using computer and enables at least 20 frames per second (fps) reconstruction of at least 1024x1024 pixel values [5]. The above mentioned features make it possible to use DHM as a tool in dynamic analysis and in quality control processes. Moreover, the single acquisition characteristic together with pulsed laser sources facilitate stroboscopic imaging and enables displacement measurement. In the field of light sources, and specifically in the DHM technique, the light sources can be combined and thus offer certain micrometers of vertical measurement range with same nanometer resolution. In order to compare DHM with analog microscopy, the following features should be taken into account. In DHM, the photochemical process is not involved, which results in faster magnitude and an ability to present video rates. In the basic DHM set, the required items are only the CCD and computer in comparison to analog microscopy, where the darkness and reagents are necessary. CCD also provides very high sensitivity resulting in reduction of exposure time by orders of magnitude. The short time required to exposure in the DHM set is also reducing the necessity of set stability in comparison to analog microscopy, so boards, where the set is placed are not required. As for pros and cons, the DHM is characterized by low resolution, where the analog microscopy is better by two orders of magnitude. Furthermore, the spatial frequency is limited to only a few degrees in angular size, while in analog microscopy the full range (1800) is achievable [8]. In the field of wavelengths, in analog microscopy, numerous holograms are produced, so the perfect image requires to be illuminated by diverse wavelengths, which is impossible. So, in most cases, the object is illuminated only by a single wavelength resulting in creation of multiple image abbreviations. In the case of DHM, the superposition requires only a few numerical arrays [7]. In DHM, the arrays are not limited, resulting in the ability to compensate existing image aberration. Furthermore, there are other methods to compensate image deformation. As an example, the matched reference wave and multiwavelength optical phase unwrapping enables precision (in nanometers) phase imaging without any difficulty in comparison to widely used software-based unwrapping technologies. This is important, especially in microorganism microscopy. In conclusion, in this field, DHM can offer advantages by furnishing a label-free, minimally invasive and particularly sensitive technique of imaging exquisite shifts in the physiological and physical states of microorganisms and their properties.

II. Technology Implementation: from Prototype to Industrial Applications

The requirements for prototypes and a mass producing systems can be quite different. For example, for prototypes, the flexibility of the setup is often important, for industrial use repeatability is key. The following properties are typically different between a prototype and a productized version:

- Cost of materials
- Use of standard parts
- Calibration procedure (speed, ease of use)
- Acquisition Speed
- Repeatability of measurements

These points can be addressed from the management and from the technical perspective. Both from the field of manufacturing and from the field of software development generic methodologies have been developed.

Project Management

The Design for Manufacture and Assembly (DFMA) methodology [9] [10] covers mostly these two items from the list above. Although this is typically aimed at far bigger systems than an optical microscope, the general line of thought is applicable.

The lean manufacturing approach [11] has broader coverage. It describes not only the productization of a single product but also the subsequent refinement for follow-up models. Although lean is aimed at large corporations and complex systems, the core concepts apply to smaller systems as well. The 5 principles of lean are:

- Identify the value (for the customer)
- Map the value stream, trace production steps to identify waste
- Flow, make sure the production runs smoothly, without interruptions
- Pull, only make when requested, “just in time”.
- Perfection. Continuously iterate and improve on both the product and the process

A development model from the software world is the V-model [12]. The key concept is to alternatively go from high-level definition to low-level implementation, and then back up from module testing to system testing.

Technology

The technical perspective on productization focuses on the last three properties of the list at the top of this section. Industrial calibration procedures are typically not published. In case of TEM microscopes some information is available [13]. This again covers a far more complex system than this project intends to build, but a few observations are relevant.

- Calibration software should be documented and have tests
- The calibration procedure should be automated to achieve reproducibility

The general observations on calibration procedures are made [14]: Although it is convenient to start with approximations. This both hinders robustness and accuracy of the result. This suggests that for a good calibration procedure, a good simulation of the physics is as well required. In the prototype phase, it

is not necessary to get the highest system throughput. For the final product this is important. The V-model [12] gives a handle, on the way to approach this. In multiple iterations of simulation and validation an accurate model can be developed. Maximizing the acquisition speed becomes an optimization problem, in which key system parameters such as the light source can be adjusted without necessity to build it. The repeatability of measurements is important for all use-cases, which require a quantitative results. Although, there is overlap with calibration. For example, the mechanical stability of the system also comes into play. No specific literature on this topic was found, but the general approach of the V-model holds it. By iteratively building and refining a simulator, misconception analysis of all the system parameters can be performed. This can be used to identify the main limitation of the system measurement reproducibility. Besides design considerations, also the performance affects reproducibility and reliability. OnSemi describes best practices for handling image sensors in one of their application notes [15].

Overview of existing designs

Different digital holographic microscope (DHM) implementations are overviewed. We picked up twenty designs well representing by the diversity of models. We divided them into three major groups: (1) research, (2) simple, and (3) product groups.

In the "research" group we can find the most advanced designs, however, these devices are not well tested under different conditions. In many cases the advancement in resolution is based on well-known optical rules, thus reproducible [16] [17] [18] [19]. In some cases mathematical tricks are used, thus usability in our scenario should be tested [20] [21] [22]. Some methods are purely usable only in a controlled lab environment [21] [23].

In the "simple" group we find solutions, which are made mainly for science purposes and optimized for price availability. All of them using 3D printed cases or plastic prototype boxes. All of them follow the simple in-line architecture without lens, mono wavelength light source, and provided reconstruction algorithms with no real super-resolution capabilities.

All devices in the "product" group have off-axis architecture. While off-axis models need more precision and have more places, where we can lose resolution, the design allows us to analyze dense samples (oppositely; in-line holography is good only for sparse sample imaging, where we searching for point-like objects on the picture, and the remaining part is empty). All level products devices have one wavelength or dual-wavelength light source. Resolutions are defined as raw value without super-resolution algorithm applied. These devices probably possess extra software/hardware modules, which using the sample displacement method in order to achieve sub-pixel shifts. They are capable to increase their resolution above the raw parameter. However, these features are publicly not presented.

RESEARCH

Name & Ref.	Year	Light Source	Image Sensor	FOV	Architecture	Super-resolution	Lateral resolution	Notes
Field-portable reflection and transmission microscope [24]	2011	331nm LD + 3um pinhole	2.2um 5MP MT9P031	3.4 x 2.6mm	Off-Axis In-line, 1.67x magnification	No	2um	
Fiber Optic Array On-Chip DHM [16]	2011	23 piece 633nm LED via 105um fiber optic cables (BW 20nm)	2.2um MT9P031I12STM;	24mm2	In-line lensless	Light position shift	800nm	An array of LEDs place above the sample and blinked sequentially
Increased space-bandwidth DHM [20]	2013	372, 470, 480, 530nm LED	6.8um / 1.12um	18cm2 / 20mm2	In-line lensless	PSF + deconvolution	1.74um /225 nm	Image is up-sampled and deconvoluted
Gigapixel nanoscopy on Chip [21]	2013	550, 372nm LED	1.12um	20mm2	In-line lensless	PSF + deconvolution + microlens	300nm /225nm /<100nm	Self-assembled micro-lens made on top of small objects with immersive oil
Tunable Vapor-Condensed Nanolenses [23]	2014	480 nm LD BW=3nm +monochromator (Newport 74100)	1.1 um	20mm2	In-line lensless ~300um distance	PSF + deconvolution + microlens	20nm	Micro-lens formed from PEG material evaporated and tuned with heat regulation
Synthetic Aperture On-Chip DHM [17]	2014	700 nm (+ 472, 532, 632nm demo)	1.12um	20mm2	In-line lensless	LISA	250nm	Variable-angle illumination and aperture synthesis
Wavelength Scanning Superres DHM [18]	2015	supercontinuum laser 480-513nm	1.12um Sony IU081	20mm2	In-line lensless 100-500um above the lens	Wavelength scanning and multi-height method	250nm	The method uses result summation iterative refinement with forward and backward projections
High numerical aperture holographic microscope [25]	2015	785nm, 50mW LD	6.7 um 1280 x 1024 PCO Pixelify	90 x 90um	Off-Axis with lens 60x	No	~300nm	Olympus CX41 microscope is modified. The sample measured in oil
2π-DHM [22]	2013	405nm LD	1280x960 Mono 8 Basler 102f	50 x 50um	In-line with lens 63x	Synthetic aperture	90nm	

ONR SI-DHM [26]	2019	633 nm He-Ne laser	Basler acA3800-14 um CMOS sensor 512x512	710 x 710um	In-line with lens 10x / 0.25NA	Structured Illumination	755nm	3D-printed frame
NSF DHMIC [27]	2017	HeNe laser / 635nm LD	5.2um 8MP 8bit DCC1545M /1.4um cellphone	104um / 260um	In-line with lens, 40x	No	900nm /1.2um	Optical breadboard / 3D-printed frame
3D Brownian Tracking DHM [19]	2015	532 nm Nd-YAG, 80mW	4.4µm 12bit 1626 x 1236 Basler acA1600	340 x 260um	In-line with lens, 20x	For localization	~500nm 2nm position	Olympus IX71 microscope is modified

Table 1: Overview of existing designs (Research)

SIMPLE

Name & Ref.	Year	Light Source	Image Sensor	FOV	Architecture	Super-resolution	Lateral resolution	Notes
Teph12/DIHM [28]	2019	430m, BW=15nm + 3um optical fiber and 405nm LED, 150mW + 15um pinhole	1.12um 3280 x 2464 color IMX219	3.6 x 2.7 mm	In-line, lensless	No	1.55um 3.9um	3D-printed frame
Unal DLHM [29]	2020	650nm LED, 5mW with lens	2.2um 2592x1944 Aptina MI5100	3.56, 1.34, 0.61 mm2	In-line, lensless	No	3 um	3D-printed frame
HuangXiwei lensless chip [30]	2020	587nm + 100um pinhole	1.12um 3280 x 2464 color IMX219	3.6 x 2.7 mm	In-line, lensless	Iterative Mudanyali et al.	<5um	0.5mm glass glued to image sensor
Hand-held low-cost DHM [31]	2012	470, 525, 625nm RGB LED, 350mW + 5um pinhole	1920x180 color Logicool C910 webcam	Not defined	In-line, lensless	No	17.5um	OpenCV used for camera, and CWO++ library was used for the reconstruction

Uconn shearing DHM [32]	2017	635nm LD	1.67um 3840 x 2794 Basler acA3800-14um, 2784	270 x 195 µm	Sharing interferometer 40x ocular	No	1.1um	Used for augmented reality view

Table 2: Overview of existing designs (Simple)

PRODUCT

Name & Ref.	Year	Light Source	Image Sensor	FOV	Architecture	Super-resolution	Lateral resolution	Notes
Holomark HO-DHM-UT01 [33]	2017	650nm LD, 5mW	2.4um 3088 x 2076 IMX178LQJ-C	185 x 124um	Off-Axis +lens 40X, 0.65 NA	Only with extra module	1um	
Schaefer Italy / LynceeTec DHM T2100 [34] [35]	2017	666nm, 794nm 0.01nm/°C 1µW/cm2	Not defined	66x66 µm 5x5 mm	1.25x to 100x	Only with extra module	300nm	
Essencedesign DHMS1 / Trimos TR SCAN 3D DHM [36]	2020	665nm, 850nm	Not defined	250x250 µm	Off-Axis +lens 10x	Only with extra module	500nm	

Table 3: Overview of existing designs (Products)

III. CBRN standards and protocols

A standard is a document, established by consensus and approved by a recognized body, that provides, for common and repeated use, rules, guidelines or characteristics for activities or their results, aimed at the achievement of the optimum degree of order in a given context [37].

European Standards (ENs) are documents that have been ratified by one of the three European Standardization Organizations (ESOs), CEN, CENELEC or ETSI [38]

Type of standard	Definition
Basic standard	Wide-ranging coverage or contains general provisions for one particular field.
Terminology standard	Concerned with terms, accompanied by their definitions etc.
Testing standard	Concerned with test methods, sometimes supplemented with other provisions related to testing.
Product standard	Specifies requirements to be fulfilled by product or group of products, to establish its fitness of purpose.
Process standard	Specifies requirements to be fulfilled by a process, to establish its fitness of purpose.
Service standard	Specifies requirements to be fulfilled by a service, to establish its fitness of purpose.
Interface standard	Specifies requirements concerned with the compatibility of products and systems at their point of connection.
Standard on data to be provided	Contains a list of characteristics for which values or other data are to be stated for specifying the product, process or service.

Table 4: Type of standards (defined in ISO45020)

Standardization activity of establishing, with regard to actual or potential problems, provisions for common and repeated use, are aiming to achieve the optimum degree of order in a given context. In particular, the activity consists the processes of formulating, issuing and implementing standards [37]

Why standardization is needed for bio-detection, security and in CBRN field? A number of EU policy statements stress the need to harmonize disaster preparedness in Europe in order to improve resilience [39] [40]. Agreed standards and technical guidance are needed to provide the necessary tools to deal with current and future crises. A large-scale, major CBRN event may require cross-border and interdisciplinary cooperation, where effective interoperability is vital. European standards can ensure that, the same specifications are adopted in all Member States, supporting the coordination of national responses. Harmonization of terminologies, methodologies, testing and evaluation procedures, data formats and measurement methodologies and the services can enhance detection capabilities and reduce costs.

In the CBRN field, most Member States use different standards, which are a barrier to achieving interoperability. To achieve a common understanding of terms related to CBRN area, CEN Technical committee TC/391 with Working Group 2 -CBRNE developed the guidance [41]. The EU CBRNE Glossary includes approximately 820 entries, prepared and reviewed by a panel of EU member states experts in the different areas. It is available in 24 languages and is also accessible via the online EU portal. EN ISO 22300:2018 'Security and resilience – Vocabulary' has been also developed and will be updated this year [42]

The testing and evaluation of systems (bio detectors) were developed to detect biohazardous substances in order to better enable inter-comparison of results across all instrument types. It is challenge for government agencies and industries involved in biosecurity programs [43]. There are no European or International standards in the area of testing and evaluation of Biological Threat Detection and monitoring systems and only very limited coverage in other standards (e.g. ASTM standard) ASTM WK46895 "New Specification for Standard Specification for Nucleic Acid-Based Systems for Bacterial Pathogen Screening of Suspicious Visible Powders" standard is specific to nucleic acid-based technologies and only addresses bacterial pathogens, not viruses or protein toxins [44].

SPADA (Stakeholder Panel on Agent Detection Assays - SPADA) established by AOAC (Association of Official Analytical Collaboration International) has produced performance standards (Standard Method Performance Requirements - SMPRs) for the 10 most important biohazard pathogens. These standards are aiming to help method developers in studies design and users in evaluating potential studies. This SMPRs describe minimum performance requirements set by the world's most respected experts.

Standardization in the area of performance requirements is complicated by the fact that there is no single ideal sensor sensitivity for the detection of CB pathogens. Most of them depend on acceptable false-positive rates and other detection properties. Sensors with different sensitivities can play a useful role in the control of CB pathogens. The main challenge related to biological threats is the ubiquity of naturally occurring biological agents in the environment and the high level of complexity due to the diversity of biological agents.

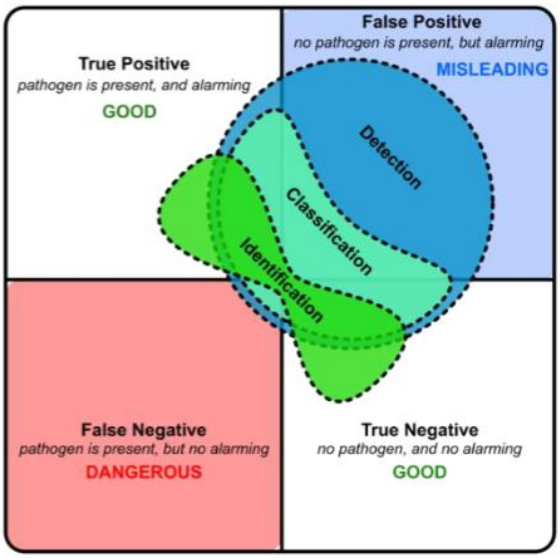


Figure 1: Bio-detection performance assessment matrix

Carrano [45] proposed that, the sensors should be evaluated with Receiver Operating Characteristic (ROC) curves. ROC curves capture the performance trade-off among sensitivity, probability of detection, and false-positive rate. It implicitly captures the performance trade-off with regard to response time. Besides these key sensor metrics, the report suggests to include additional attributes such as unit, operation cost, maintenance, reliability, size, weight and power consumption, which can be presented in spider charts.

There are NATO operational standards that can be used in crisis situations: NATO AEP 66, AJP 3.8 STANAG 2352, AEP-10, AEP-66, but there is a lack of operational protocols for equipment (deployment protocols) or standardized biological threat analysis and reporting procedures. Available standards in the CBRN field are outdated and sometimes irrelevant (ENCIRCLE report, September 2020).

It is also important to create the conditions for interoperability in the field of sampling. In this field, a number of sampling standards have been developed for environmental reasons but they are not applicable for security and CBRN sampling. (International standardization documents, ISO 16000-16: 2008 and ISO 16000-18: 2011) specify, respectively, the requirements for long-term (0.5h to several hours) indoor sampling of molds by air filtration (ISO 16000-16, 2009) (97) and the requirements for short-term (1–10 min) indoor sampling of molds by air impaction on solid agar media (ISO 16000-18, 2011).

Standardized procedures, such as the EN 45000 series (includes requirements for laboratory settings, analytical techniques, analytical performance criteria) can be appropriately applied in sectors such as environment or health, but these procedures can only be applied to a limited extent as quality assurance and quality control rules in the CBRN field (e.g. biological, natural hazards such as climate hazards and pandemics or terrorism).

Standardization is well advanced in the areas of risk assessment and biosafety and biosecurity planning. There are few unified guidelines for biosafety. For example, the BMBL and WHO Laboratory Biosafety Manual. Sections II and VI of the BMBL provide a good foundation for risk assessments and the development of biosafety and biosecurity plan. The threat of biological attacks, infectious agents can be located in multiple locations and therefore industry standards would be needed. In their absence, it is important that institutions pay attention to overall bio risk management [46].

ISO 35001, Bio risk management for laboratories and other related organizations, is the first International Standard for bio risk management system. It defines the requirements and guidance for laboratories or any other organization that works with biological agents in order to control and reduce the risks associated with their use. ISO 31000:2018 provides guidelines on managing risk faced by organizations. The application of these guidelines can be customized to any organization and its context.

Pre-standardization is strongly recommended for research projects and technology developers at an early stage of design. The STAIR4SECURITY project created a pre-standardization enabling platform and governance system designed to support the coordination, research and innovation and standardization for preparedness and response in the area of Disaster Resilience. But the results and experience of other EU projects can also be used in the pre-standardization process. Results and experience from previous H2020 projects such as RESISTAND, DRIVER+,IN-PREP, RECONASS, IDIRA, ZONeSEC and INACHUS can be also exploited to understand standardization challenge.

CEN/CENELEC are actively coordinating the standardization process. There are several CEN/CENELEC and ISO/IEC Technical Committees where the topic of detection can be raised. CEN/CENELEC/JTC 4 Service for fire safety and security systems

- CEN/CENELEC/JTC 13 Cyber security and data protection
- CEN/TC 325 Crime revention through building facility and area design

- CEN/TC 391 Societal and citizen security –
    - CEN/TC 391/WG 3, Crisis management/civil protection, T
    - CEN/TC/391/Working Group 2 -CBRNE
- CENELEC/TC 79 Alarm systems
- CEN-CLC Sector Forum on Security
- ISO/IEC/JTC 1/SC 27 Information security, cybersecurity and privacy protection
- ISO/TC 262 Risk management
- ISO/TC 292 Security and resilience
- ISO/TC 283 Occupational health and safety management
- ISO/TC 329 Consumer incident investigation guideline

In 2021, CEN and CENELEC will create a constructive dialogue among all stakeholders on standardization in the defense sector, in full transparency with the European Defense Agency and the NATO Standardization Office (annual plan, CEN/CENELEC).

NATO AEP 66, AJP 3.8	ALLIED JOINT DOCTRINE FOR CHEMICAL, BIOLOGICAL, RADIOLOGICAL, AND NUCLEAR DEFENCE	Provides North Atlantic Treaty Organization (NATO) strategic and operational commanders with fundamental principles for the planning, execution, and support of NATO operations for which the threat and/or risk of intentional or accidental use of chemical, biological, radiological, and nuclear (CBRN) substances are either assessed or exist
STANAG 2352	Chemical. Biological. Radiological And Nuclear (CBRN) Defense Equipment - Operational Guidelines	To provide a NATO agreed document detailing CBRN defense equipment requirements based on the main threats from CBRN weapons or devices and potential hazards originated (resulting) from toxic industrial materials (TIM) within operational area in general and deployed NATO forces in particular.
AEP-10	Allied Engineering Publication 10: NATO Handbook for Sampling and Identification of Biological and Chemical Agents (Procedures and Techniques)	Development of a capability for the timely sampling and unambiguous identification of biological, mid-spectrum and chemical warfare agents in a battlefield environment or in operations other than war. The aim is to confirm their first use by the enemy and thereby support timely politico-military decisions concerning an appropriate NATO response to such actions
AEP-66	Allied Engineering Publication 66 Edition 1: NATO Handbook for Sampling And Identification Of Biological, Chemical, And Radiological Agents (SIBCRA	NATO Handbook for Sampling and Identification of Biological Chemical and Radiological Agents (SIBCRA) and of the CBR FORENSIC SAMPLING TRAINING CONCEPT (FRTC) are important tasks which were identified by the JCBRN Capability Development Group (CDG) and passed on to the subordinate CDG panels.
RFI-ACT-SACT-12-02-2,	NATO Standard CBRN Sensor Interface,11 January 2013	(RFI) to support establishment of a NATO Standard CBRN Sensor Interface

Table 5: CBRN - B related standards

IV. CBRN Bioterrorism

Biological weapon is the one of the oldest weapon of mass destruction used during military conflicts. In ancient times, in VI century B.C., the Assyrians contaminated the water sources using the ergot or hellebores during their retreat. Furthermore, in old times, the common action was to poison the arrowheads using poisonous plants or animal carcass. Scythians, were soaking the arrowheads in the blood of dead people, while the Carthaginians were pulling the poisonous snakes into enemies' boats. The Macedonians were leaving dead soldier and their horses in order to create the epidemy in enemies' troops. On the territory of ancient Mesopotamia, the archaeologist was found the traces of epidemy caused by *Francisella tularensis* as a probable action done by Hittite, which were chased their sick animals on enemies' territories [47]. In middle ages, the biological weapon was used also very often. It should be mentioned about, the siege of Kaffa fortress, where the dead corps were catapulted inside and resulted in plague epidemy [48]. The same action were performed by Russians during Russian- Swedish conflict in Reval city. In 16th century, Spanish conqueror Francisco Pizarro ordered to give out clothes to Incas people contaminated by the Smallpox virus resulted in approx. 3 million of death cases. The same action was performed by the governor of New Scotland Jeffrey Amherst, which ordered to give out the blankets also contaminated by the Smallpox virus [47]. In 18th century, the same strategy was performed by Tunisians during siege of La Calle and by Napoleons troops in Mantui in Northern Italy [48]. In 19th century, a lot the cases of using the pathogens in conflicts were reported. During the US civil war, the Union troops were fallen sick from the food and water, which they received, while they were stationed on Confederates territories [47]. As a result of these cases the order of forbid to taken any food and water was issued. In general Sherman diary, is also mentioned about Confederates physician - Luke Blackburn, who organized the disposal of Smallpox virus-contaminated clothes to Union troops. The 20th century is also full of cases of usage of pathogens in conflicts. During World War I, the German was trying to cause epidemy of cholera in Italy and Romania and plague in Russia. There are evidence that, the German agents in the USA were using the *Burkholderia mellei* to infect the horses, which were transported to France as US support [47]. Japan was also involved during 1930 – 1945 in the development of biological weapon. Their Unit 731 commanding by general Ishii, was using the local Manchurian population in experiments with biological agents. Other Japanese Unit 100, was performed the same experiment in North-west China. In the 1940s, also the US developed their own biological program [48]. The two Centers were established, in Camp Detrick and Camp Frederick. They were planning to used *Helminthosporium oryzae* to destroy Japanese rice fields. Also, in 1940s, the British government experimented with Bacillus anthracis spores on Gruinard island resulted in island quarantine up till 1986, when the 520 tons of formaldehyde was used to destroy Bacillus anthracis spores [49]. After World War II, both Soviet Union and US were developing their biological programs. During this time, the North Koreans accused US of using *Brucella suis* in Korean peninsula conflict in 1950–1953. In 1969, the president Nixon issued the decree ending the US biological program but it is worth mentioning that during this program in only one Centre Camp Detrick approx. 60 000 laboratory animals were used. This can illustrate the scale of the program. In recent past, worth mentioning is the Sverdlovsk (Yekaterinburg) incident in Russia in 1979, where the incidental spray of anthrax spores into the air caused approx. 68 death cases [49]. As terrorist act, the usage of biological agents is also documented. In 1978, the Georgi Ivanov Markov, the Bulgarian dissident writer was killed by ricin administer by a dedicated mechanism placed in an umbrella.

Nowadays, biological agents still posing a daily threat to humans, animals, and plants in Europe. Distinguishing intentional release of B-agents from natural outbreaks is difficult and requires knowledge and detailed competencies in the field of not only of B-agents morphology but also in identifying, tracing, and characterization them in samples. Moreover, the existing detecting technologies are suitable to laboratory environment and not reflecting real-life conditions, so the discovery of terrorism incident, which include biological agent is very challenging [50].

Considering the possible modes of possible terrorism incident, it should be mentioned the following:

Spraying into the air.

According to the literature: releasing of 100 kg of Bacillus anthracis spores in the vicinity of agglomeration can be compared to attack using the hydrogen bomb [51]. The number of the death cases will be very high and the economic loss resulted can be estimated on approx. 20 billion EUR.

Food contamination.

In the recent past, the luncheon bar was a target of such incidents using Salmonella strain by Shree Rajneesh sect and resulted on numerous cases of food poisoning. The number of the cases and economic loss will be lower in comparison to releasing the agent into the air, however it should be mentioned of easiest of executing of such terrorist act.

Water contamination.

In ancient times water contamination was chosen as an effective method of enemy destabilizing in ongoing conflicts. There are well-known cases of water poisoning, especially during army retreats. In the present times, the contamination of water sources is also considered an effective method of terroristic incidents. In comparison to previously described modes, in relationship to the number of cases and economic loss, the water contamination is placed between biological agent spraying into the air and food contamination mode.

Unconventional mode.

The recent past example of unconventional mode were the letters containing Bacillus anthracis spores in the USA in 2001. Despite the fact that, only 22 persons were infected and only 5 of them died, antibiotic therapy was administered to non-infected 32000 people. The panic and disorganization were tremendous: ransoming the medicines, protective equipment and changes in human routine behavior (movement limitations and home isolation). Furthermore, after US incident, among all EU countries were observed numerous incidents of letters addressed to governmental institutions containing according to sender Bacillus anthracis spores. The mentioned incidents were pranks; however all dedicated services were placed in the highest alert status. The economic losses resulted from these incidents were very significant.

V. Application of Machine Learning Methods in Digital Holographic Microscopic Image Analysis

Image-processing and pattern-recognition techniques combined with various types of classifiers are often used as effective tools for automatic recognition of bacteria species and strains in laboratory images [52] [53] [54] [55] . Unlike conventional microscopy, holographic microscopy measures optical field images using laser-based interferometry. Thus, in addition to the amplitude images, which are available from conventional microscopy techniques, holographic microscopy quantitatively measures the optical phase delay maps dictated by the refractive index (RI) distribution of the sample [56]. As the RI distribution is governed by the structural and chemical properties of the sample, holographic microscopy enables the simultaneous measurement of morphological, dynamical and chemical feature related properties of cells [57].

The first step towards particle characterization in holographic microscopy is to reliably detect features of interest and precisely localize them within a recorded video frame, to enable subsequent analysis. Poor localization slows the following analysis and can prevent fitting algorithms from converging to reasonable results. In this field, machine-learning algorithms can improve feature detection and precise object localization in holographic video microscopy [58] [59]. Interestingly, even when runtime is more important than precision for an application, neural network-based solutions have advantage over heuristic methods, as the speed-gain can be more than an order of magnitude [60]. This might be important in the case of real time or quasi real time biomedical video stream analysis.

In certain application specific use cases, iterative fitting and finetuning computational methods help to decrease uncertainties and increase precision when determining and classifying the motion pattern of bacteria. Extracting additional parameters of bacteria, like size, orientation and refractive index distribution properties improves the fitting of the generative spherocylindrical model of E.coli during scattering which decreases the difference between the measured and simulated holograms. Under these specific conditions, the certainty of the computational method is higher if the number of object-specific parameters is lower [61]. The above computational imaging approaches enable quick and specific motion pattern analysis of bacteria and other type of cells by digital holographic microscopy in contrast with traditional microscopy methods (for example confocal fluorescence microscopy), where the acquisition time is longer, and the sample quality can be influenced by photobleaching and photocytotoxicity.

Classification in computer vision is a crucial problem to build and train a classifier that maps an input image to the corresponding classes especially regarding the first layers of the decision tree. A classification problem includes training and testing. During training, the images with the corresponding ground truth class labels are used to train the parameters of a classifier in a supervised manner. An optimization procedure typically accompanies a loss function and the corresponding learning rule. The learned characteristics are used to automatically identify the newly measured images (test data). The performance is quantified by comparing the predicted and ground truth (reference) labels. Machine learning methods in holographic microscopy enables the classification and identification of cells and tissues for rapid screening and diagnostic purposes.

The pioneering study combining machine learning with DHM was proposed by Javidi et al. [62] to recognize two algae species based on Gabor wavelet-based features (that represent both spatial frequencies and

local properties). In their further studies, shape-tolerant feature-extraction methods were proposed [63] [64]. In the last decade, machine learning combined with DHM has been applied to a variety of samples: bacteria [65] [66] [67] yeast [68] lymphocytes [69], cancer cells [70] [71], [72] [73] [74] sperm cells [75] stem cells [76], air quality [77], and the semi-transparent red blood cells that are hard to detect with brightfield-microscopy [78]. Focusing on bacteria recognition and classification, we found two studies on supervised learning. Jo et al. (2015) used DHM combined with Fourier transform light scattering (FTLS) technique to measure the light scattering pattern of individual bacteria, demonstrating the capability of this technique to distinguish four rod-shaped species in a mixed sample (*Bacillus subtilis, Lactobacillus casei, Synechococcus elongatus* and Escherichia coli). In their study principal component analysis (PCA) was employed to represent a FTLS map as a linear combination of uncorrelated principal patterns. Bedrossian et al. [67] designed a linear logistic regression pixel classifier to identify and track bacteria (*Bacillus subtilis* and *Colwellia psychrerythraea*) from DHM images obtained from different concentration of bacterial samples. At the location of the particles of interest, a pixel feature matrix is designed to train the classifier. The following features were employed: difference of pixel values (in x), local image gradient, local standard deviation, absolute difference of pixel values (in z), local neighborhood median value, total image standard deviation. With the classifier trained, the coordinates for all particles of interest in the dataset are found and a nearest neighbor particle tracking algorithm is employed to identify identical particles across time sequences. Jaferzadeh et al. [78] compared 2D and 3D morphologic features to distinguish between 4 different shapes of erythrocytes (human red-blood cells). Describing complex 3D cell bodies with scalar features is a complex and computation demanding task, therefore it is important to specifically explore and quantify the usefulness of each metric that is fed into the classifier. The computed 3D features were as follows: surface area, average cell thickness, sphericity index, MCH (mean hemoglobin concentration related constant) and two newly introduced features extracted from the ring section of the erythrocytes. The study revealed, that relying only on 2D features leads to worse classification performance, while adding 3D features help to achieve as low as 1-2% error rates.

Despite the increasing number of classification-based studies in holographic microscopy, most of these rely on hand-designed feature extraction. A convolutional neural network (CNN) has been proposed by [79] for the automatic identification of bacteria from three-dimensional holographic imaging. In this study optical diffraction tomography (ODT) was applied to obtain 3D refractive index (RI) maps of bacteria specimens. For the classification purpose of various species of bacteria, they trained a densely connected convolutional network (DenseNet) and a wide residual network (WRN). For optical screening of anthracis spores, Jo and colleagues [66] developed a CNN algorithm, named HoloConvNet to accurately distinguish Bacillus anthracis spores from other Bacillus species in label-free holographic microscopy. Interestingly, the network automatically exploits inter-species dry mass differences, that may arise from subtle structural attributes, without teaching the network to calculate dry mass from phase images. This result highlights the learning capability of CNN which is able to automatically extract features, enhancing the chemical specificity of DHM imaging. The network, with the same architecture and learning rules, was also trained for diagnosing the pathogen Listeria monocytogenes showing a surprisingly high diagnostic accuracy (85%).

Employing recurrent neural networks (RNNs) in combination with CNN would also address inter-frame relations and can be useful for analyzing 4D data [80].

While in most use-cases machine-learning is applied to the already reconstructed hologram, neural networks can help with the reconstruction itself as well. Jianglei et al. [81] reasons, that executing a trained CNN takes shorter time then an iterative, physics-based propagation algorithm. The authors even built a DHM device, which uses CNN-based reconstruction to prove the applicability of their quantitative phase-reconstruction method applied on the images of living mouse osteoblastic cells. Besides computation speed, the other most crucially important metrics are accuracy and precision. According to the consequence of the principles of supervised learning, the trained neural network cannot be more accurate than the original training data. Still, the above proposed solution reaches 0.94 similarity (SSIM) score, which means very high fidelity to the output of the propagation algorithm. It is important to mention, that the efficiency of a neural-network based reconstruction method is the highest if the features extracted from the bacterial dimensional and morphological properties are included in the training data. Therefore, the importance of a complex and carefully designed image database is crucial in digital holographic microscopy-based image reconstruction and classification projects.

The resolution of the raw hologram is obviously limited by the pixel size of the detector, but specific post-processing methods -commonly applied in super-resolution imaging- can increase the overall image resolution output [82]. Several image-processing and image-converter tasks can be thought to neural networks [83] [84] to enhance the image quality. Holograms can be converted to brightfield images, where in addition to the cross-modality conversion the AI is able to remove noise and out-of-focus artifacts from the resulting image [85].

On the image below: following digital backpropagation in 3 different depths, CNN converts holographic images to brightfield-microscopy-like output, while removing noise and out-of-focus objects (illustration from: [84]).

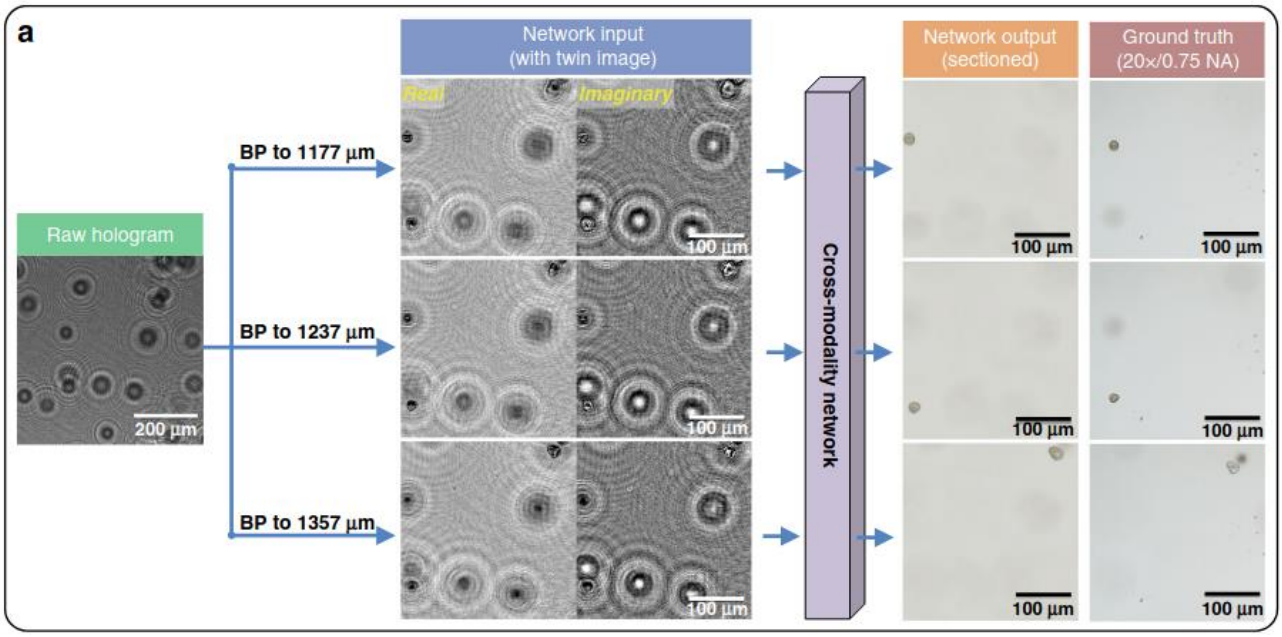


Figure 2: Digital backpropagation in 3 different depths

In quantitative phase imaging and holographic imaging related applications, digital staining is another example of using AI to recognize content. Instead of manual histological staining, the neural network algorithm can couple the reconstructed phase-image with color code which represents material components or tissue elements [83] [84]. CNNs can also support autofocusing in digital holographic microscopy [82] [86] and can improve image quality captured under low-intensity contrast and low-SNR conditions [87].

Based on the reviewed scientific and technology-driven literature we can conclude that the accurate design and implementation of machine learning methods combined with the appropriate reference image database will make holographic digital microscopy more accessible to medical doctors, biomedical scientists and CBRN experts enabling easy, rapid, and accurate diagnosis of pathogens.

VI. Microorganism Detection Limits

The limit of detection (LOD) of microorganisms depends both on the microorganisms and on the technique used. The LOD can be defined as the lowest number of microorganisms that can be detected by using a particular technique. In the case of pathogenic microorganisms, the detection technique used should detect at least the infectious dose of the specific pathogen, as this dose can vary among different pathogens. One of the crucial elements in the detection of microorganisms is the proper sample preparation, to avoid false positive and negative results. Samples can be divided on environmental (outdoors/indoors) and clinical samples based on their source. In case of environmental samples, airborne contaminants as pollen, dust or other physical particles (e.g., microplastics) can interfere with the potential pathogen detection and the accurate assessment of the sample. In clinical samples, the number of expected contaminants is reduced in comparison to the environmental ones; however, specific knowledge concerning sampling methods is required, therefore the sampling must be performed by health professionals. The techniques used for the detection of microorganisms are diverse, and each of them presents a particular limit of detection and advantages/disadvantages.

Cultivation method

According to the literature, the cultivation method is considered a fair method of microorganism detection. The detection limit ranges on approximately one organism per analytical portion of a sample, which is equivalent of 0.04 CFU per g. However, this method is time consuming. It is estimated that E. coli, for instance, needs around 12 hours to replicate from 10^7 to 10^8 cells, so it doubles every 20 minutes. For other microorganisms, the standard time of culture is ranging from 24 to 48 hours. Furthermore, in the cultivation method the sterility, equipment, reagents and human factor play essential role. The false positive outcome can be obtained easily as a result of secondary contamination and false negative results due to incorrect preparation of materials.

Molecular techniques

The molecular techniques such as conventional PCR or quantitative Real-time PCR (qPCR) are the most sensitive method for pathogen detection. The detection limit of Real-time PCR ranges approximately around 10 cells/ml of the sample with median limit of 450 cells/ml in the case of single assay. In the case of environmental samples, it ranges from 10 CFU/g - 3.2×10^8 CFU/g of soil – the median limit 1.2×10^4 CFU/g for soil samples. It should be underlined that the detection limit strictly depends on the sample pretreatment and the extraction process. The difference of detection between pretreated and untreated samples is estimated to be approximately three orders of magnitude in favor of the pretreated samples. The explanation of this phenomenon is the presence of PCR inhibitors in the soil, which will interfere with the reaction. In the case of air samples, the detection limits are similar, but the presence of PCR inhibitors is lower in comparison to soil samples; therefore, the sample pretreatment/extraction processes are less critical. However, two important factors are the volume of air which will be filtered and the collection method of air particles. In the case of air collected by Coriolis air samplers, the detection limit is higher than when using only filters. In case of water samples, the detection limit is like the mentioned above; however, pretreatment/extraction processes are also important since water samples contain large number of contaminants that can interfere with the PCR.

Immunoassays: enzyme-linked immunosorbent assay (ELISA) and electrochemiluminescence (ECL)

The detection limits range from 10^2 cells/ml to 10^6 cells/ml in the case of ECL. The four orders of magnitude difference are dependent on the kind of ECL technique, as some are more sensitive such as the immunomagnetic ECL (IM-ECL) or the aptamer-magnetic bead-ECL. In the case of environmental samples, the detection limit is almost the same; however, it should be underlined that a sample pretreatment/extraction process is needed, especially in the case of soil and water samples. As for the PCR technique, this process is less important for analysis of air samples. Regarding the ELISA, the detection limit ranges from 0.01 pg mL−1 to 100 ng mL−1 and is also strictly dependent on the presence of clear antibody/antigen in the sample. The sample pretreatment/extraction process is also crucial, especially for environmental samples.

Biosensor assays

The detection limit of biosensors is one of the poorest. It is estimated to be on the range of 3.2×10^8 CFU/g in the case of soil samples. However, in this case the sample extraction method is estimated to be

the easiest one, as no human involvement is needed. In many cases it relays on an automatic process. It should also be underlined that, due to automatization, this appears to be one of the safest detection methods.

Mass spectrometry including Raman spectroscopy

The mass spectrometry and Raman spectroscopy according to numerous literature publications appear to be the least-sensitive methods. Their detection limit was estimated to be in the order of 1.0×10^7 and 8.0×10^7 cells/ml respectively.

Microscopy techniques

Microscopy techniques offer a tremendous potential of direct microorganism observation. However, they require a trained eye in order to differentiate microorganisms among themselves and from contaminants. There are different kind of microscopy techniques. Among them, immunofluorescence is one of the most specific ones due to the detection of specific microorganism antigens by fluorescent-coupled antibodies. As for other immunoassays, it requires thorough environmental sample pretreatment/extraction process in order to eliminate or lower the presence of contaminants, which can interfere with the assay and result in false negative or positive results. The detection limit was established on 10^3 CFU/g for soil samples. However, it should be noted that the type of soil is also important. The above presented value concerns sand samples; in garden soil sample the limit is higher and ranges 10^4 CFU/g. The simple explanation of this phenomenon is the possible aggregation of microorganism in garden soil samples, which definitively influences the detection limit. Regarding electron microscopy using negative staining, the detection limits are in the range of 5×10^4 particles per ml. A disadvantage of the microscopic methods is that the morphological features of observed microorganism may not be sufficient for their specific identification.

For Digital Holographic Microscopy (DHM), it is possible to combine the technique with artificial intelligence to train the system for microorganism detection and differentiation from contaminants based on their characteristics and features. It is estimated that the DHM detection limit ranges from 10^3 cells/ml in laboratory samples to 10^4 cells/ml in environmental samples. On this basis, the WHO recommended limit of detection (1 CFU/100 ml of sample) will be easily reached and due to deep learning process, the device will be able to identify different kind of microorganisms. However, this technique presents limitations for bacteria and virus detection, due to their low contrast and small size, respectively. Some technical developments are being implemented to improve the resolution of DHM and, therefore, the limit of detection. One example is the use of high refractive index materials such as polyethylene glycol (PEG) that deposits around particles, increasing the size and scattering cross-section [88]. The use of extracellular dyes in the sample medium and a dual-wavelength DHM has been used to measure the refractive index and cellular thickens in yeasts [89]. Surface fluorophore labelling has been also reported; however, it is limited to isolated microorganisms, therefore more difficult to apply for environmental samples [90]. On the other hand, the use of smaller wavelength light, as UV light (266 nm) as a light source increases the forward scattering from nanoscale objects, making possible to detect sub-30 nm particles [91]. The UV light is also strongly absorbed by biomolecules as nucleic acids and proteins, further enabling high-contrast imaging of biomolecules. The mentioned implementations together with imaging processing (e.g., deconvolution, pixel super-resolution) methods and artificial intelligence makes this

technique very promising for applications in biomedical sciences and environmental monitoring, such as bacterial or viral load measurements, air and water quality monitoring.

VII. Cell Identification by DHM

Although conventional light microscopy has been the standard tool for microbial identification over centuries, technologies based on lens less DHM combined with machine learning are being developed for faster and cheaper label-free microbial identification [92–94]. Other techniques based on label-free identification of biological objects have been reported, as a microfluidic system for bacterial pathogen classification based on their size and shape [95].

In DHM, instead of recording the projected image of the object, the images are reconstructed digitally from a recorded hologram obtained by collecting the information from a reference planar (or near planar) light and the wave front originated from the recorded object (holographic interference pattern). Both the amplitude and the phase of the light are obtained for each point. The change of the phase of light can be reconstructed from one plane to another, thus spatial wavelength modulation can be calculated for each imaging point. This gives information about the optical distance and the refraction ratio of the matter at a given position, thus depth 3D information about the investigated object can be calculated [96]. Since the image formation in DHM is very different than in conventional light microscopy, a deep/machine learning training from reference images of different nature and size/morphology is needed for their subsequent classification and identification.

The resolution of DHM is limited, as for conventional light microscopy, by the diffraction of the light source, although it is also limited by the pixel size of the sensor and by the signal-to-noise ratio. Although the lateral resolution of DHM is equivalent to conventional light microscopy, super-resolution values can be achieved by improving the effective numerical aperture (NA) with computational techniques. By using pixel super-resolution and hologram deconvolution, lateral resolutions of 225 nm [97] and of 90 nm [98] have been reported. Nowadays, with new elements as 0.8um - 0.7um pixel-sized CMOS chips (IMX686, S5KGW1, S5KGH1) and laser diodes with 1 nm spectral bandwidth (e.g., OSRAM PLPT9 / PLT3, ROHM RLD), a lateral resolution of ~70 nm is theoretically possible to reach.

Cells and viral particles, as well as some other biological (e.g., pollen, spores) or physical (microplastics) particles, have particular refractive properties both in air and water according to their structural and biochemical characteristics, which can be also used in DHM for deep learning and object classification. As references, the refractive index (RI) at 500 – 600 nm wavelength is 1.33 for water droplets; for bacterial cytoplasm, ~1.36 - 1.38; for viruses 1.4; and for microplastics is ~1.47 - 1.64 [99,100]. As mentioned before, bacterial spores are highly refractive, and it was one of the features used for identification of Bacillus anthracis spores by DHM and deep learning [101].

As DHM is a non-invasive technique, 3D images from live objects can be reconstructed with minimum photodamage. Moreover, as the light phase is obtained, it allows the visualization and quantification of transparent objects with low contrast, such as living cells and intracellular components. Therefore, several applications for imaging different cell types have been reported to image mammalian live cells, such as hepatocyte cells [102], primary cortical neurons [103] or endothelial cells [104].

Although the application in microbiology can be more challenging, the resolution limit of DHM allows the detection of many microbial cells, such as fungi, microalgae, protozoa, and virtually all bacterial species, although one of the smallest one, *Mycobacterium*, is 0.2 um wide and therefore close to the current resolution limit of the technique. Rappaz and collaborators, for instance, studied the cell cycle of the fission yeast *Schizosaccharomyces pombe* [105], derived from the information about dry mass production rate and surface density in wild-type and a mutant fission yeast strain. The group of Javidi reported several studies on the imaging of microalgae [92], and more recently, they developed a deep learning, label-free cell classification method based on refractive index values used to classify microalgae cells based on their lipid content [106]. DHM and de-noising algorithms have been also used for tracking *E. coli* cells in a dense suspension [107], as well as for tracking 3D trajectories and speed of *E. coli*, *P. aeruginosa* and *Agrobacterium tumefaciens* cells [108], and a machine learning algorithm was developed for the detection and tracking of *B. subtilis* and *Colwellia psychrerythraea* at different concentrations [109]. To enhance the tracking and resolution of bacteria by DHM, an approach using fluorescent dyes and protein labelling was reported [110]. The contrast enhancement was enough to resolve intracellular structures in *B. subtilis*; however, it was not enough for the resolution of flagella in *Vibrio alginolyticus*.

Although a biological-object classification is possible by using DHM, the identification of specific pathogenic microorganisms might be more challenging. In a step further in microbial cell identification, Jo and collaborators [101] developed a holographic screening method by using deep learning to identify *B. anthracis* spores. For this, individual *Bacillus* spores were imaged for the training, and the network was then able to distinguish *B. anthracis* spores from other *Bacillus* species spores only based on biological traits encoded in the images. Interestingly, the classification of other bacteria, *Listeria monocytogenes*, was possible by using the same method. In another study using a similar approach, the detection and classification of three live bacterial species, *E. coli*, *Klebsiella aerogenes* and *K. pneumoniae*, was performed by following their growth in plates [111].

As a major part of human pathogenic viruses is on the limit or below the theoretical lateral resolution of DHM, they are therefore under the limit of detection of this technology. Although the refractive index of some viruses has been determined by other techniques [99], as well as the classification of viral-infected from non-infected cells [112], the individual classification of individual viral particles has not been reported yet.

The integration of additional modules such as microspectrophotometer, laser induced-autofluorescence (LIF) or surface plasmon resonance coupled with specific antibodies labelling might be needed to increase the identification capacity of the system, in particular for the identification of microorganisms presenting a biological threat, such as Bacillus anthracis or viruses such as SARS-CoV2.

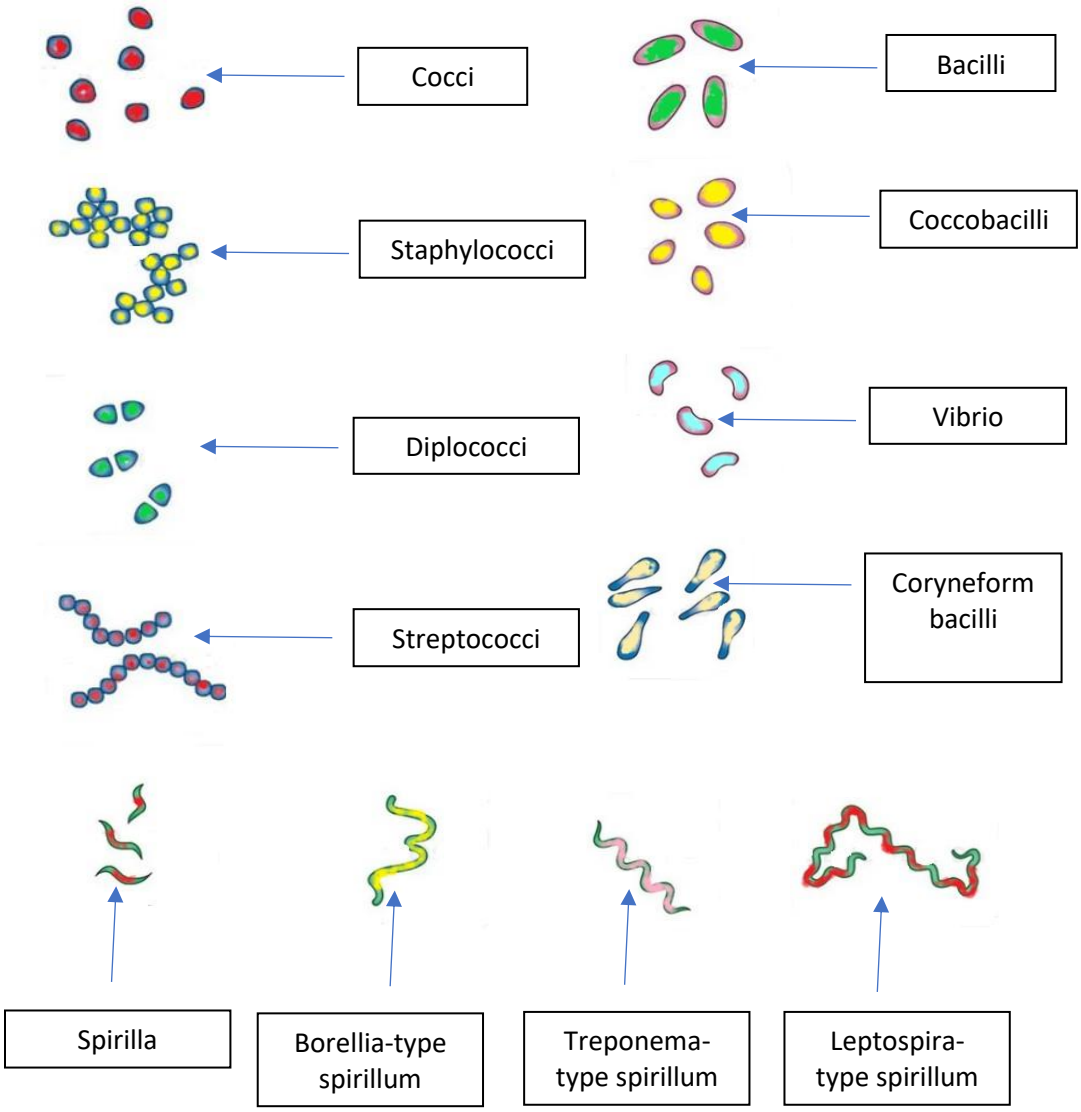
VIII. Bacteria Size and Shapes

Microorganisms are the structures, which can be characterized by their diverse structure, shapes and sizes. Bacteria as a part of microorganism can also be characterized using above mentioned ways. Bacteria are the single cell organism, which lack organized nucleus, however they possess thick cell wall. The cell wall thickness is responsible for their shape but also determines their flexibility and other features. Bacteria are divide into two groups based on their response to the Gram's staining. These two groups are: Gram positive and Gram negative. The difference between these two groups are based on the structure of the cell wall. The Gram positive bacteria possess single layer cell wall, while the Gram negative is equipped with the double layer cell wall [113]. In regards to the bacteria size, the average is range 0,2µm to 2µm, which allow to be visualized by microscopy techniques [114]. The diversity of bacteria shapes allow to identify them within their genus and distinguished from other bacterial genera. On the basis of bacteria shapes, they can be classified into 4 categories: spherical bacteria, spiral bacteria, rod-shaped bacteria and filamentous bacteria. Besides 4 main bacteria shapes, the irregular shapes are also present: appendage, pleomorphic, star-shaped, sheathed, stalk, spindle-shaped, lobed, trichome-forming or rectangular bacteria. Examining the bacteria arrangement, they may group in pairs (diplo) in group of four (tetrads), in grape like (staphylo), chain (strepto) [115].

Coccus bacteria. The bacteria are usually occur in pairs. Diplococcus as two joined cells. The name originating from double and coccus as berry. Usually, they are Gram negative bacteria. Tetrad bacteria. The bacteria usually form the square of four bacteria. Staphylococci bacteria. The meaning of the name comes from the grape like berry. They are mostly Gram positive bacteria. Streptococci bacteria. The meaning of the name comes from chain berry. The bacteria arrangement simulate chain, where one cell is attached to another. *Sarcinae* bacteria. The bacteria arrangement forming packets of 8 or more cells attached. Rod-shaped diplobacilli bacteria [116]. The bacteria are short and occur in pairs with arrangement side by side to each other. Rod-shaped streptobacilli bacteria. The shape of the bacteria is similar to rods in chain. They are Gram negative. Rod-shaped coccobacilli bacteria. The shape of the bacteria is placed between rod shaped and spherical. The forming rods are short and can be easily mistaken with spherical shape. Rod-shaped palisade bacteria. The shape of the bacteria arises during the cell division process and leads to the creation of palisade formation. The shape is similar to picket fence or to some Chinese characters. Comma shaped bacteria. The shape of the bacteria reminds comma not completely bent or twisted. They are usually Gram negative bacteria. Spirilla bacteria. The shape is spiral and flagellated. They are Gram negative bacteria. Spirochete bacteria. The shape is spiral and simulates long helically coiled cells. Filamentous bacteria. The shape is long and looks like filament, which can form network name mycelium. Rectangular-shaped bacteria. The shape is box like. The bacteria from this group are extremely halophilic. Appendage bacteria. These group contains heterogeneous collection of bacteria. They differ from other bacteria on reproduction mode (budding). They can be either motile or non-motile. The motile form are usually flagellated [117].

Pleomorphic bacteria. These bacteria can change or alter their shape under the influence of environmental factors. The same refers to their sizes. Star-shaped bacteria. These group of bacteria are flat in their shape look like a six pronged star. Sheathed bacteria. These bacteria form the filament form, which is wrapped in similar to sheath structure. Stalked bacteria. They usually appear as cell equipped

with stalk, which arise from one end of the bacteria. This arrangement appear during the bacteria binary fission process, when bacteria divide asymmetrically. Spindle shaped bacteria. The bacteria possess the fusiform



appearance with ends tapered and bulged middle. Lobed bacteria. The bacteria shape is lobe like, irregular and flagellated. Trichome bacteria. The bacteria forms a chain consisting their vegetative cells, sometimes they can be enclosed.

Figure 3: The schematic diversity of bacteria shapes

Bacteria are prokaryotic unicellular microorganisms lacking a nucleus or membrane organelles. Bacterial size can vary in diameter from 750 nm for the giant bacteria Thiomargarita namibiensis, to ~0.2 um for the Mollicutes class of bacteria. However, the average size of cultured bacterial cells is 0,2 – 2 um diameter and 0.5 – 5 um in length, which is also the size range of the great majority of pathogenic bacteria, such as Salmonella Typhi, Yersinia pestis, Bacillus anthracis of enterohemorrhagic Escherichia coli (EHEC). The size

scale of these microorganisms allows to detect them by light microscopy. The small size of bacteria is in part the evolutionary result of the minimal volume needed for their internal biochemistry functions and of the competition for nutrients and its intracellular diffusion [118,119].

Although the best-known individual bacterial shapes are the rods (E. coli, Bacillus), spheres (Streptococcus, Staphylococcus) and spirals (Helicobacter), they come in the most diverse flavours: commas (Vibrio); corkscrew (Leptospira); filaments (Corynebacterium); stalks (Caulobacter); among many others [120]. At the same time, bacteria can grow isolated or in aggregates (Staphylococcus), palisades (Corynebacterium), chains (Streptococcus), or biofilms (Pseudomonas aeruginosa). Most bacteria have a peptidoglycan layer forming the cell wall, which has a key role in maintaining cell shape and supporting osmotic pressure [121]. That is why some bacteria lacking a cell wall are pleiomorphic, like the case of Mycobacterium. The bacteria cell wall can be single-layered or multi-layered, which confers different properties such as a differential permeability for Gram staining dyes. This led to the classification of bacteria in two large groups: Gram negative with a single peptidoglycan layer and an additional outer membrane, such as E. coli, and Gram positive with a multi-layered peptidoglycan and a single cell membrane, such as S. aureus. In addition to the cell wall, other differences can be found at the surface level structure, as the presence of a capsule surrounding the cell wall (e.g., Klebsiella, Acinetobacter); mycolic acids (Mycobacterium); corynemycolic acids (Corynebacterium); or the S-layer (Clostridioides difficile). On the other hand, some Gram-positive are endospore-forming bacteria, and among them some serious pathogens of humans and other animals are found, as Bacillus anthracis or Clostridium botulinum. The endospores have characteristic features, among which being strongly refractile structures under the light microscope [122].

It is worth noting that, although several genera of bacteria can be deduced from the cell shape, a specific morphology can be shared among pathogenic and non-pathogenic bacteria from the same genera and even from the same species (e.g., non-pathogenic E. coli and EHEC). The morphology can also vary with growth rate, nutrient availability or differentiation. Moreover, prokaryotes with different genealogies and cell wall composition (Archaea) share similar morphologies with bacteria [118].

In order to prepare bacteria for microscope visualization, the different techniques of staining should be consider. Starting from simple staining (using single stain): direct (positive) and indirect (negative) and ending on differential staining (using two contrasting stains): separation into groups - Gram stain, Acid Fast Stain and structure visualization – flagella stain, capsule stain and spore stain. Furthermore, in staining protocol, the fixation (methanol, heat) is important due to the fact that allow to adhere to the slide. Slide washing in staining protocol is also important, it will allow to remove some contaminants. The microscope visualization allows to differentiate bacteria among their genus.

Viruses, on the other hand, are genetic elements of DNA or RNA that can replicate only inside host cells. They have a protein capsid containing the virus genome, which is predominantly helical and icosahedral, and many of them are surrounded by an outer layer called the envelope. Their size is typically between 20 - 200 nm, around one or two orders of magnitude lower than bacteria and 100-1000 times smaller than average human cells [123]. SARS-CoV2 virus or influenza virus, for instance, are 100 nm in diameter. Due to this small size, they are below the light microscope resolution limit (Abbe’s diffraction limit: 200 nm) and

are generally observed by electron microscopy. However, they can be detected by fluorescence microscopy when using specific staining or by using super resolution microscopy techniques.

IX. Sampling Methods

For the purpose of complete analysis of existing literature concerning the scope of sampling airborne pathogens. Main findings: Sampling airborne pathogens is a hot topic of our time (2021), in the middle of the COVID19 pandemic. However, novel coronavirus is not the only deadly pathogen circulating globally. In our literature search we have identified a very wide range of existing pathogens as well as potential future pathogens as well which may be sources of an upcoming pandemic. There is an abundance of existing technologies in the field, even existing products are available, both for CBRN and medical applications. However, many of the existing technologies have shortfalls and issues which make them difficult for wide scale use. Our research is focused on finding niches which can point us in the right direction to be able to identify optimal sampling strategy and working concept model implementation.

Air sampling.

Air sampling has a long history. Detection and quantification of environmental, biological, chemical, nuclear, and radiological air contaminants are of high importance. Therefore, a broad spectrum of air collection and trapping systems were developed. The two principal types are the passive and active sampling methodologies. Then the air can be analyzed in the field, and in the laboratory. The HoloZcan project focusses on the fast, direct analysis of the collected air for CBRN related particles. Therefore, this literature review is also focused on the appropriate methods for collecting of particles with the size between 10 nm and 1 mm like viruses, prions, exosomes, liposomes, bacteria, spores, nanoparticles, cells and physical carriers of chemical, biological, nuclear, and radiological compounds, and systems. The sampling does not include aerosols with non-particulate compounds like environmental DNA, biological and chemical toxins, polymers and dendrimeric systems with stabilized DNA and RNA systems, and other non-particulate CBRN compounds.

Air Sampling

1. The passive sampling has become popular because of its lower cost, simplicity, and small size and weight, which facilitate long-term sampling, increased spatial coverage and applicability on air drones. However, passive samplers are less suitable for short-term sampling and are in general less accurate than active samplers because they require reliable sampling rate measurements for individual analytes under diverse environmental conditions.

2. The active air sampling allows to collect fast and localized, from small up to large air volumes with defined velocities. Combined with the appropriate trapping system the air contaminants could be sampled with high recovery.

Collecting of the air

The main active air collecting method are the air suction with ventilators, membrane, or spiral air pumps located behind the particle trapping system to avoid potential particle absorption or contamination from the pump system. The air flow ranges between 1 and 100 L/min depending on the type and size of the

particle capture system used. For sampling systems should be used non pulsative pumps to remain stationary conditions on the particle trapping site. The pump should be allowed highly stabile air flow over the defined time schedules. The start and stop of the air flow should not produce pressure peaks on the particle trapping part. The airflow system should be calibrated or adapted to the different air pressure conditions to allow to sample defined mass of air. The constancy of the air humidity and temperature is also important for reproducible sampling over a longer time. Under field conditions, it could be necessary to warm or cool the air and dry or add humidity the air in the system before it reaches the particle trapping system. It could be necessary to install an air mass flow measurement and regulation system. The surfaces of the system should be not adsorptive like covered with a “Lotus effect”.

Trapping of the particles

The particle trapping systems are based on a variety of different physical-chemical principles:

1. Trapping of particles with absorbers or filter systems.

2. Kinetic impactor and direct absorption on smooth plastic, glass, metal, ceramic surfaces, impacting into gelatin, rigid cell growth medium, porous foam, filter systems.

3. Coriolis air concentration systems. The cyclonic sampling mechanism is based on the principle of a liquid-scrubber aerosol collector with tangential impingement into liquid.

4. Electrostatic sampler that collected aerosols in a liquid.

Control of the sampling conditions

During the air sampling process, in particular the biological particles can be damaged by mechanical or chemical stress, rendering them undetectable and leading to a longer sampling time to produce a detectable sample, which hinders the rapid monitoring of the particles. Therefore, the sampling, needs to be non-destructive, protecting the particles from deformation and degradation.

Conclusion of the sampling methods

The best compromise between sampling efficacy, no destruction, the possibility to optimize the sampling method for further processing seems to be the trapping into liquid by a Coriolis system. This allows low and high air flows, the application of protecting and surface tension modification compounds. For quantification it is necessary to create a laboratory test installation and appropriate calibration sets with particles to be analyzed.

Conclusion

We have searched the literature in an extensive range of corresponding databases. An exhaustive database was assembled of the relevant articles, technical files, both scientific and industrial/commercial. The cyclonic sampling mechanism has been identified as an optimal method for the purposes of the HoloZcan project. We have also studied the core imaging facility in the project- holographic microscopy- and its potential interfaces to sampling. Our focus was mainly on-air sampling and involved substances (e.g. exhaled vapor/exhaled breathing concentrates). We have studied articles regarding the deposition of airborne pathogens on imaging detectors or interfaces to imaging detectors, such as microscopic plates.

In our work we have focused on preparations for the next tasks, including the sampling strategy formulation as well as planned developments of working concept models. Standards and existing frameworks in air sampling (both CBRN and medical) have been studied extensively and shall be considered during upcoming developments.

X. Exploitation in Medicine

The HoloZcan project will use digital holographic microscopy (DHM) as the core technology of a new biothreat detection system, together with an air sampler to detect microorganisms either in environmental air or more specifically, in human exhaled air from patients.

On the one hand, holographic microscopy allows rapid, accurate and real time analysis thanks to a robust, portable system associated to artificial intelligence power; on the other hand, air collection is a versatile human-adaptable, cheaper, and far less invasive device than conventional sampling methods from respiratory tract, thus allowing easy and iterative sampling.

Collecting air or more specifically breath samples from patients for diagnostic purpose is a new and fast-growing field in medicine. So far, many studies have been focused on volatile organic compounds (VOC) as biomarkers, having some new applications in health care such as cancer detection [124], or diagnosis of infectious diseases such as *Helicobacter pylori* - already translated in current practice for many years [125] or as *Clostridioides difficile* for which a proof-of-concept already exists [126].

In this project, we intend to directly detect microbial elements and DHM technology could have a major application to assess the presence of SARS-CoV-2 particles in patients exhaled air.

This method can indeed be exploited in three main situations:

- Diagnosing patients in a very rapid manner in emergency services, in order to perform real-time diagnosis, or in services at high risk of cross-contamination between patients, such as surgery, to avoid previous time-consuming and costly RT-PCR on nasopharyngeal swabs.
- Evaluating on-site transmission risk from individuals in specific conditions where they are gathered in confined places for some time (hospitals, airports / train stations, other public service locations, spectacle rooms / museums or monuments / culture spaces, workplaces, schools)
- Evaluating the transmission potential of the environment itself in the above locations, to assess contamination of environmental air, for example, before opening these places or to regularly assess its safety at various times of opening.

These specific applications proposed for COVID-19 transmission control within the HoloZcan project may also be extended to other pathogens causing respiratory diseases, such as:

- Viruses: *Paramyxoviridae; Orthomyxoviridae; Coronaviridae; Adenoviridae; Parvoviridae; Polyomaviridae*
- Bacteria: *Corynebacteriaceae; Legionellaceae; Streptococcaceae; Mycobacteriaceae; Pseudomonadaceae; Chlamydiaceae*

Indeed, the HoloZcan system has a dual medical/defense aim, as some of the mentioned pathogens can be the cause of biological threat/bioterrorism, such as MDR and XDR Mycobacterium tuberculosis (particular strains already known as resistant to antibiotics).

DHM technique has already been used to detect other infectious diseases in vitro or in human samples, such as *Plasmodium*-infected cells [127], or infection by *Babesia microti* [128], and it has even been reported for SARS-Covid-19 detection in infected patients by analyzing morphological changes in their red blood cells (RBCs) [129]. Other viral infections, such as vaccinia virus (VACV), herpes simplex virus (HSV), and rhinovirus (RV), were revealed by DHM in different cell types according to oscillations in the infected cell volume [130]. The reduction in the refractive index of macrophages infected with *Salmonella typhimurium* also allowed the classification of macrophages between non-infected and infected ones [131].

Moreover, not being limited to microorganisms, this technology can also find easier, large applications in the diagnosis of diseases requiring cutting-edge cellular imaging technology, such as cancer or red blood cell disorders. Indeed, both biology (marine biology, parasitology, cell biology) and human diagnosis research (tissue 3D organization and dynamics) are very active fields on these research fronts and several proof-of-concepts have already been published.

There are, for example, many reports over the last two decades using DHM and 3D methods for red blood cells (RBCs) classification [78]. Since both the biophysical properties and the mechanical characteristics of RBCs membrane play a fundamental role in the physiological and pathological condition of the erythrocytes, a variety of microscopic methods have been used to study and classify the morphological features of these cells according to their shape or membrane features [132] [133]. For example, successful and reproducible time-lapse DHM measurements were used to describe the spatial and temporal membrane fluctuation dynamics of healthy RBCs within the 30.8±4.4 nm to 35.9±8.9 nm range [132]. Moreover, pixel-to-pixel level numeric computational methods have been applied to quantify the temperature dependence of the RBCs membrane fluctuation intensity to characterize the surface distribution properties of healthy human erythrocytes [134], as shown in Fig 5. This type of analysis can be applied to regular RBCs counting [133] or for detection of diseases such as sickle cell disease [135].

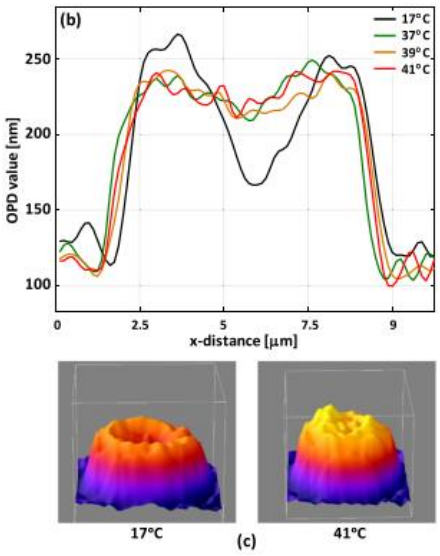


Figure 4: Numerically computed sphericity coefficients, pixel intensity,

adapted from Jaferzadeh et al., 2019. Panel b shows the cross-section of an RBC under different temperatures and panel c the 3D representation of an RBC at two different temperature values.

Furthermore, DHM allows the early detection of morphological changes and apoptotic cells both in adherent [136] and suspension cells [137]. Therefore, the technique can be adapted for the analysis of various cellular dynamics, such as drug-induced changes in cells [138], to monitor laser microsurgery on cells, for quantitative evaluation of the damage and repair of cells, for drug screenings, and to monitor cellular organelles in real time.

DHM was also applied to determine the inflammation degree both in experimental colitis [139] and in human biopsies [140], as well as for sperm quality analysis [141] [142], enlarging even more the possible applications in biomedical sciences and diagnosis.

Finally, in addition to human medicine, DHM could also be easily applied to veterinary medicine and animal disease transmission studies, particularly in the case of zoonoses which are prone to induce new large epidemics or pandemics, as it was respectively the case in 2014 – 2015 with Ebola large epidemics and since decades for influenza pandemics, like 1918 flu after the first world war.

XI. Supplementary Modalities

With digital holography the transmission and phase shift of a specimen can be measured. In practice the phase shift gives the most information on the sample. The principle of holography requires a monochromatic light source. There are other imaging techniques which have the potential to enhance this information.

Spectral Holography

Spectral Holography extends the monochromatic holographic principle to multiple wavelengths by combining multiple acquisitions over time. These wavelengths can be used for phase unwrapping [143]. Since the measurements are time multiplexed, image registration is required for non-stationary specimen.

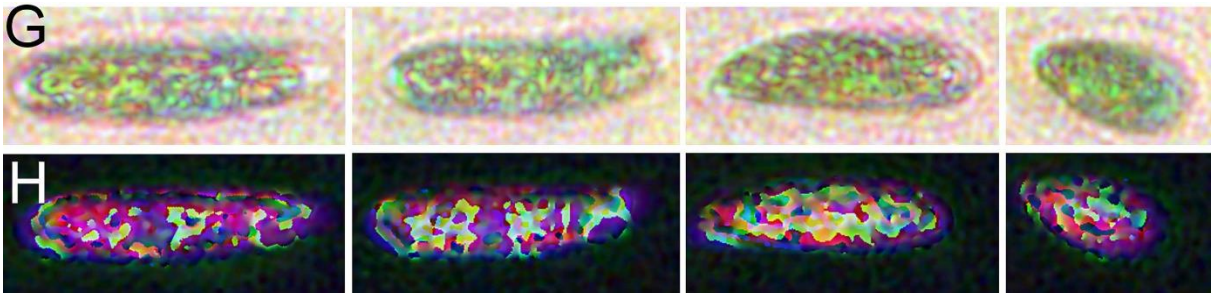


Figure 5: Amplitude (G) and phase (H) reconstruction of Euglena using spectral holography [143].

Spectral Holography can also be used to reduce the coherent noise [144]. By having a tunable light source, the wavelength can be slightly varied to affect the coherent noise, but not the absorption of the specimen. By averaging over the reconstructions of multiple acquisitions, the coherent noise is reduced.

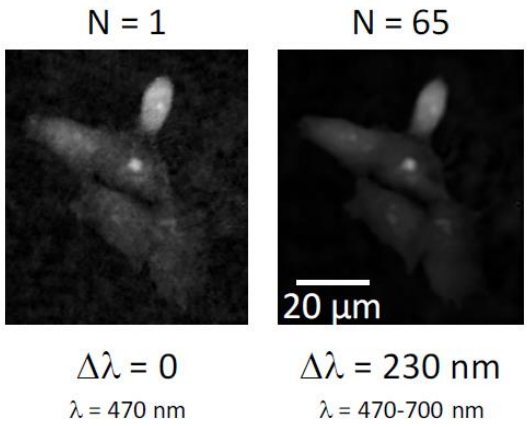


Figure 6: Noise reduction using spectral holography [144]

Multi-Modal and correlative microscopy

Besides transmission and phase-shift, fluorescence is another property of specimen that can be of interest. By combining these modalities, a volumetric reconstruction can be made [145]. The fluorescence signal makes it possible to discriminate pigments, the holographic modality has a higher spatial resolution. The combination therefore gives more information on the specimen than either one of alone. In [146] the authors combine x-ray holography with diffraction and stimulated-emission-depletion (STED) microscopy. In cardiac cells of rats they show that the combination of modalities is needed for accurate detection of the filaments. In [147] holography is combined with Raman spectroscopy, where both methods are non-destructive for the biological sample.

Since holography requires coherent light, a combination with fluorescence requires a setup with multiple light paths. Figure 3 shows the work of [148]. It has multiple optical paths, which allows the system to capture different modalities simultaneously. The authors expected the system to be able to image rapidly moving phenomena in living 3D structures.

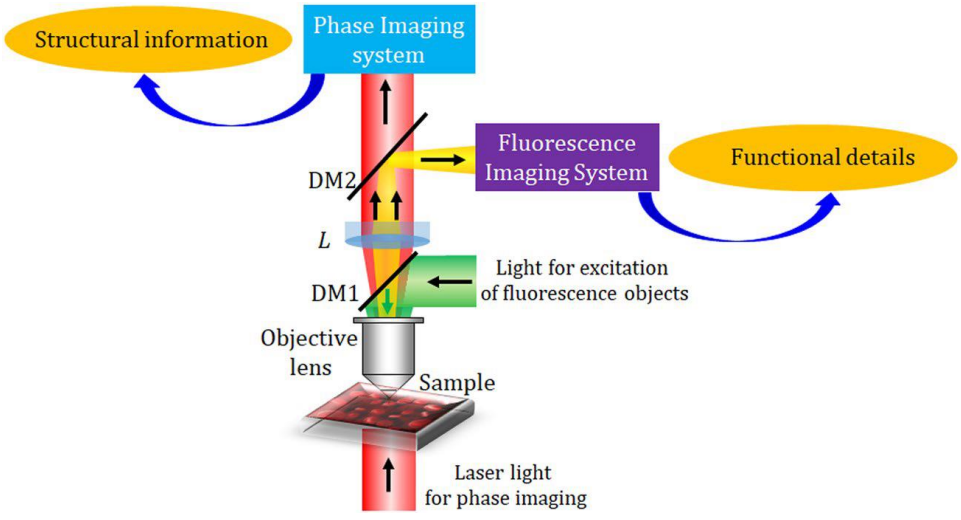


Figure 8: Schematic of a multimodal system for recording off-axis fluorescence and phase digital holography [148]

Reflective Holography

For biological samples, holography is typically used in a transmissive setup. From a physics and mathematics points of view, reflective holography is very similar. Even though the exact setups might not be applicable, it is an interesting field to study same similar solving techniques apply. In setup is shown a combined with the Gerchberg-Saxton reconstruction algorithm [148], which is one of the basic reconstruction algorithms for transmissive holography as well.

XII. Microscopy Contaminants

In environmental samples apart from microorganism, various other biological particles are present. It should be mentioned about dust mites, various pollens, molds, dander etc. The mentioned particles will distort the identification process using microscope visualization. Identifying them as contaminants and differentiating from microorganism is an essential element in proper interpretation of sample content. In order to proper analyze the environmental samples content using the microscope visualization, should be taken into account that, the contaminants possess their own characteristic, which allow to differentiate them from microorganism and among themselves. The contaminants characteristics should be the process of machine deep learning executing simultaneously with microorganism characteristics learning process. It should also be mentioned that, comprehensive analysis of microscopy contaminants and their characterisctics is based on the envirnment, where the sample, where taken.

The contaminants, especially the dust can present different morphology and size. The majority of dust particles are spherical-like shapes with the average size of approx. 2 µm [149]. However, larger and smaller dust particles can also be found and the shapes can also be irregular. Furthermore, as it was mentioned, depending on places, where the sample, where taken, the dust particles can possess different chemical compounds. The traces of potassium, sulfur, calcium, silicon, magnesium and others. The presence of of chemical compounds will inteact with dust particles and influence on the their shapes. As an example, it should be mentioned that, the sulfur, calcium will influence on creation of anhydrite or gypsum component in dust particles and will change the dust shape to quadrangular [150]. Other chemical compounds will also influence of the regular size and shape of dust contaminant [149]. The environmental samples taken in places rich with calcium and silicon will change the shape of regular dust particle to flake-like shape.

The presence of other contaminants – *pollens* is also depending on the surrounding environment but also on time of the year. The enlarge presence of pollens is observed in spring and early summer season, while their number lower during the fall and winter season [151]. The shape of most pollens is mainly prolate, however some species can have sub-prolate to spheroidal prolate shapes [152]. The sizes ranges from approx. 4.5 µm to approx. 200 µm, so only the small size pollens should be consider as microscopy contaminants, which can interfere with the microscopy microorganism visualization. The pollen also are different from microorganism in microscopy visualization due to their texture. In well prepared sample, the pollen texture is granulated and consists discrete sub-circular clusters, which are visible [152]. Below on the Fig 9, the examples of spectrum of common microscope contaminants (pollens) visualized by laser induced fluorescence.

The molds. The molds are the microscopic fungi, which can be found anywhere and especially in environmental samples. Furthermore, it's impossible to eliminate the molds from the environmental sample. In order to identify molds and differentiate them from microorganism the following feature should be consider: colonial forms, surface color and pigments production. Within the size of molds, it should be mentioned that range from 2 µm up to 50-100 µm. Underlying, the fact that small molds e.g. *Aspergillus spp*. ranging from 3 till 6 µm, while the large ones 6-50 spp. e.g. *Coccidioides spp*. The shapes of molds also vary starting from regular e.g. *Dermatophyte spp*., ribbonlike *Zygomycetes spp*. ending on simulating link sausages as is observed among yeast forms: *Candida spp*.

The dander. Dander is created from microscopic skin flakes. The dander is commonly present in environmental samples, especially the dog dander. In order to differentiate them from microorganism, the following features should be taken into account. Human dander: in shape looks equant, polyhedral and with low or no birefringence. Dog dander: identity by elongated shape and birefringence, sometimes similar in microscope visualization to sawdust. Cat dander: less common especially in environmental samples. In shape looks more triangular and elongated in comparison to human dander and with rounded corners. Bird dander: in shape is crescent or "C" shaped. The size of dander ranging from 5 µm till 50 µm with the average size of 10 – 20 µm [153].

The protists. The protists are single-celled organisms, which can be found in different habitats. In most of the cases, the protists are associated with aquatic habitat, however in other ones, they can be also found [153]. They are characterized by very diverse structures observed in microscope. The average size of protists range approx. 25-30 µm, however it can be found smaller and bigger. Differentiating them from a microorganism (bacteria) is not problematic when the human factor is involved. However, in the case of machine learning, the protists should also be part of the learning process in order to provide their microscopic signature allowing to differentiating them from others.

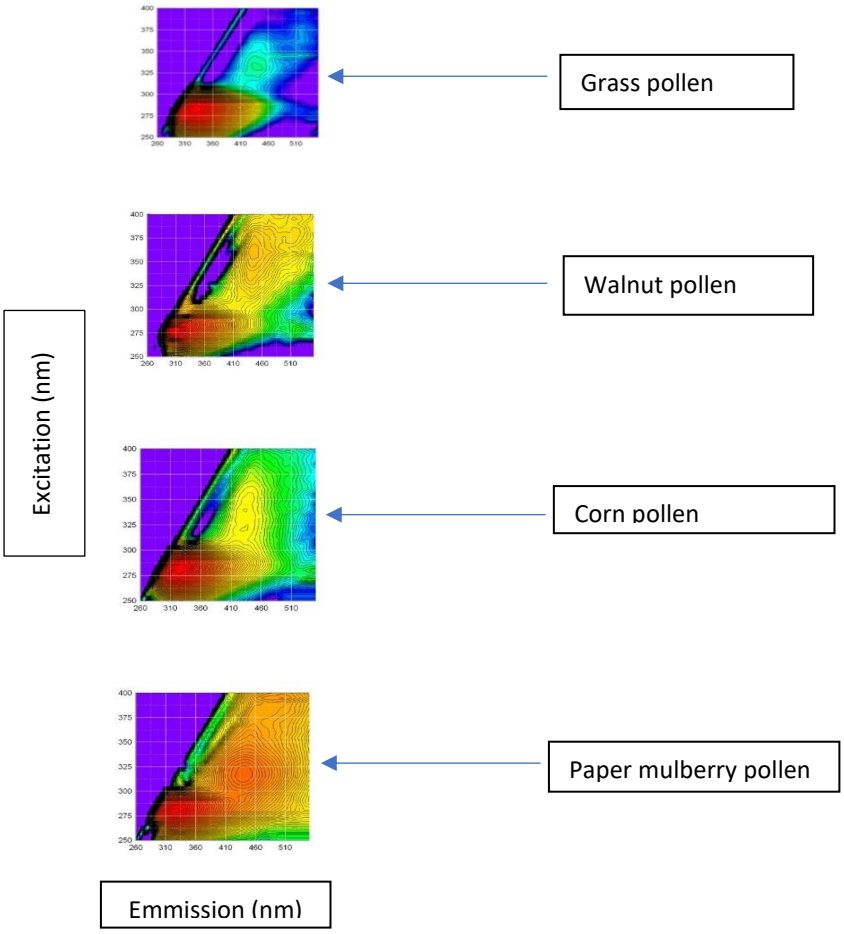


Figure 9: The examples of induced spectrum fluorescence of common pollens

..II. Emerging Technologies on Biodetection

In the past decade, several technological advances allowed to design and elaborate of new bio-detection technologies. Miniaturization brings to reality the most expected today's requirements, i.e., efficient field-portable devices for airborne threats. A closely associated element to the detection capability is the use of IT, especially artificial intelligence in the whole process. The use of IT and AI granting both, the quick detection and the development of pathogen signatures database, which enhanced characterization and recognition capacity. Several ongoing researches are of high comparative interest and besides the technique selected, the number of targeted scenarios, suggesting the significant improvement in detection capabilities [154] . It is challenging to compare among offered technologies as a wide variety of considerations will certainly need to be applied. Among the most noticeable technologies are the following ones.

Real-time detection

Real-time detection area made possible to identify a variety of substances, including the possibility to pinpoint single cells in a short lapse of time. This approach combining infrared and visible sensors, ultraviolet, and terahertz lasers in order to perform the identification of biological aerosols remotely [155]. Other technologies with long-term perspectives are Surface-Enhanced Raman Spectroscopy (SERS) [156] [157], Single Particle Aerosol Mass Spectrometry (SPAMS) or the hand-held portable LED microarray reader [158].

Nucleic acids

Nucleic acids allowing to identify a biothreat agent based on its deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) [159]. Within this area of research, several technical solutions exist and are further developing. First is Polymerase Chain Reaction (PCR) including digital and quantitative PCR. Second, pathogen sequencing as a definitive confirmation of the presence of pathogen in tested sample including the identification of whole sample content – metagenomic sequencing procedures. Third, technologies aiming on hybridization of the sample nucleic acids to an array or beads. Fourth, technologies utilize loop-mediated isothermal amplification approaches that enrich target sequences without the time and equipment-intensive temperature cycling used in PCR. All mentioned techniques based on nucleic acid are reliable and precise but require equipment and reagents, knowledge, full human involvement in detection protocol and operators' experience [160].

Proteins

Technologies based on pathogen proteins identification. Antibodies or aptamers permit to bind to target proteins. With spectrometry techniques, particularly matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), the detection of protein or peptide profiles characteristic of biological agents becomes a reality [161]. This technology allows profiling the characteristic of specific phenotypes, such as antibiotic resistance. With the spectrometry techniques, a bacterial colony can be used for a test screening. Other improvements are observed in a whole new discipline called proteomics, which not only can identify pathogens on the base of their protein content, but also permits to study of the whole pathogen proteome or their subfractions, resulting in obtaining the information concerning the landscape of expressed proteins and their modulations.

Protein abundance

Protein abundance technologies was introduced almost 40 years ago, however recent technology increase the development of new tools. Tools for mRNA expression come to be the mainstream in technology development. The information taken from mRNA and protein levels are interrelated and can be used for the identification process and for absolute understanding of pathogen cell works [159].

Whole-of-agent properties

New developed whole-of-agent technologies allow to study pathogenicity, aerosol properties, and agent/host interactions. Furthermore, the technology is used in microscale cultures observation in order to seek antibiotic susceptibility [159].

Microscopic identification

The presence of pathogens in samples using the microscope techniques was used since the invention of microscope. The microscope detection can be characterized by their rapidness, minimum requirements in sample preparation and in most cases direct detection (culture-free). However, the technique is full of limitation. The observed technology progress narrows these limitations and creating the opportunity to usage the microscope technique as a basic tool in pathogen detection. Furthermore, combining the microscope technique with software allowing the deep learning possibility of pathogen shapes and sizes allowing to not only pathogen detection but also offers limitation of human involvement in the process. This kind of opportunity creates e.g., digital holographic microscopy (DHM) with deep learning software, which offers not only pathogens whole cell detection but also pathogen components and morphology visualization [154].

Enabling technologies

Enabling technologies is based on cross-cutting technologies. The idea is based on microfluidics for the purpose of automated identification with less samples and reagents. The technology uses a selection of methods such as loop-mediated isothermal amplification and SERS for detection and fluorescent in situ probes hybridization for confirmation. Many of these approaches are conductive to multiplexing. The development of improved readout techniques is another approach. As an example, the dropleton-thermocouple silhouette quantitative PCR systems seem to have a quicker reaction. Others, concatemers of fluorescent protein-binding aptamer research aiming to make binding events detectable to the naked eye and bacteriophage technology with thousands of coat proteins engineered to bind to biological agents and amplifying the signal [154].

Other technologies

Focus on developing single-cell assays in order to detect single molecules, designing the compact flow cytometers, or to create smartphone-based detection systems. Improvement in detection technologies is directly associated with supporting sample collection and processing, system configuration as well as detection protocols [160].

Sample collection and processing

Aiming to improve air handling (including particle characterization), viable and nonviable sample collection both with and without filters, sample integrity during transport (for systems, in which collection and analysis occur at different locations), and methods for pathogen extracting from the sampling device. Examples include technologies allowing the pathogen viability in aerosols and wearable monitors for counting, collecting, and measuring aerosol particles [162] .

System configuration

Aiming to develop the technology allowing providing the information concerning the number, locations, and types of sensors necessary in environmental detection system [163].

Identification protocols

Including technologies aiming to develop strategies and protocols for relaying the outputs of an environmental bio detection system to decision-makers [154].

Last, needs to be mentioned that, the end users' requirements and expectations are changing. They expect simplicity, portability, precision, no human involvement in the detection process and a cost-friendly budget [160]. Below, the five end users' essential criteria's:

- the ease of operation both indoors and on the field (power supply, reagents, additional IT equipment, and training time)
- the dimensions and weight of the detector
- the quality, efficiency and limits of analytical results obtained
- the necessary lead-time to diagnostic, up to quasi-instantaneous surveillance systems with a detect-to-warn mission
- the total exploitation costs including maintenance

Finally, the emerging technologies on bio-detection should be affordable for personal use by ordinary man, beyond the scope of primary technology end users: law enforcement, military, medical facilities, laboratory, scientific, industrial personnel.

XIV. Users' Needs and Technology Implementation

An overview of the user's needs includes both end-users' practitioners (first responders) and the beneficiaries from scientific field. Their active continuous involvement in the technology development will define the crucial needs of the best and most suitable detection technology. The end user's involvement in elaboration of new technology can cover many different approaches. Particularly, the involvement should be started in early phase of technology development. Obtaining user's satisfaction is the main result of their involvement. On the other hand, users' centered design will allow emphasizing technology usability and the typical methods of obtaining it. These are; task analysis, prototyping, evaluation of technology usability. The end user's involvement has pros and cons [164]. As for pros it should be mentioned: feedback information; as cons, lack of end users' information concerning what is the designing process. Both of these pros and cons are concerning technology preparation phase. In the further phase of technology development (design phase), the benefits of their engagement are: identifying the interaction issues, their practical view and ideas. As the obstacles: little or no consensus among end users' groups from particular organization, providing new concepts, which sometimes are difficult to fulfill. In the last phase (the evaluation phase) as the pros it should be mentioned: their comments, feedback, suggestion acceptance [165].

As for designers and builders, medical and engineering professions have the key role to play in the closest possible combination [166]. Their sphere of innovative influence within their respective organizations has become a direct part of their daily professional routine. These recommendations find their place within future Standard Operating Procedures and Policies. They are active in International Organizations, Governmental structures, Higher Education and Research facilities, and Private companies. The design method often preferred when moving from expectations to device is reverse engineering [167]. It will have a major role to create ready for building solutions to medical issues. Selected user participants need to demonstrate two key features. One being a thorough understanding of the type of pathogens/threats to be identified. This requires persons having examined databases and carried out personal research on the agents. The second need is the understanding of the fundamentals of bio-engineering [168]. To avoid

manufacturing and field failures, the science of reliability engineering is considered [169]. In addition, the study of human factors, safety and risk analysis, and new testing techniques, will be included. Basic design principles need to be put in perspective with imagined scenarios drawn from real/potential cases [170]. It is vital to get participants on board from the beginning of the research [171]. The use of a multidisciplinary pool of users' approach, enables to foster the alignment of technology, science and design. Along with project facilitators, the multidisciplinary team comprises a fair representation from across the range of users. The type of involvement does include meetings either face-to-face or online [172]. The format of focus-groups has also been identified as one helping in the formulation of needs [173]. The use of translators is often a method utilized to concentrate the quality of results gathered [166]. The users' input is voluntary and must not be too cumbersome given their intensive workloads. Through the workshops and observational work, a series of paths for investigations appear. This gives direction and impetus to further expand the research. It does also raise expectations of what may come from the research and within what time frame [164]. The communication strategy of releasing information regularly about the progresses and milestones of the technological development becomes a mean to conduct an ongoing consultation process [171].

Scope of involvement

With a clearly defined list of deliverables required for each development phase, the design process will be set on a tracked development pathway [169]. A group of users brings its strongest output if kept together from inception stage until the final product. This takes the format of truly collaborative research and development [174]. The concept of reverting to users leads interestingly to a larger dialogue encompassing possibly a second-stage interaction in the form of feedback involvement during testing phases. Association of expertise to product development may draw further complementary research from an Academic institution for example [175]. This would even result in a possible partnership for a specific product line range. In order to clearly catch users' expression of their needs and formalize the expectation for design purposes, an accurate analytical scheme must be established and followed. Only synthesized and detailed views can support progress toward functional end-product [171]. One of the direct consequences of a close interaction with users will heavily impact and facilitate the parallel development of training material and procedures. Ultimately, from the question and answers exchanged all the evaluation criteria will be gradually emerging for a convenient later use when running later rounds of product review and improvement.

Finally, "Customers 'designed" products is one theoretically structured pathway commonly utilized. It is referred to as "Six Sigmas" and it has demonstrated its efficiency in improving quality to bring it to full clients' satisfaction and expectation [176]. This development process of new and expected medical device technology-based detectors can only benefit from a generic theoretical framework, where development scenarios and device exemplars are the objects of a thorough discussion. Hopefully, enough users from a wide representation can be assembled to achieve the quality objectives [177].

Conclusion

The aim of the literature review concerning state-of-the-art (SoA) in the field of bio-detection was to provide a comprehensive analysis of existing technologies and their application. It is necessary from the project perspective to evaluate existing methods and trends in pathogen detection. The quick, simple and reliable method of pathogen identification plays a crucial role in the confirmation of pathogen presence and directly influences public/law enforcement/medical services activity. The existing and commonly used technologies are based mainly on the identification of the presence of pathogen nucleic acids. These methods provide fairly reliable results. However, as all existing technologies, nucleic acids based detection technologies possess imperfections. It should be mentioned about the time necessary to obtain the results, the costs, the necessity of serious human engagement in reaction set up, equipment and most important knowledge and experience from device operators to interpret obtaining results. The usual cost of a single RT PCR reaction is range approx. 100 – 150 EUR per reaction. The current epidemiological with SARS CoV 2 situation virus clearly showed that, in days when all EU member state countries executing tens of thousands to couple hundred thousand reactions per day. This ratio is serious and overburden state or in the case by individual payment domestic budget. Other technologies based e.g. on immunochromatographic methods are widely used in pathogen detection. They can be characterized by fairly low price, low human involvement, however their reliability is far from good level. Furthermore, they are very sensitive to the presence of inhibition substances in tested sample and this way they can produce false positive or negative results.

The proposed project application of digital holographic microscopy in the field of bio-detection appears to be very promising. The technology in not new, however, observed progress in all fields of technologies opening the doors in the efficient application of this technology in pathogen detection. In comparison to other detection technology, it offers quick, fairly reliable results with unitary low cost per single detection. Furthermore, combining existing DHM with ability of connected software deep learning process allow to limit the human involvement in the detection process. The other worth mentioned benefit of DHM technology with deep learning software is technology openness, which allows to continuous learning process and inclusion of new emerging pathogen signatures, which definitely appear in near future. Moreover, the observed technology progress will also allow miniaturizing the elaborated technology, which creates the opportunity of widely using of this technology. In final, the performed comprehensive literature analysis emphases, the necessity of introduced a new tool in pathogen detection and the DHM with deep learning capability appear to be sufficient and fulfilling the existing expectations from detection technology.

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