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Surface Enhanced Raman Spectroscopy (SERS) of Bacillus endospores – The search for the ideal SERS substrate

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Agenda

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Introduction

Raman and SERS of Bacteria

Research on SERS of Bacteria at IOE MUT

Way forward

Summary

Terrorist bioattack threat

Paris, France

Brussels, Belgium

Nice, France

Terrorist bioattack threat

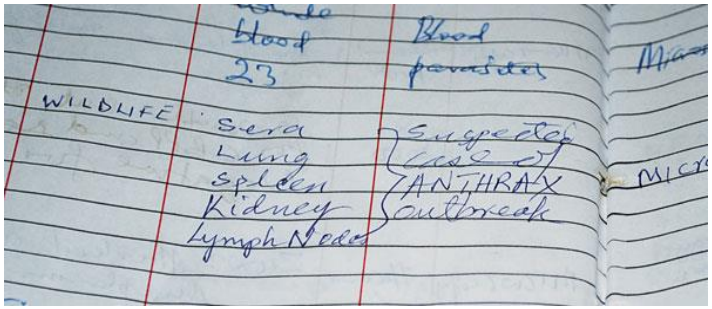
Biosecurity Effort Expands To Africa - Chemical & Engineering News 2011

The Cooperative Threat Reduction (CTR) program - U.S. effort to secure deadly pathogens to prevent their use in bioterror attacks.

Some of the world's deadliest diseases—including the Ebola, Marburg, and Rift Valley Fever viruses—occur naturally in Africa, a volatile region of the world where civil upheaval and terrorism are widespread.

A Ugandan facility that Senator Lugar visited lacks the proper tools to handle samples carrying deadly diseases such as anthrax and Ebola virus.

A handwritten logbook at a lab in Uganda notes a "suspected case of ANTHRAX outbreak."



Terrorist bioattack threat

The examples of the categories A, B and C bioagents according to the Centers for Disease Control and Prevention (CDC)

Category A Diseases/Agents	
Anthrax (*Bacillus anthracis*)	Botulism (*Clostridium botulinum* toxin)
Plague (*Yersinia pestis*)	Smallpox (variola major)
Tularemia (*Francisella tularensis*)	Viral hemorrhagic fevers (filoviruses [e.g., Ebola, Marburg] and arenaviruses [e.g., Lassa, Machupo])
Category B Diseases/Agents	
Brucellosis (*Brucella* species)	Epsilon toxin of *Clostridium perfringens*
Food safety threats (e.g., *Salmonella* species, *Escherichia coli* O157:H7, *Shigella*)	Glanders (*Burkholderia mallei*)
Melioidosis (*Burkholderia pseudomallei*)	Psittacosis (*Chlamydia psittaci*)
Q fever (*Coxiella burnetii*)	Ricin toxin from *Ricinus communis* (castor beans)
Staphylococcal enterotoxin B	Typhus fever (*Rickettsia prowazekii*)
Viral encephalitis (alphaviruses [e.g., Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis])	Water safety threats (e.g., *Vibrio cholerae*, *Cryptosporidium parvum*)
Category C Diseases/Agents	
Emerging infectious diseases such as Nipah virus and hantavirus	

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Biodetection

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Cell Culture
- Differential Media
- General Media
- Selective Media
- Specialty Media
- Viral Culture

Electrophoresis
- Denaturing Gradient Gel Electrophoresis (DGGE)
- Polyacrylamide Gel Electrophoresis (PAGE)
- Pulsed-Field Gel Electrophoresis (PFGE)

Flow Cytometry

Immunological
- Cellular Analysis and Notification of Antigen Risks and Yields (CANARY)
- Electrochemiluminescence
- Enzyme-linked Immunosorbent Assay (ELISA)
- Fluorescence
- Lateral Flow/ Hand Held Immunoassay
- Magnetic Immunoassay
- Surface Plasmon Resonance
- Western Blot (WB)

Microscopy
- Atomic Force Microscopy (AFM)
- Fluorescence Microscopy (FM)
- Phase Contrast Microscopy (PCM)
- Scanning Electron Microscopy (SEM)
- Transmission Electron Microscopy (TEM)

Molecular
- Capillary Electrophoresis Sequencing (CES)
- Digital PCR (DPCR)
- DNA aptamer
- Evanescent Wave Fiber-Optic Biosensor (EWFO)
- Lawrence Livermore Microbial Detection Array (LLMDA)
- Microarray (MA)
- Next Generation Sequencing (NGS)
- Northern Blot (NB) RNA
- Polymerase Chain Reaction (PCR)
- Single Molecule Real-Time Sequencing (SMRT)
- Southern Blot (SB) DNA
- Zero-Mode Waveguide (ZMW)

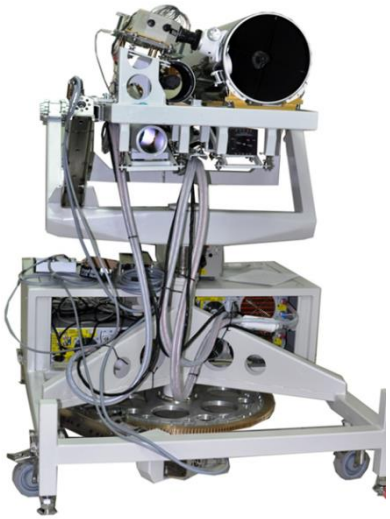
Molecular Spectroscopy
- Electrochemical Impedance Spectroscopy (EIS)
- Fluorescence
- Mid-infrared Spectroscopy (MIR)
- Near Infrared (NIR)
- **Raman Spectroscopy (Raman)**
- **Surface Enhanced Raman Spectroscopy (SERS)**
- Ultraviolet-Visible (UV-Vis)

Spectrometry
- Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF)
- Quadrupole Mass Spectrometry (MS)

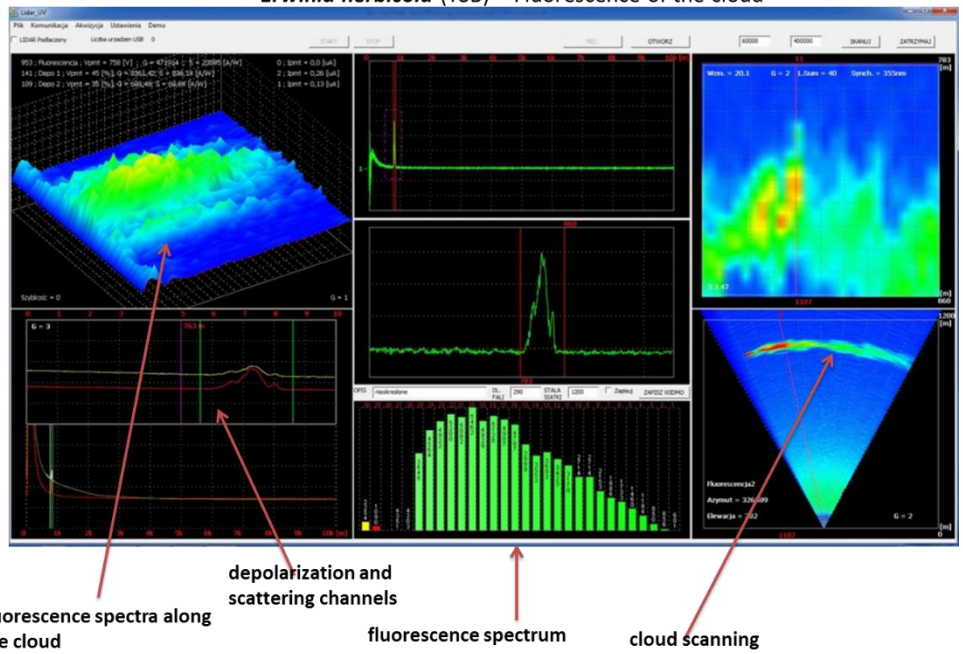
http://www.cbrnetechindex.com/Biological-Detection

Biodetection Fluorescence

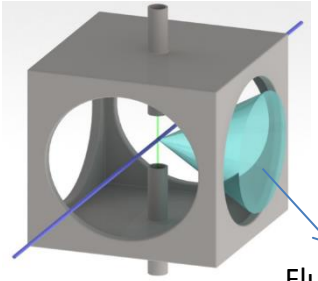
Stand off and point biodetection systems developed in IOE MUT



Erwinia herbicola (TSB) – Fluorescence of the cloud

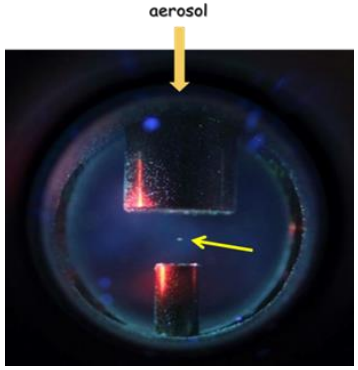


Aerosol inlet



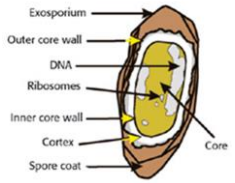
UV laser beam

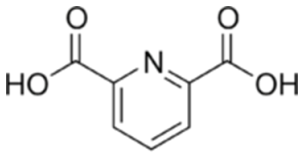
Fluorescence and scattering

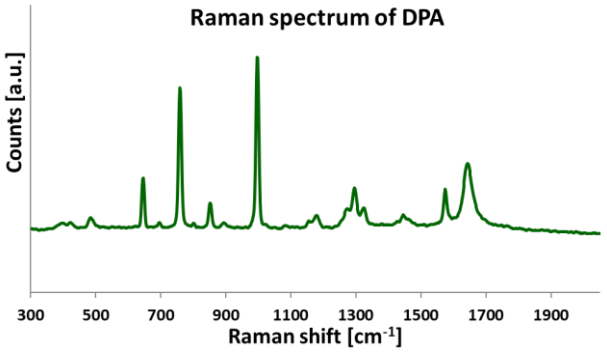


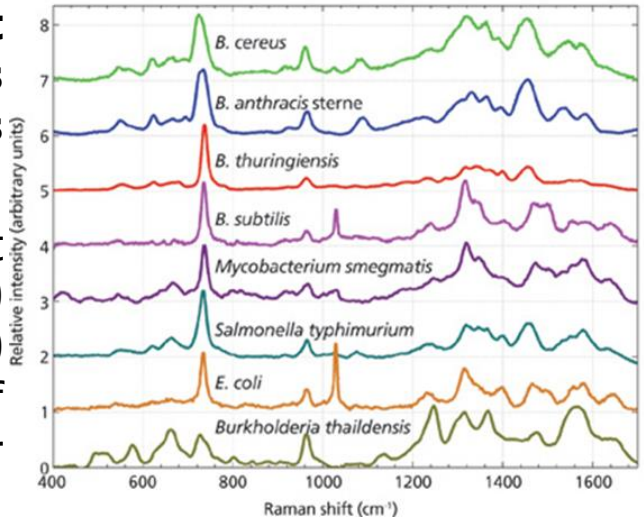
Biodetection
Raman Spectroscopy

- Immunological (i.e. ELISA) or moelcular (i.e. PCR) methods are capable of detecting B. anthracis, and in some cases differentiating Bacillus species, well below the human LD50. However, they are too slow for appropriate interventions. **Raman spectroscopy can provide almost imediate response to biothreat.**
- Spectroscopic techniques such as **Raman spectroscopy have successfully been used to identify B. endospores through the detection of the acidic and/or calcium-chelated dipicolinate ion (DPA)**. DPA represents ca. 10% of the total spore weight and is not found in natural interferents.
- **Raman spectroscopy can be used for the direct detection of DPA existing inside the spore and it has been used to detect DPA contained in a single Bacillus endospore.**
- Typically, the significant regions of the Raman spectrum that are observed within biological specimens fall within 400–2000 cm^{-1}, associated with bond vibrations of proteins (1500–1700 cm^{-1}), carbohydrates (470–1200 cm^{-1}), phosphate groups of DNA (980, 1080 and 1240 cm^{-1}) and additional cellular biomolecules.



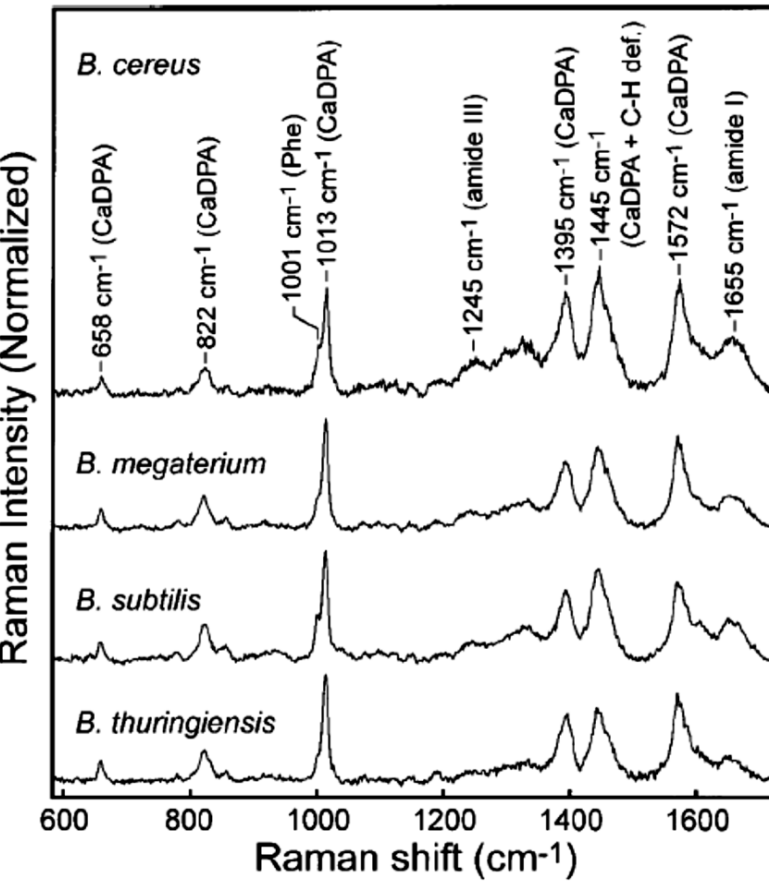






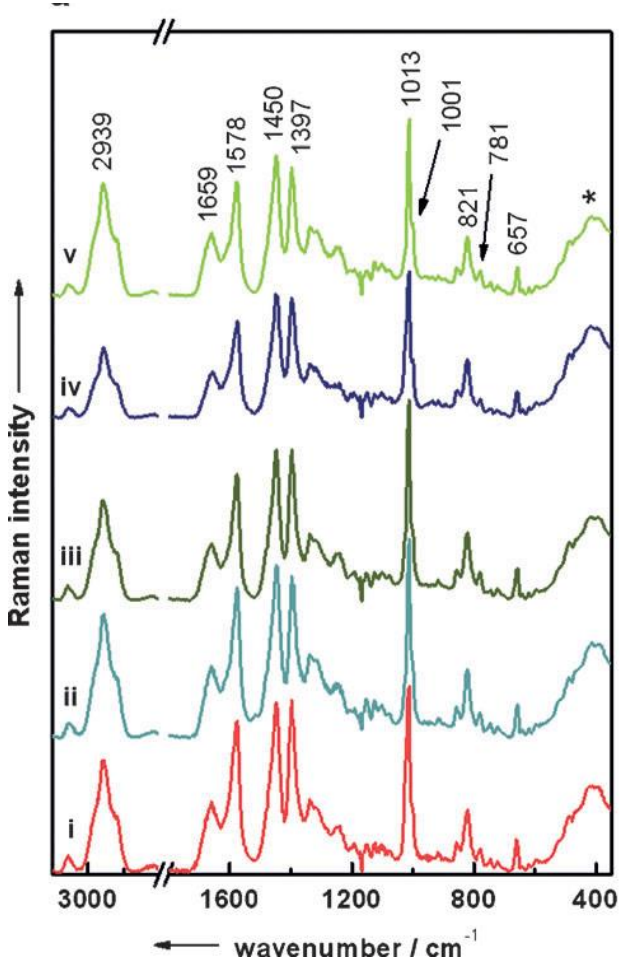
Biodetection
Raman Spectroscopy

Raman spectra of Bacillus endospores and various matrix particles. a) Background-corrected mean Raman spectra of B. anthracis (i), B. megaterium (ii), B. mycoides (iii), B. subtilis (iv), and B. thuringiensis (v).



488 nm
100x
Single spore

S.M. Lane et al. Appl. Spectrosc. 2003, 57, 868



532 nm
100x
Average of ca. 1000 spectra

J. Popp et al. Angew. Chem. Int. Ed. 2012, 51, 5339

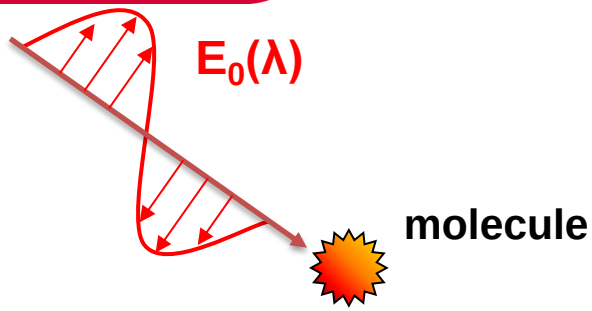
Biodetection
Raman Spectroscopy

REBS
Resource Effective
Bio-Identification System
(< $100k per system)

https://www.battelle.org/government-offerings/national-security/cbrne-defense/threat-detection/battelle-rebs

	REBS (Resource Effective Bioidentification System)
Principle of operation	By combining patented aerosol collection and optical spectroscopy (Raman Microscopy), REBS accomplishes this with new levels of accuracy, sensitivity, and speed, as well as near-zero false alarms.
Number and type of Agents	Over 100 pathogens - liquid, solid and aerosolized bacteria, viruses, toxins, and persistent chemical aerosols in ambient conditions
Collection Interval	Continuous
Assay Time	< 30 min
Limit of Detection	$< 10^6$ CFU×Min/M^3 150-200 PPL
False Alarm Rate	Identification false positive rate: ≤ 0.001%
Size	18” W × 12” D × 12” H
Weight	35 lb

Biodetection Raman Spectroscopy

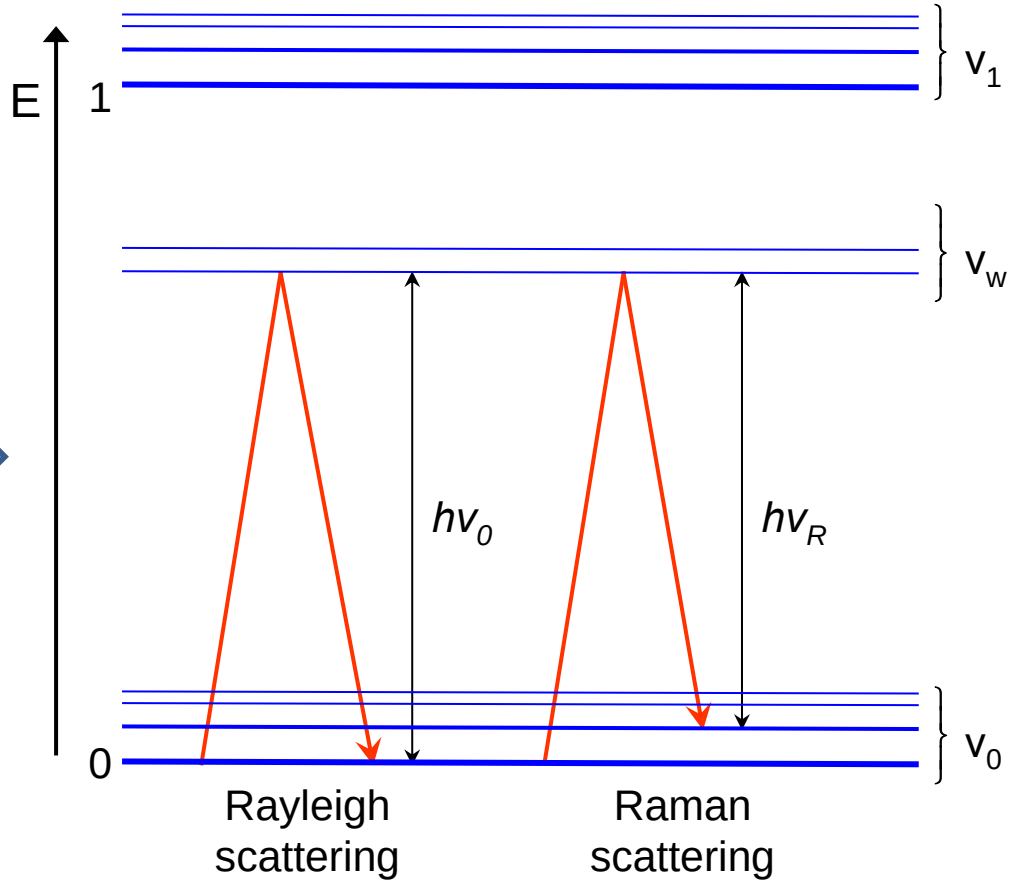


- absorption
- emission
- scattering

PROBLEM !!!

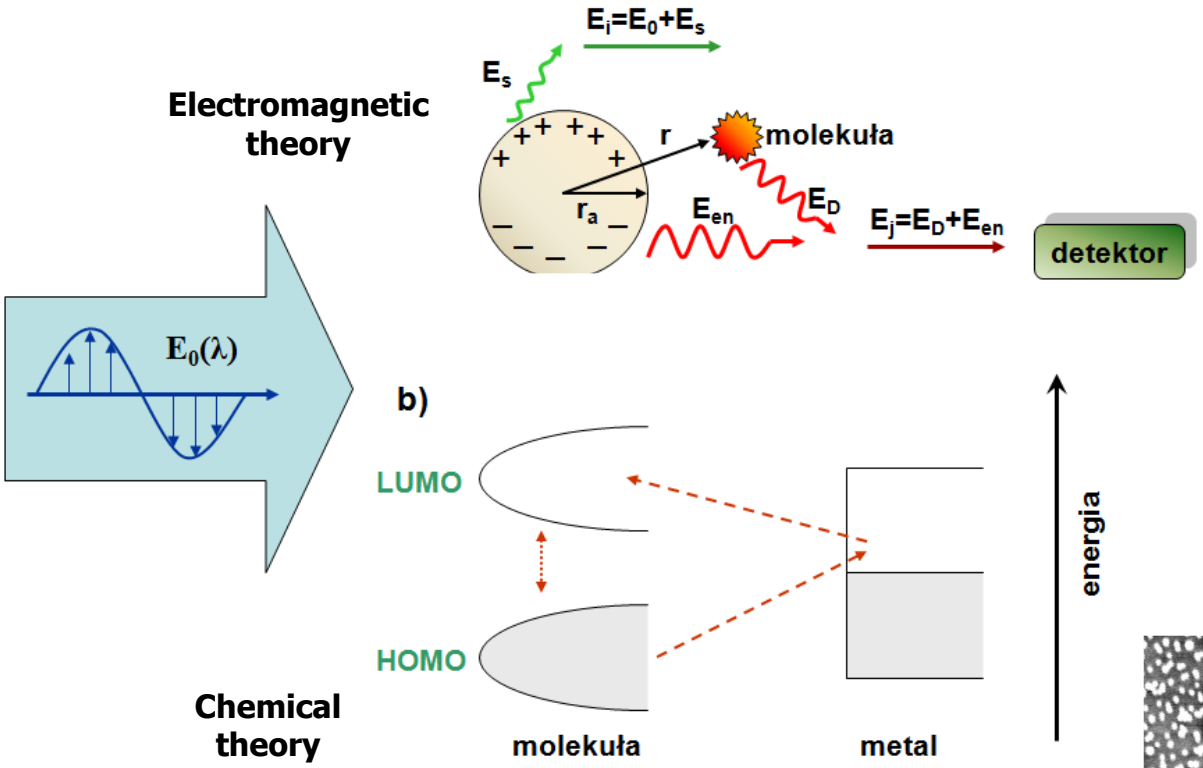
Low signal intensity

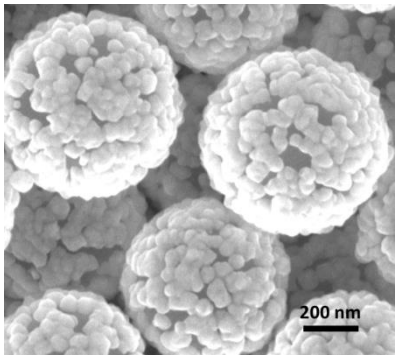
$$\frac{I_{Ram}}{I_{Ray}} \approx 10^{-4}$$

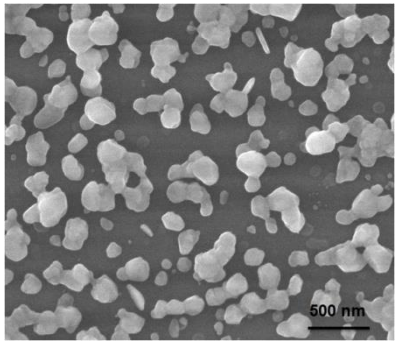


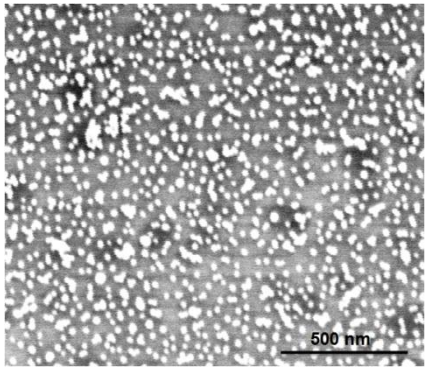
H. Barańska, A. Łabuzińska, J. Trepiński, Laserowa spektrometria ramanowska, zastosowania analityczne, Warszawa 1981

Biodetection SERS



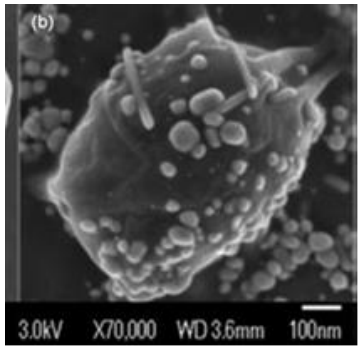




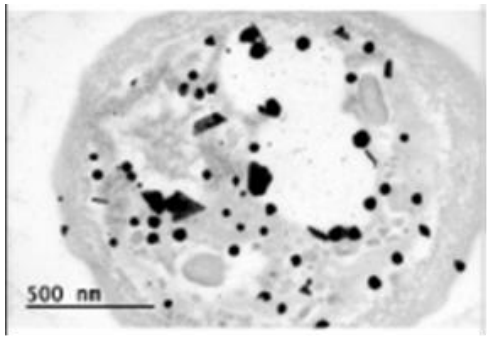


N. Chandrabhas, S. Soumik, *Nanomater. Nanotechnol*. 2012, 2, 1-13.

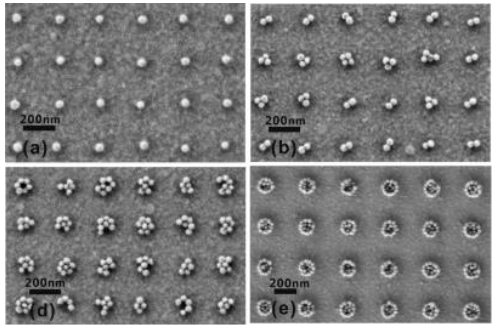
SERS of Bacteria
SERS Substrates



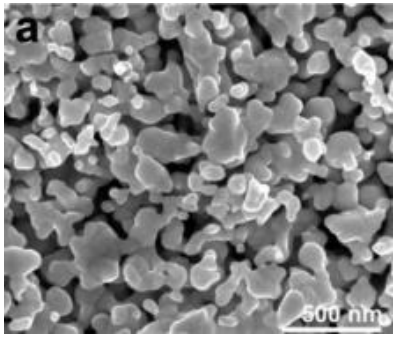
J. Raman Spectrosc. 2010, 41, 1632.



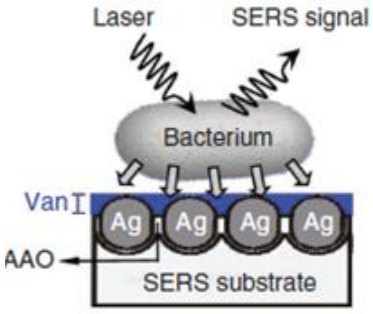
ACS Sustainable Chem. Eng. 2014, 2, 1599



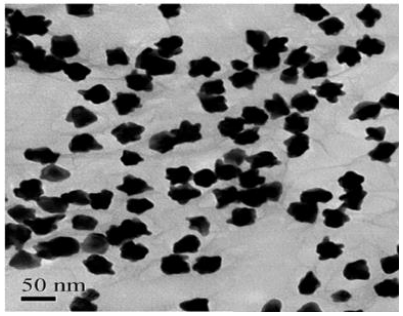
ACS Nano 2009, 3, 1190



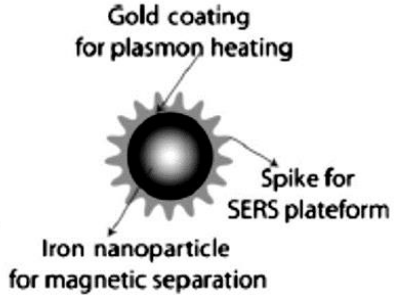
Analyst, 2014, 139, 1037



Nature Comm. 2011, 2, 538

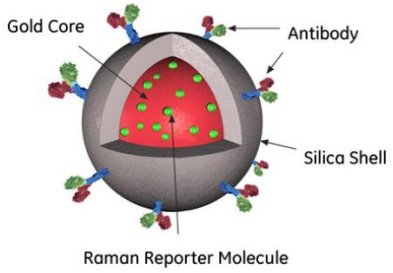


J. Phys. Chem. Lett. 2013, 4, 3813



Biosens. Bioelectron. 2012, 31, 130

Chem. Eur. J. 2013, 19, 2839



Proc. SPIE 2009, 7319, 73190C

SERS of Bacteria

J|A|C|S
ARTICLES
Published on Web 00/00/0000

Rapid Detection of an Anthrax Biomarker by Surface-Enhanced Raman Spectroscopy

Xiaoyu Zhang,† Matthew A. Young,† Olga Lyandres,§ and Richard P. Van Duyne*,†

Contribution from the Departments of Chemistry and Biomedical Engineering, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208-3113

A rapid detection protocol suitable for use by first-responders to detect anthrax spores using a low-cost, battery-powered, portable Raman spectrometer has been developed.

Van Duyne reports that the procedure developed in his lab takes only 11 minutes and is sensitive enough to detect about 2,600 anthrax spores--about one-quarter the infectious dose of 10,000 spores.

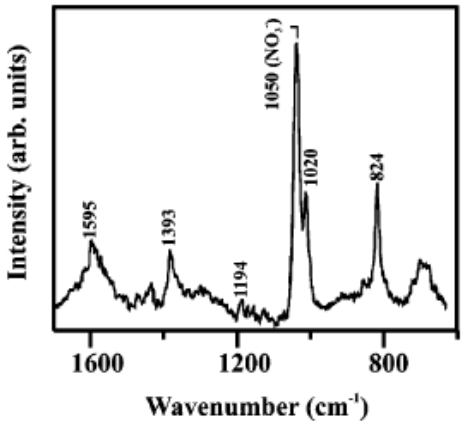


Figure 6. SERS spectrum of 2.1×10^{-14} M spore suspension (2.6×10^3 spores in 0.2 μL, 0.02 M HNO_3) on AgFON; $\lambda_{ex} = 750$ nm, $P_{ex} = 50$ mW, acquisition time = 1 min, $D = 600$ nm, and $d_m = 200$ nm.

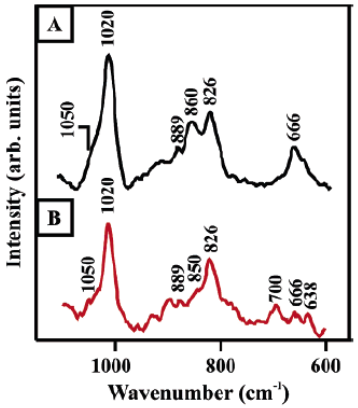


Figure 8. SERS spectra obtained by the portable Raman spectrometer. (A) SERS spectrum of 8.3×10^{-14} M spore suspension (1.0×10^4 spores in 0.2 μL, 0.02 M HNO_3) on 30 day old AgFON. (B) SERS spectrum of 10^{-4} M CaDPA in 0.2 μL of 0.02 M HNO_3 on 30 day old AgFON substrate; $\lambda_{ex} = 785$ nm, $P_{ex} = 35$ mW, acquisition time = 5 s, resolution = 15 cm^{-1}, $D = 600$ nm, and $d_m = 200$ nm.

SERS of Bacteria

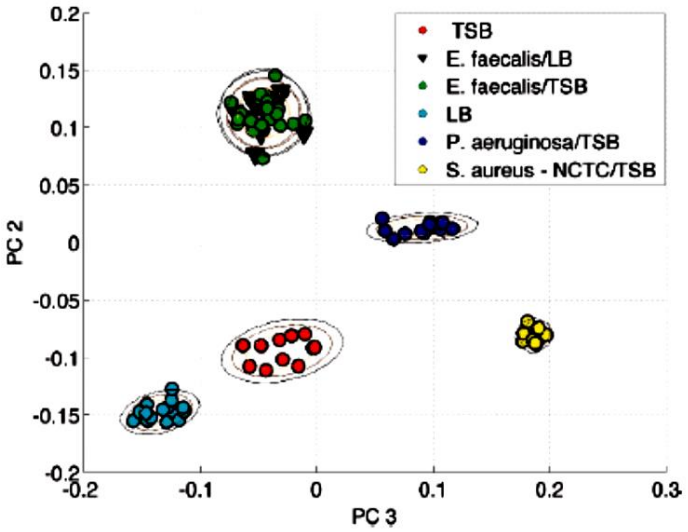


FIG. 3. Barcode-based PCA analysis of SERS spectra of *E. faecalis* grown in LB and TSB, *P. aeruginosa* and *S. aureus* grown in TSB, and growth media TSB and LB alone. The SERS spectra of *E. faecalis* grown in LB and TSB are found to be identical.

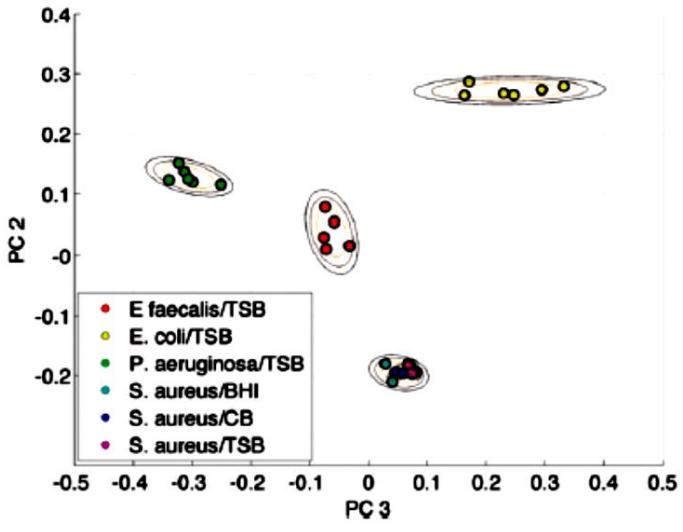


FIG. 4. Barcode-based PCA analysis of SERS spectra of *S. aureus* grown in CB, TSB, and BHI and *P. aeruginosa*, *E. coli*, and *E. faecalis* grown in TSB. The SERS spectra of *S. aureus* grown in CB, TSB, and BHI are found to be identical.

The multivariate data analysis of the SERS spectra of bacteria prepared by the standard multiple washing/centrifugation protocol shows that different bacterial species grown in the same media exhibit different SERS spectra, the same bacterial species grown in different media show the same SERS spectra, and, finally, SERS spectra of bacteria and the culture media in which they are grown are reproducibly distinct.

Ziegler et al. Appl. Spectrosc. 2011, 65, 493

SERS of Bacteria

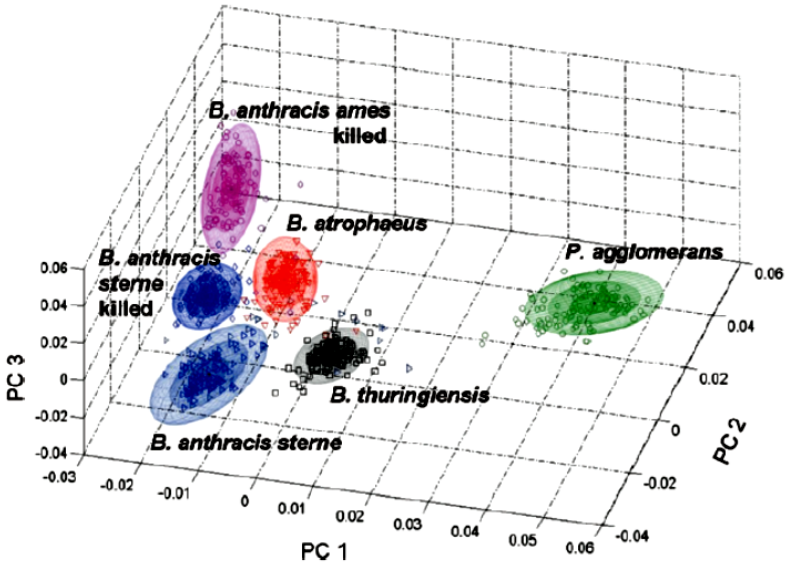


FIG. 4. PCA plot showing discrimination between five *Bacillus* spore samples and *Pantoea agglomerans*.

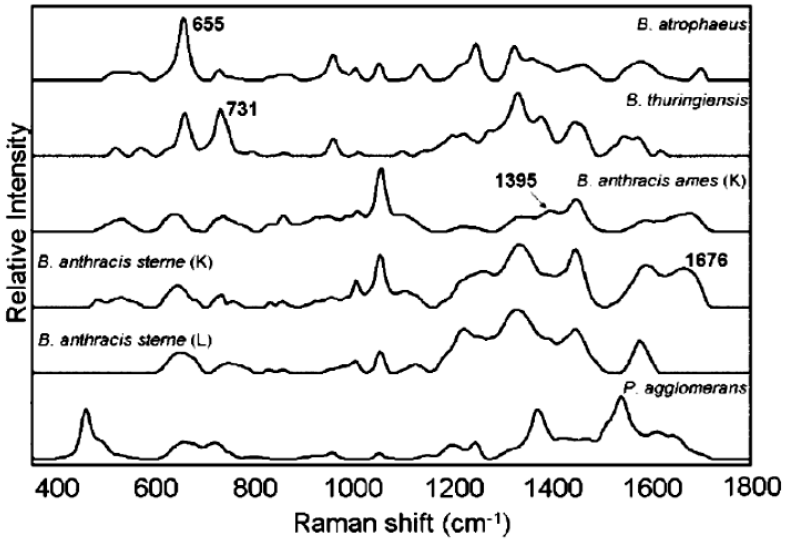


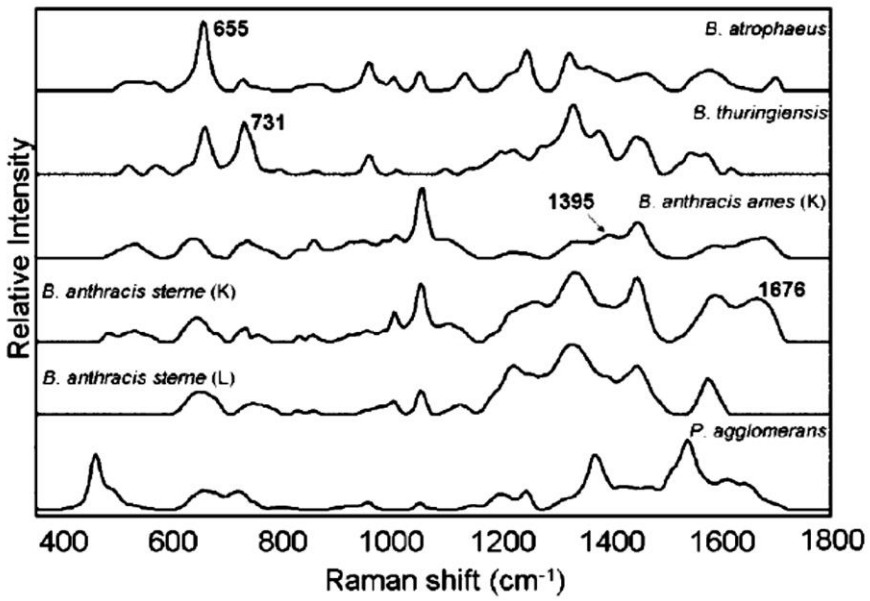
FIG. 5. Representative SER spectra of *B. atrophaeus*, *B. thuringiensis*, *B. anthracis ames* killed, *B. anthracis sterne* (K) killed, *B. anthracic sterne* (L) live, and *P. agglomerans*.

It was shown that use of PCA and SERS spectra allows to discriminate between different species – in this case different strains of Bacillus spores.

In addition, studies have shown that Gram-positive Bacillus spores can be differentiated from Gram-negative vegatative cells.

J. Guicheteau et al. Appl. Spectrosc. 2008, 62, 267

SERS of Bacteria



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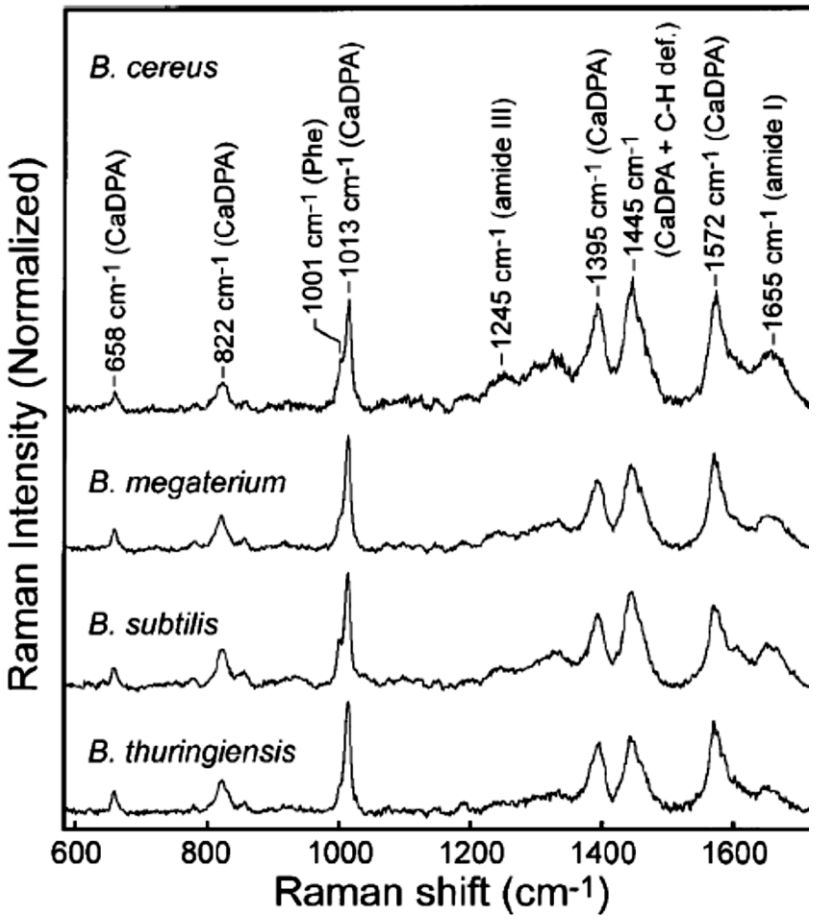
J. Guicheteau et al. Appl. Spectrosc. 2008, 62, 267

vs.



S.M. Lane et al. Appl. Spectrosc. 2003, 57, 868

SERS of Bacteria

SERS of E. Coli

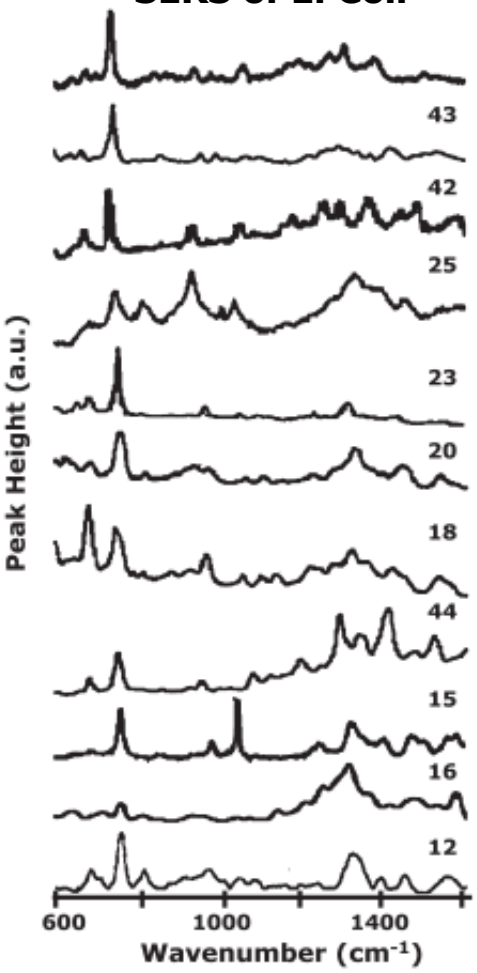
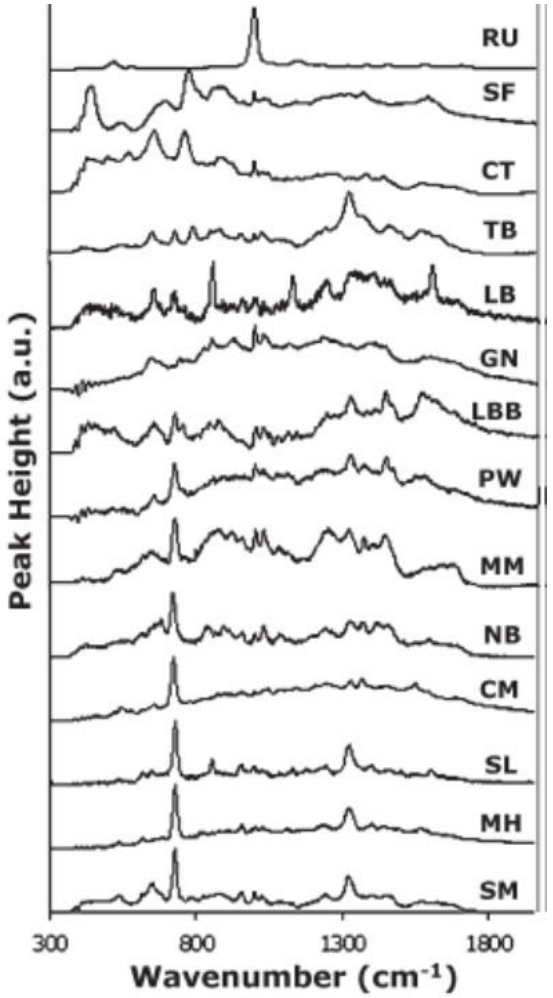


FIG. 7. Published SERS spectra acquired on *E. coli*. The spectra presented in the figure were taken from the reference corresponding to each spectrum label. Spectra were offset along the intensity axis for ease of comparison.

Marotta et al. Appl. Spectrosc. 2010, 64, 601

SERS of Feeding Media



G. McNay et al. Appl. Spectrosc. 2011, 65, 825

SERS of Bacteria – IOE WAT studies

- In 2010 we started work on the fabrication of plasmonic nanostructures for various applications.

- With delivery of new SPM-Raman System in 2010 one of our main directions for plasmonic nanostructure applications became SERS of biological materials.

- **Our main motivation was to answer question whether SERS technique is useful for fast and reliable biodetection by first responders and military personnel using both man-operable and autonomous systems.**

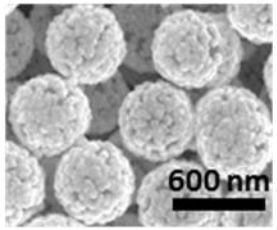
- In our studies we aimed to:

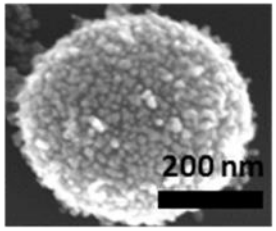
  - Find the most suitable SERS substrates for SERS of bacteria
  - Develop simple procedures for SERS analysis of bacteria
  - Determine whether it is possible to distinquish between various bacterials species using SERS technique
  - Test suitability of SERS technique for use in autonomous biodetection systems

SERS of Bacteria – IOE WAT studies
Nanofabrication

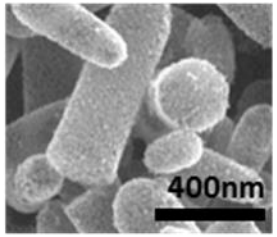
Well equipped Wet Chemistry Laboratory and Thin Film Technology Laboratory allow for fabrication of various plasmonic nanostructures and their modifications.

Core-shell structures SiO_2@Au

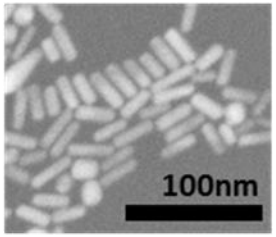




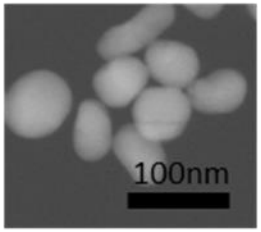
SiO_2@Au



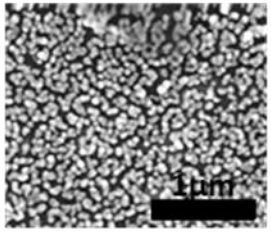
Au nanorods



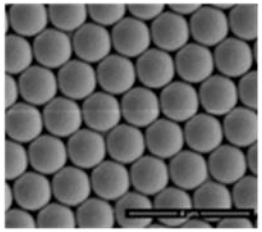
Au colloids

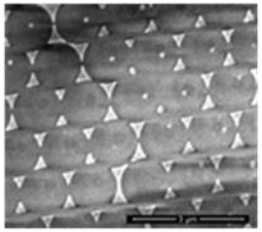


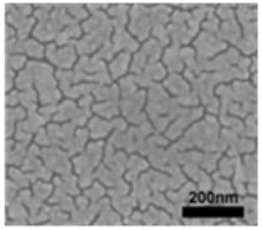
Au nanolayers

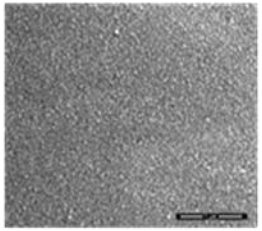


Metal island films









IOE MUT Raman System

Raman-SPM system (the multilaser confocal Renishaw InVia Reflex Raman spectrometer coupled with the NT-MDT Ntegra Spectra SPM microscope)

Lasers: 325 nm, 532 nm, 633 nm, 785 nm

Software: Wire 4 (incl. streamline HR, 3D volume mapping, Particle statistic analysis)

Detectors: Andor EMCCD (1600 x 200 pixels; readout 1515 spectra/s), CCD RenCAM (1024 x 256 pixels)

Optical Microscope: Leica Research Grade

Set of optical objectives:

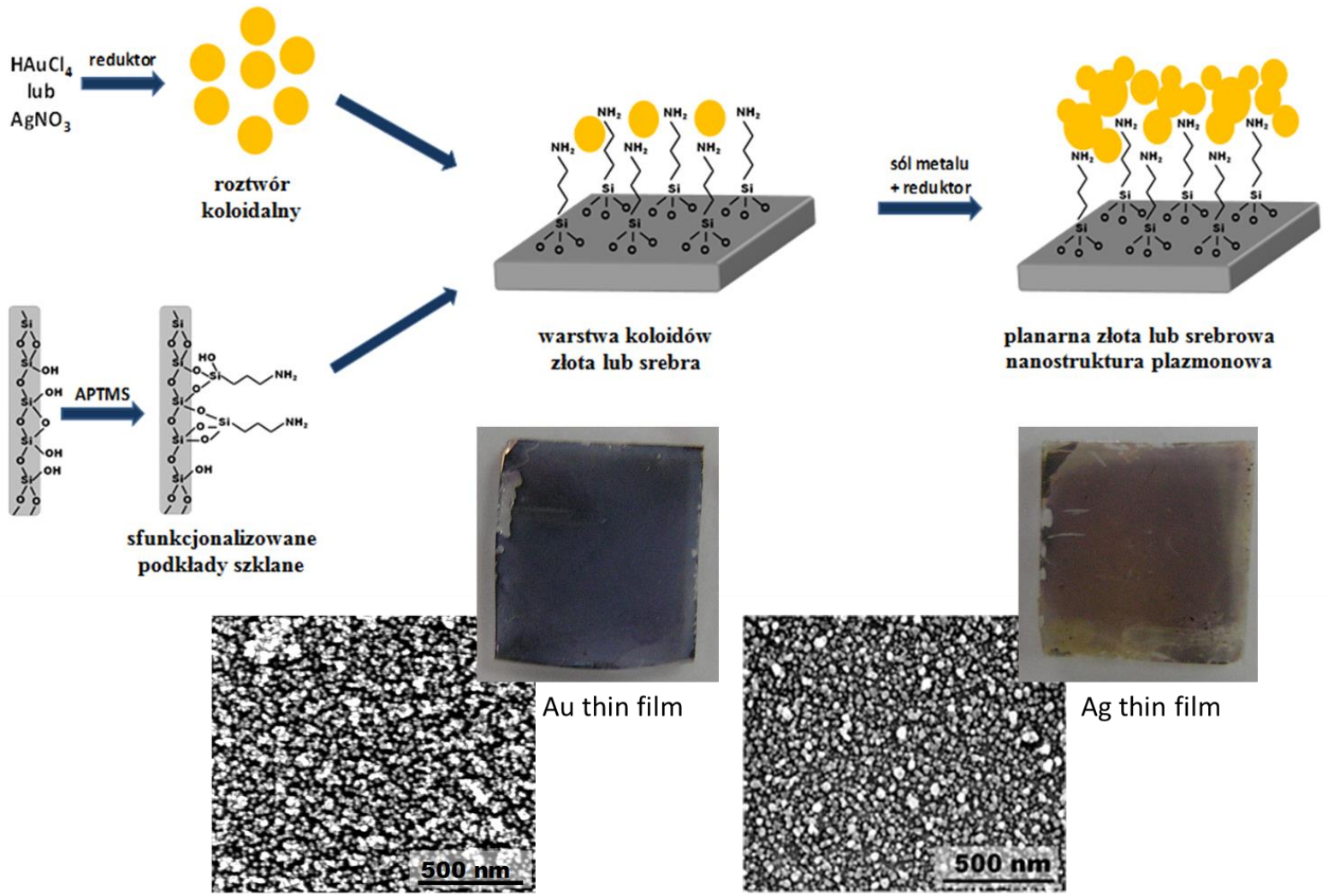
- Vis/NIR: 5X (NA 0.12 WD 13.2 mm), 20X (NA 0.35, WD 20 mm), 50X (NA 0.75, WD 0.37 mm) and 100X (NA 0.9, WD 3.4 mm)
- 100×oil immersion objective N.A. 1.25
- LWD: 20x
- NUV: 40X (NA 0.5, WD 1 mm)

XYZ- HSES Renishaw Mapping Stage

Heating/Cooling Cell: Linkam Scientific Instruments heating/cooling cell kit (-196 °C to 350 °C).

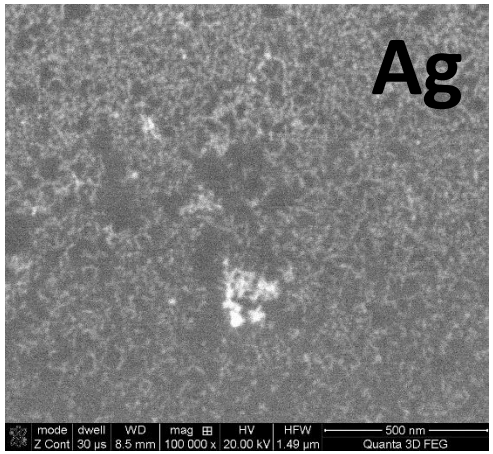
Additional features: Eclipse filter notch for low frequency Raman identification below 15 cm^{-1} both for Stokes and anti Stokes - for 532nm laser; Fibre probe CFOP for 785nm laser; Polarization kits and analyzers for 785nm laser; Multiwell-plate reader with software

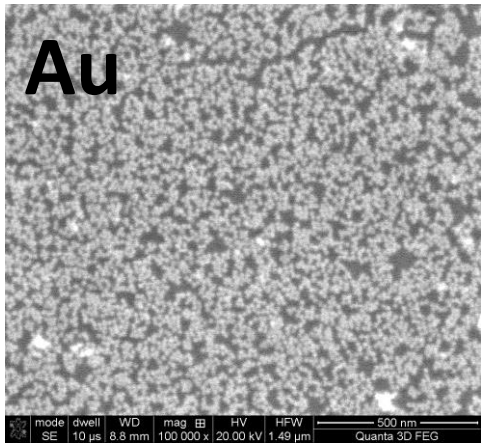
SERS of Bacteria – IOE WAT studies

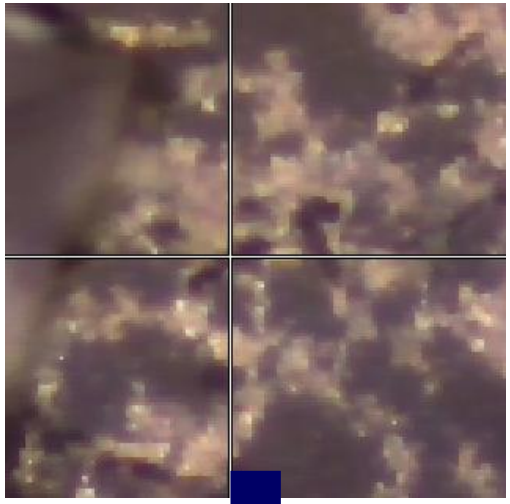


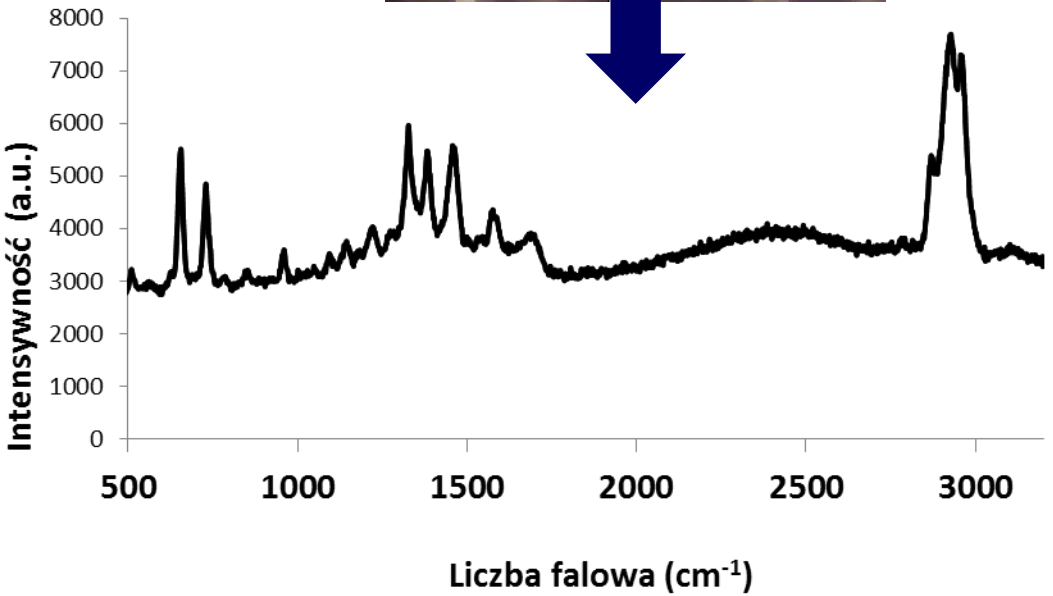
B. Bartosewiczet al. Photonics Lett. Pol. 2013, 5, 48 – 50.

SERS of Bacteria – IOE WAT studies



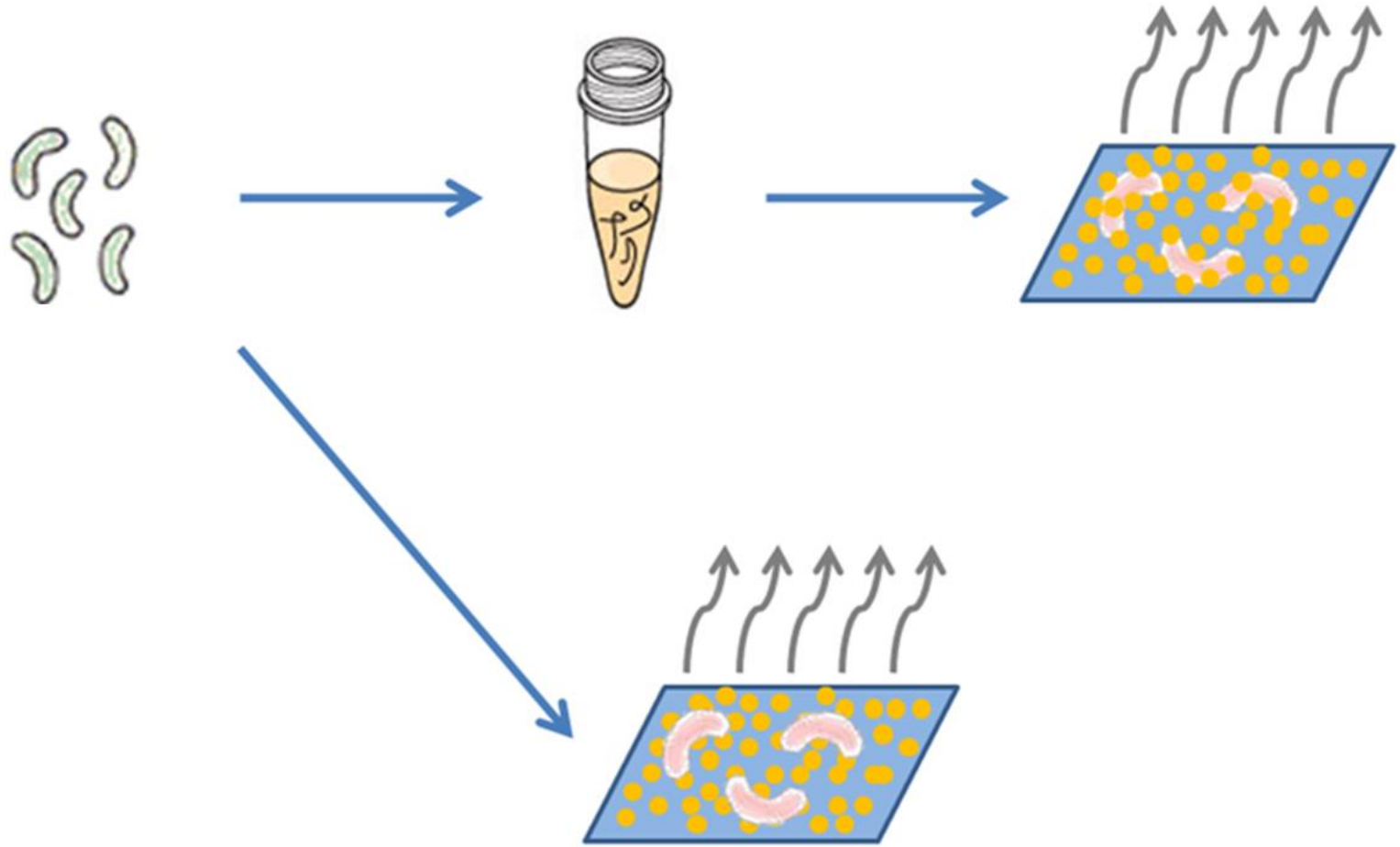




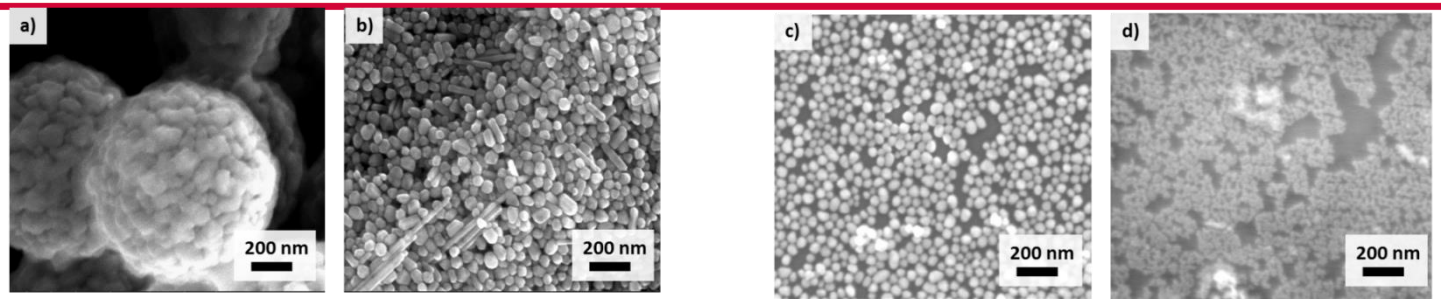


SERS spectrum of single Bacillus thuringiensis cell on Ag colloids

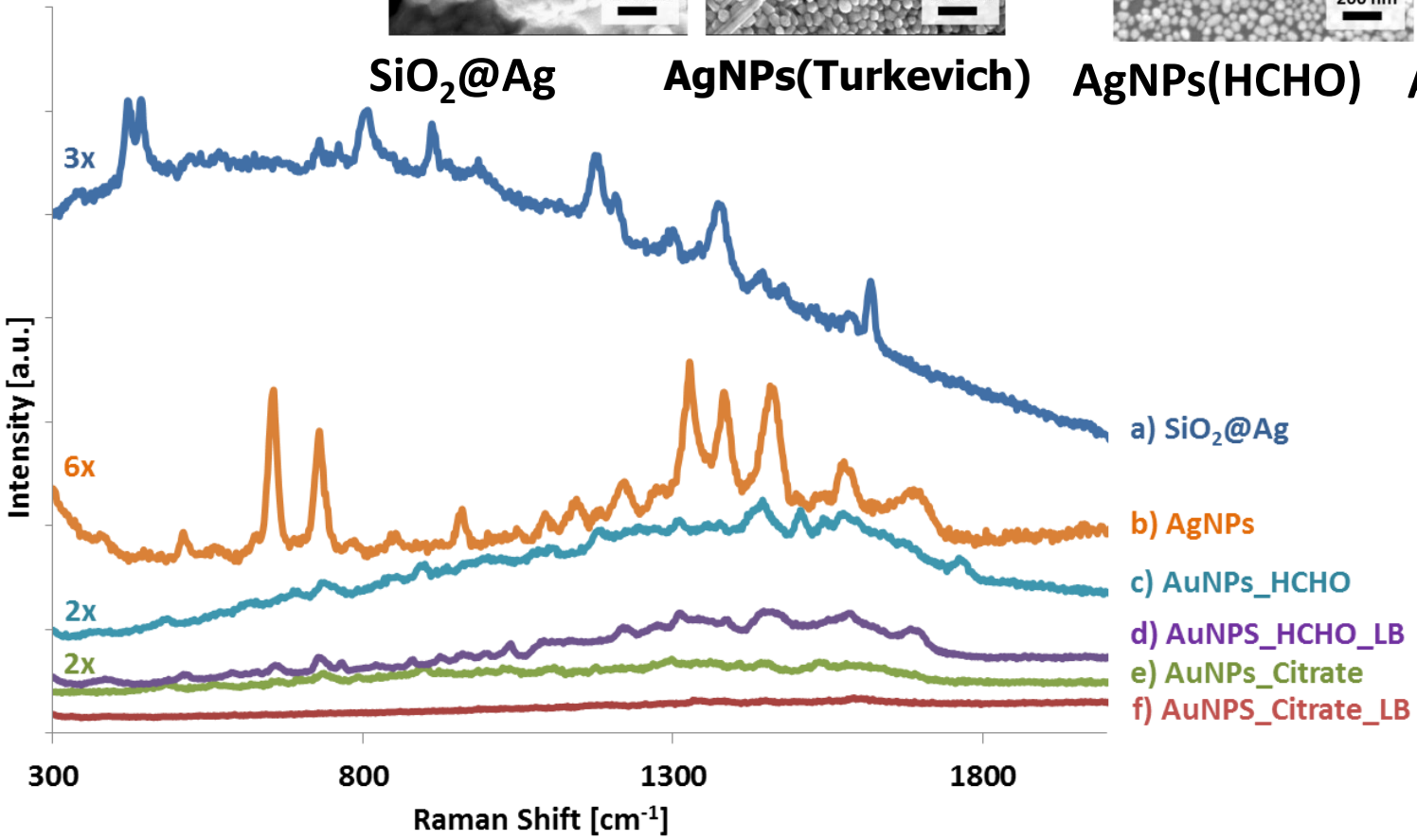
SERS of Bacteria – IOE WAT studies



SERS of Bacteria – IOE WAT studies

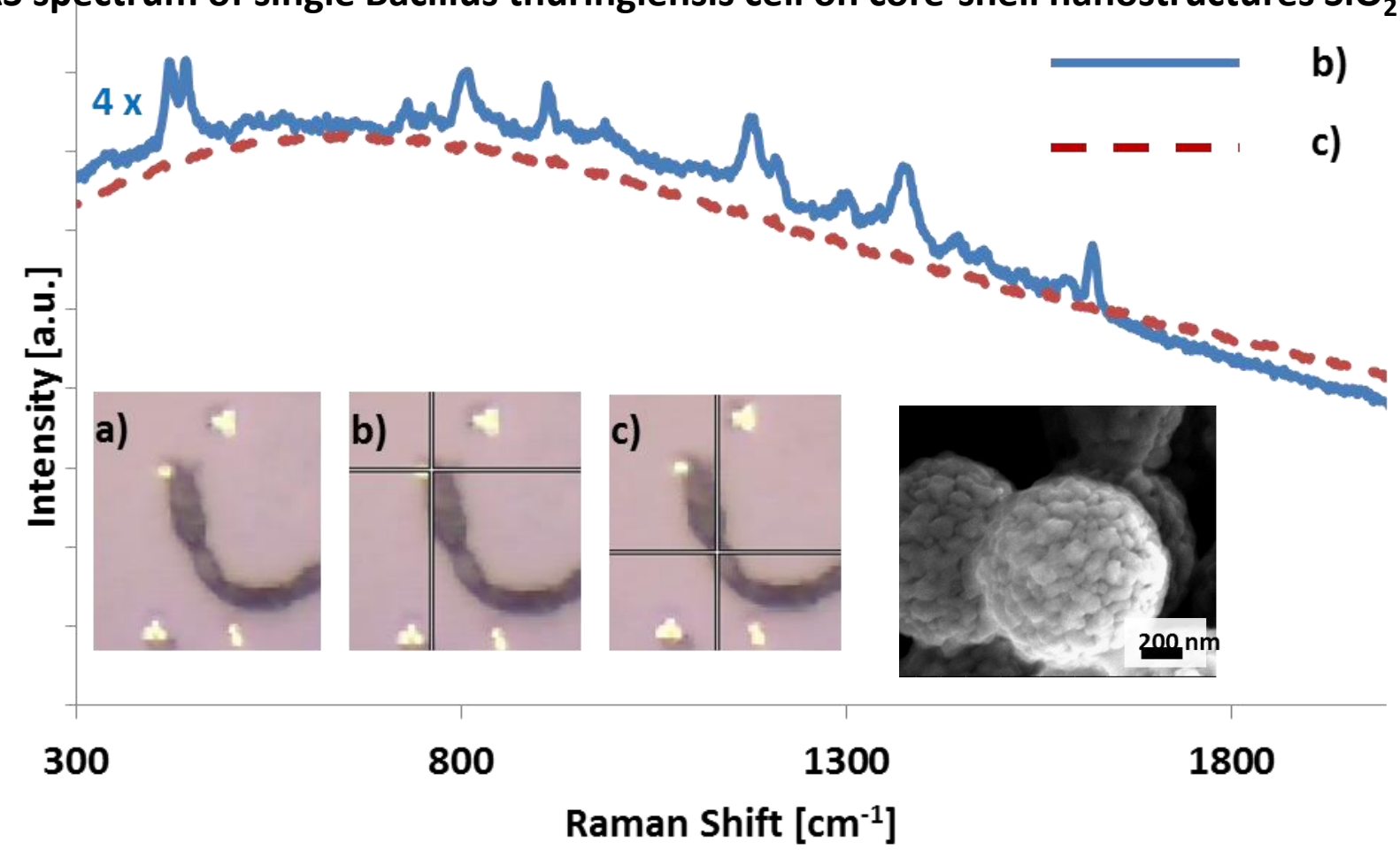


SiO_2@Ag AgNPs(Turkevich) AgNPs(HCHO) AuNPs(Turkevich)



SERS of Bacteria – IOE WAT studies

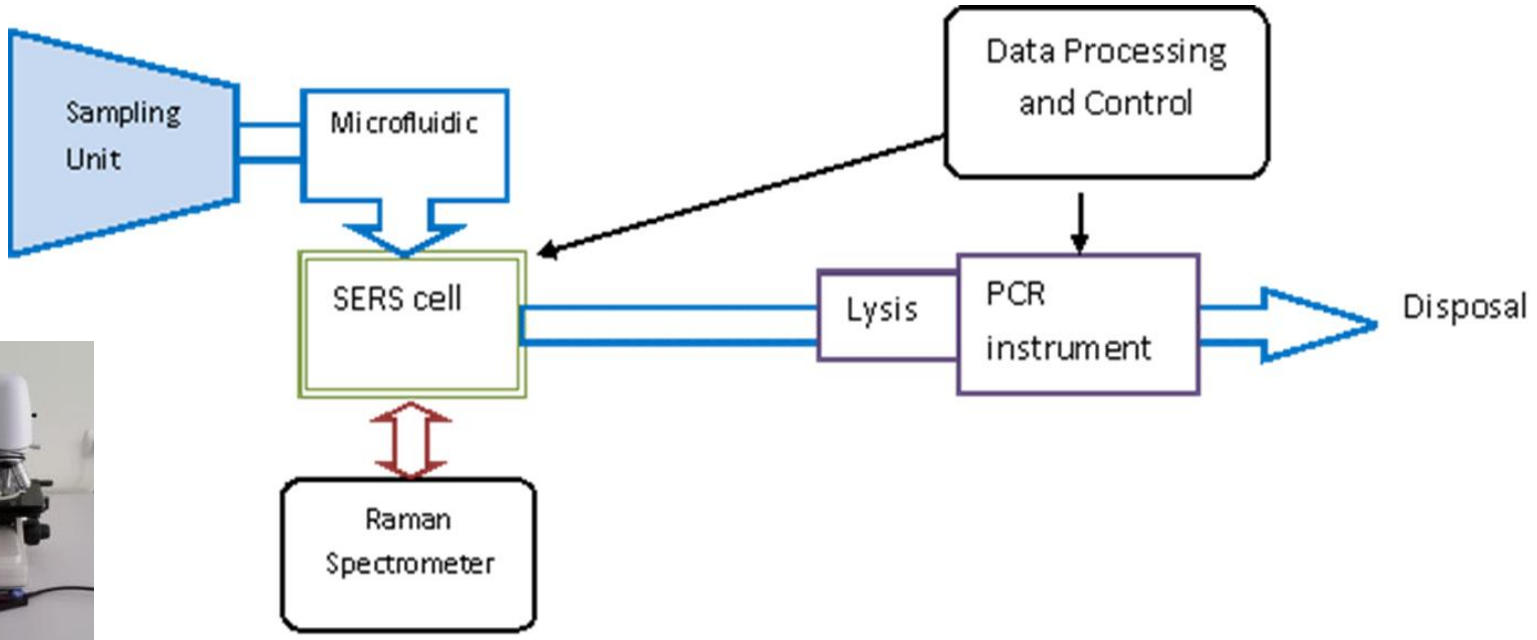
SERS spectrum of single Bacillus thuringiensis cell on core-shell nanostructures SiO_2@Ag

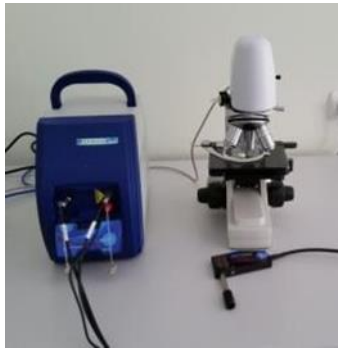


B.J. Jankiewicz et al. Biomed. Spectrosc. Image. 2014, 3, 29.

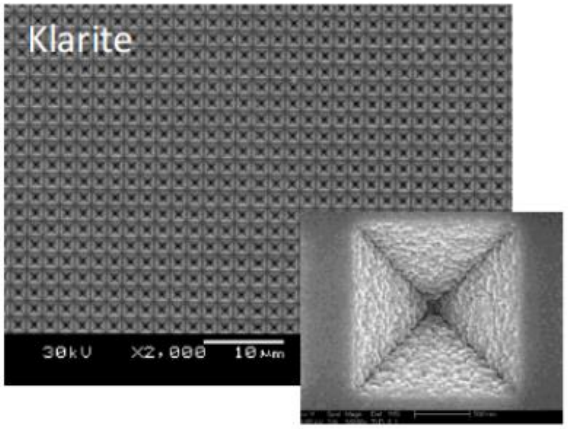
EDA JIP CBRN RAMBO

RAMBO - **R**apid **A**ir-particle **M**onitoring against **BiO**logical threats

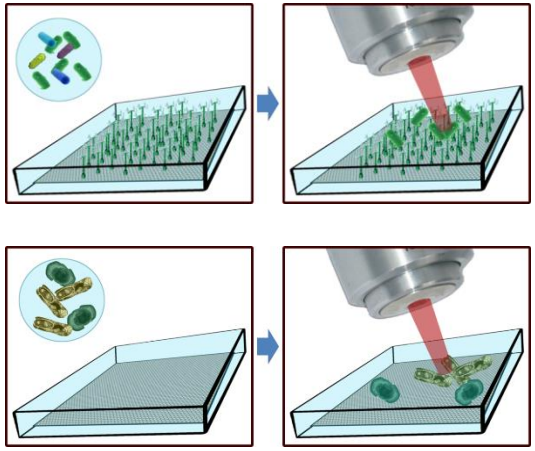




EDA JIP CBRN RAMBO



Withdrawn from the market at the beginning of project

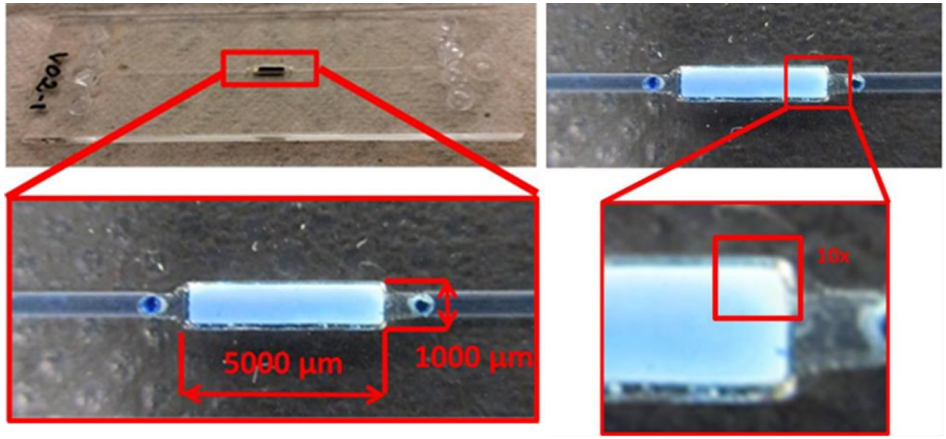


Functionalized

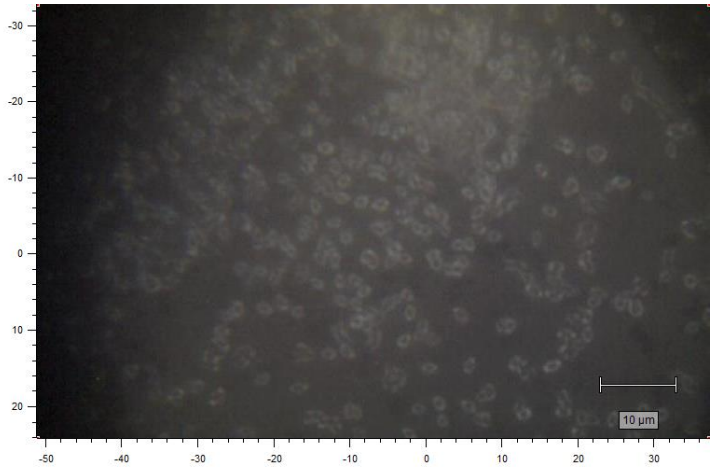
vs.

Non-functionalized

????????????



How to find (automatically) spores on the SERS substrate?

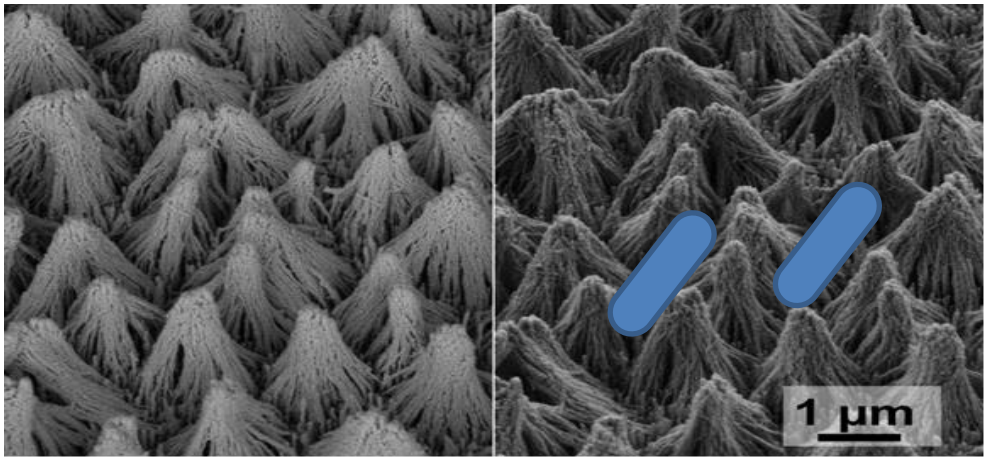


Are too many spores a problem?

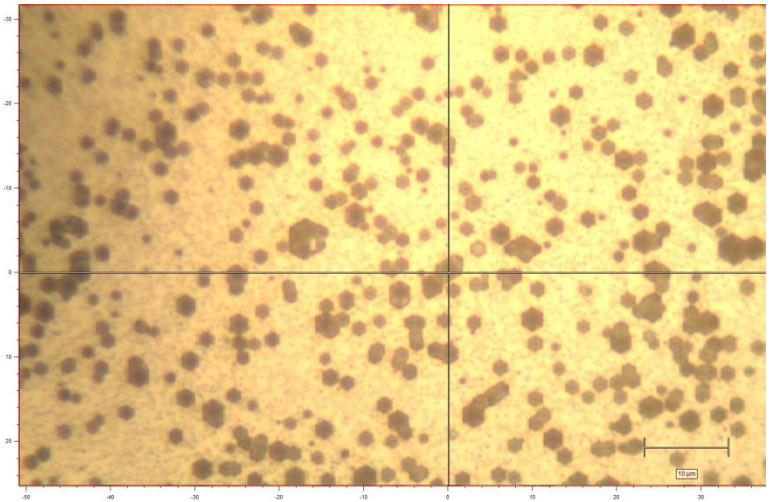
SERS of Bacteria – IOE WAT studies

Testing of other Bacillus bacteria on SERS substrates alternative to Klarite

UNIPRESS provided us GaN based SERS substrates including GaN/Au-Ag, GaN/Au-Cu and GaN/Au with pits



GaN/Au-Ag

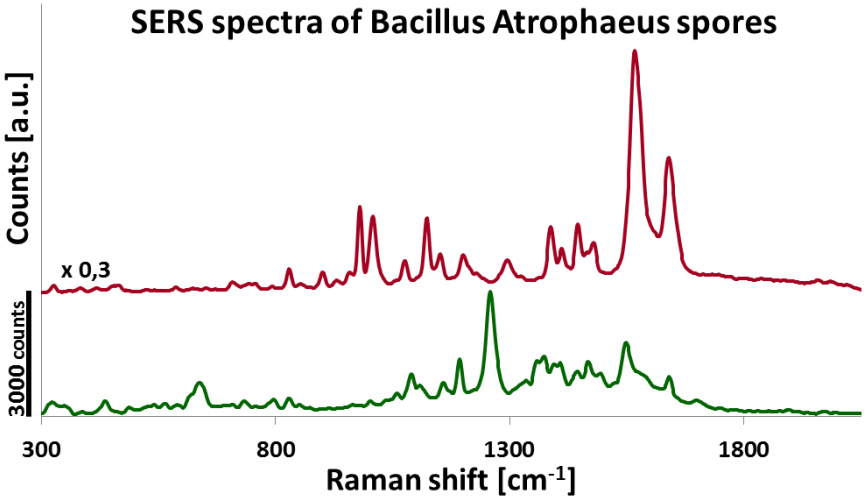


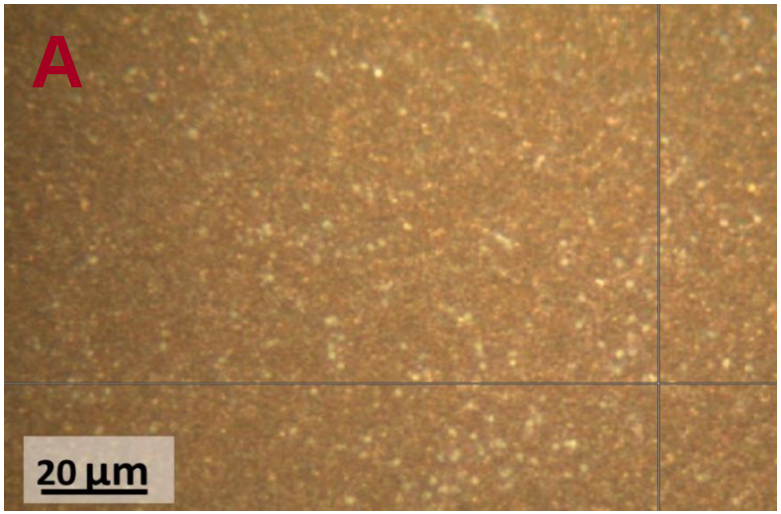
GaN/Au Pits

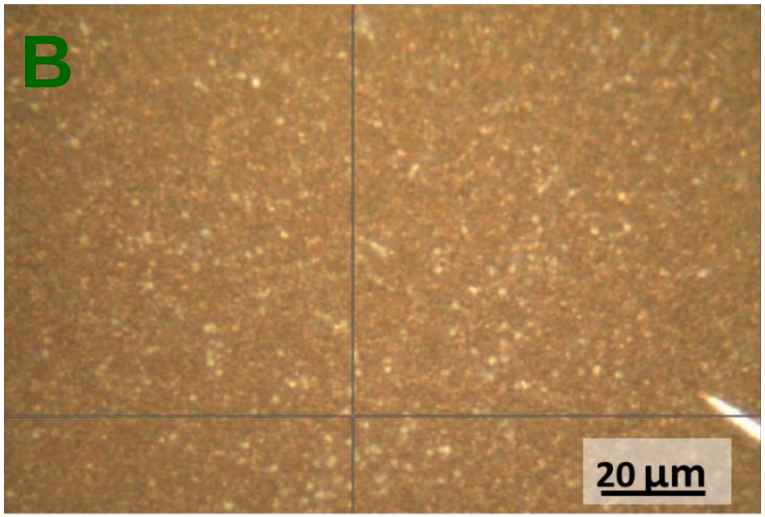
Prof. Janusz Weyher - UNIPRESS

J. Weyher et al. Appl. Surface Sci. 2016, 378, 30-36; J. Phys. Chem. C 2016, 120 (3), 1841-1846; Biosens. Bioelectron. 2015, 66, 461-467; J. Appl. Phys. 2012, 112 (11), 114327; J. Mater. Chem. 2011, 21 (24), 8662-8669.

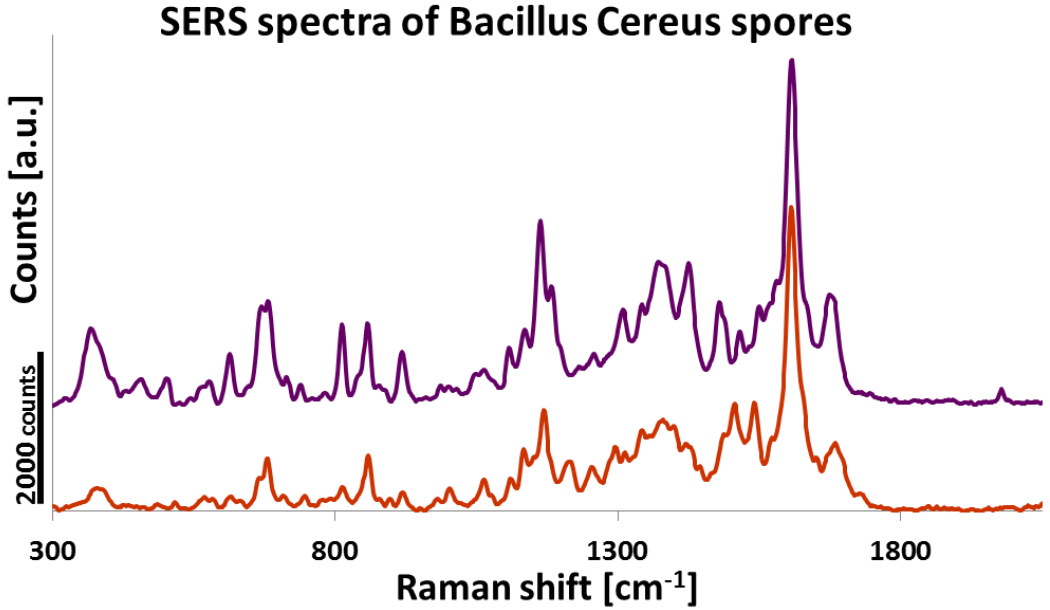
SERS of Bacteria – IOE WAT studies

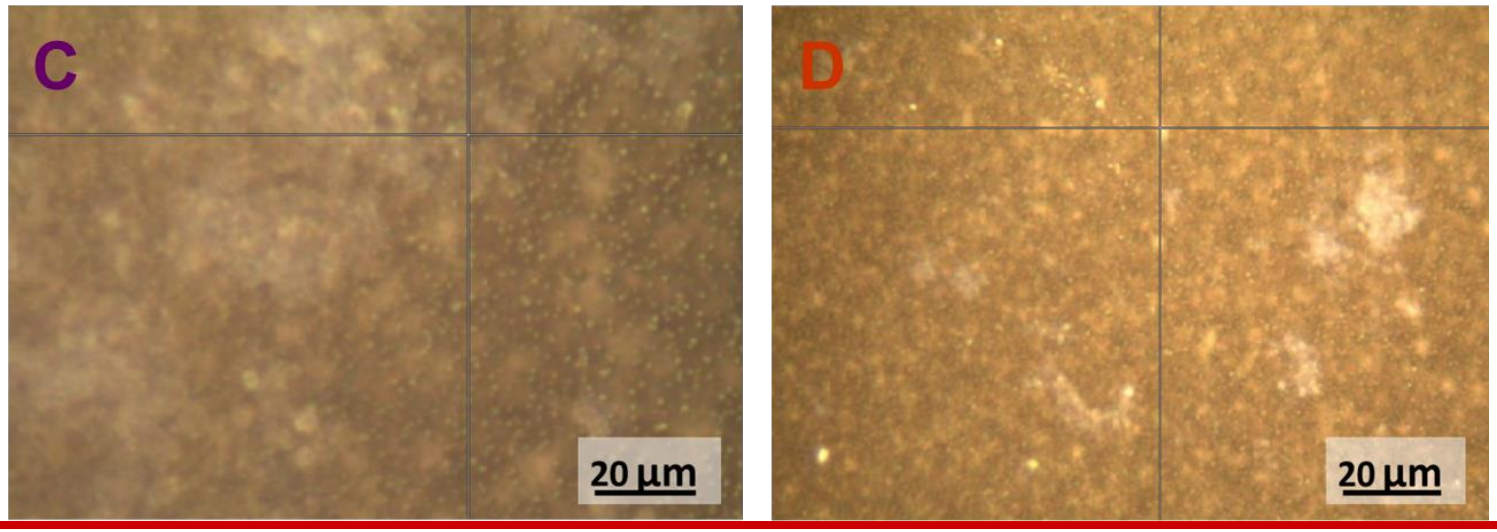




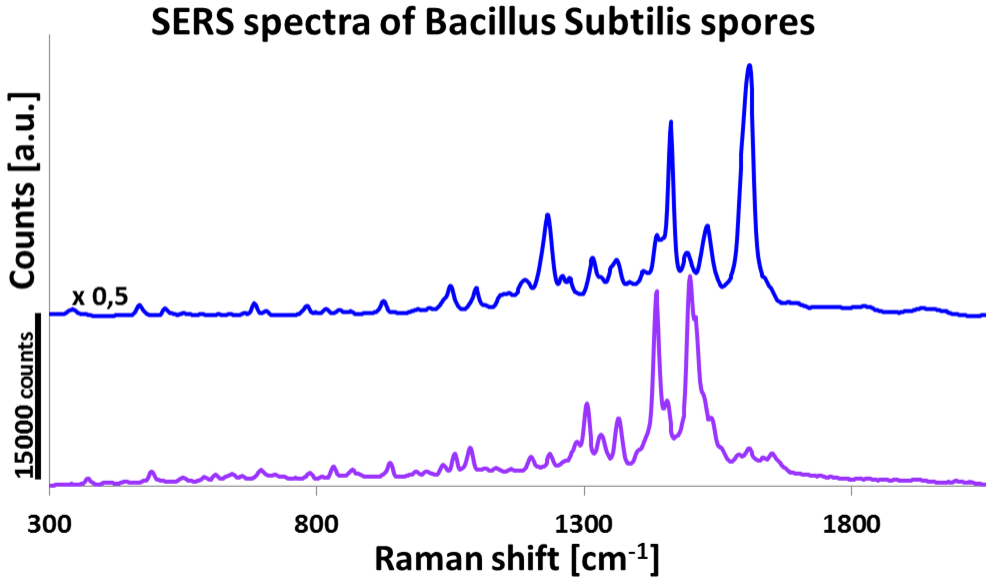


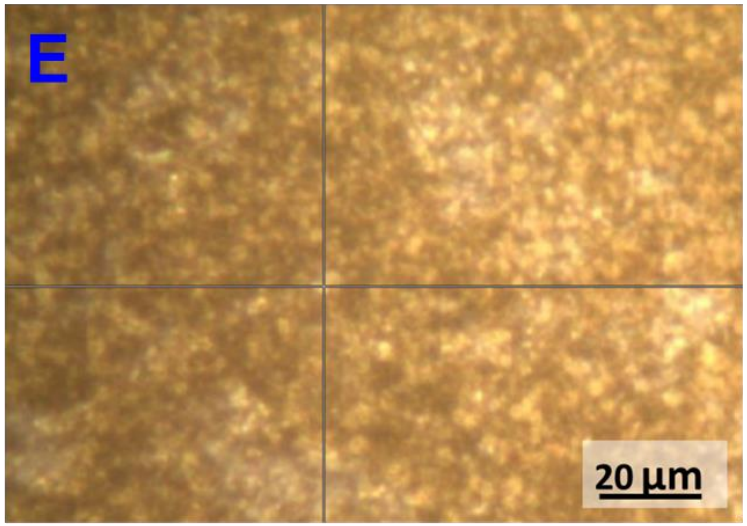
SERS of Bacteria – IOE WAT studies

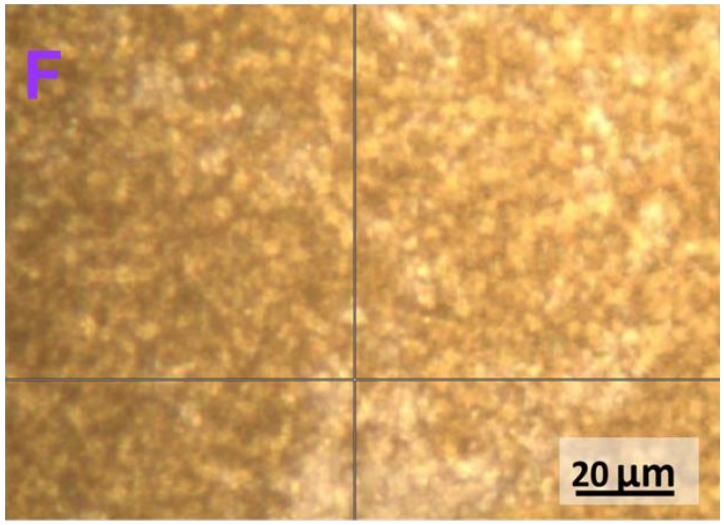




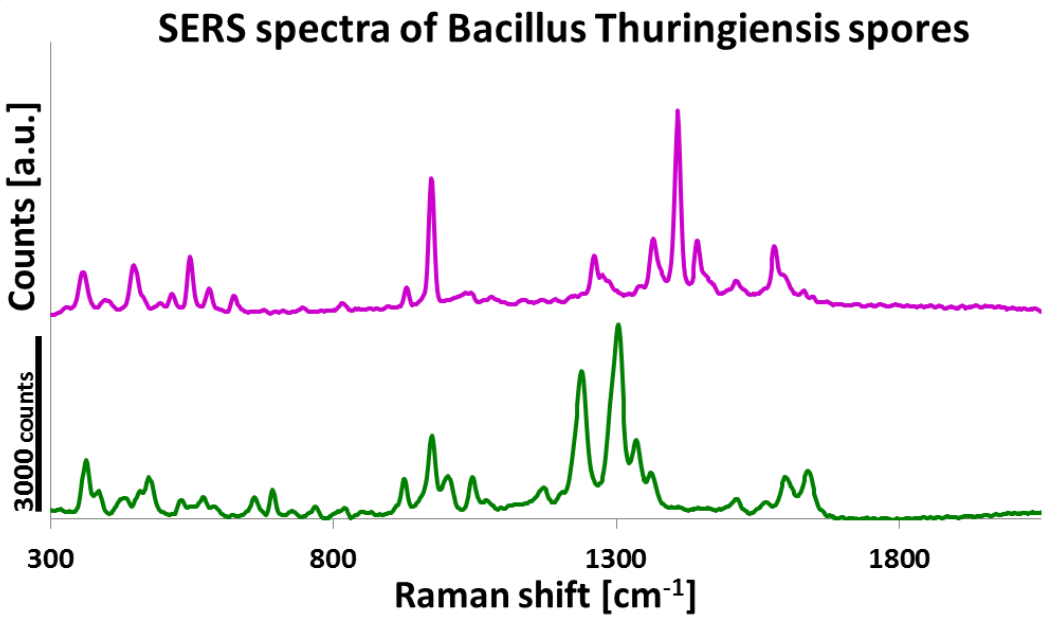
SERS of Bacteria – IOE WAT studies

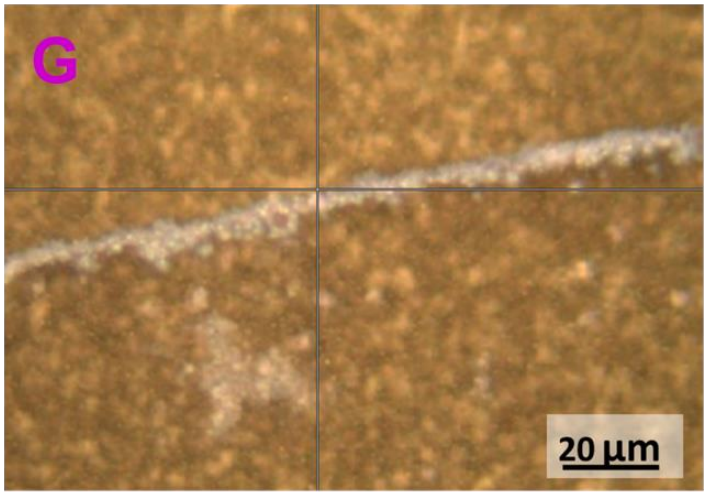


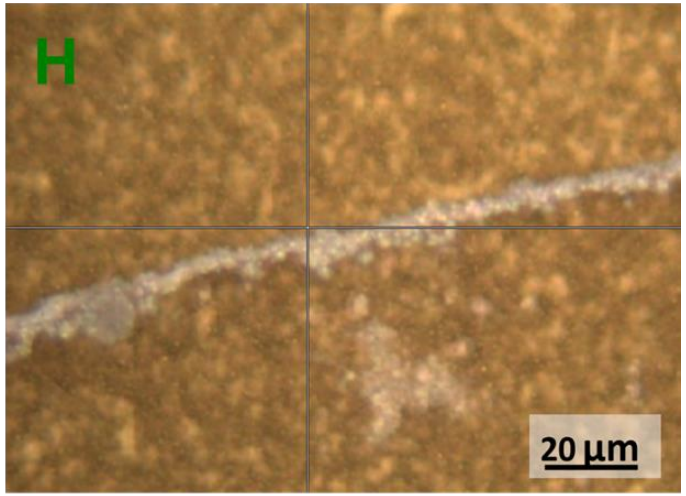




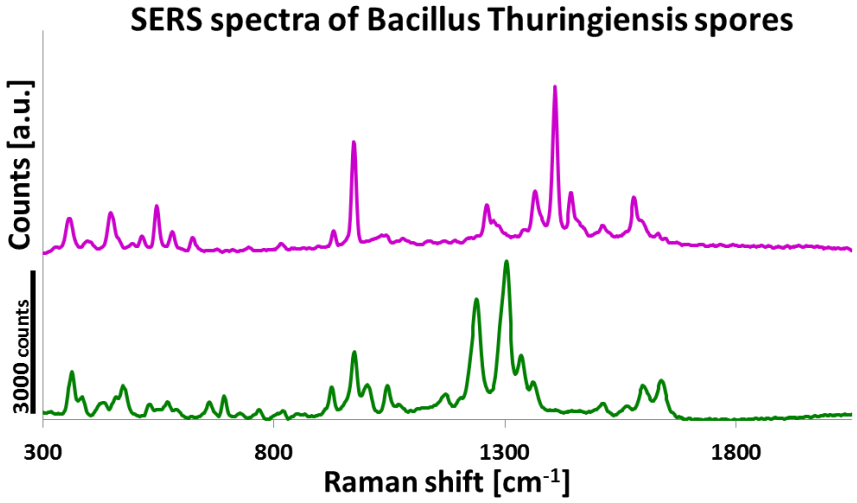
SERS of Bacteria – IOE WAT studies

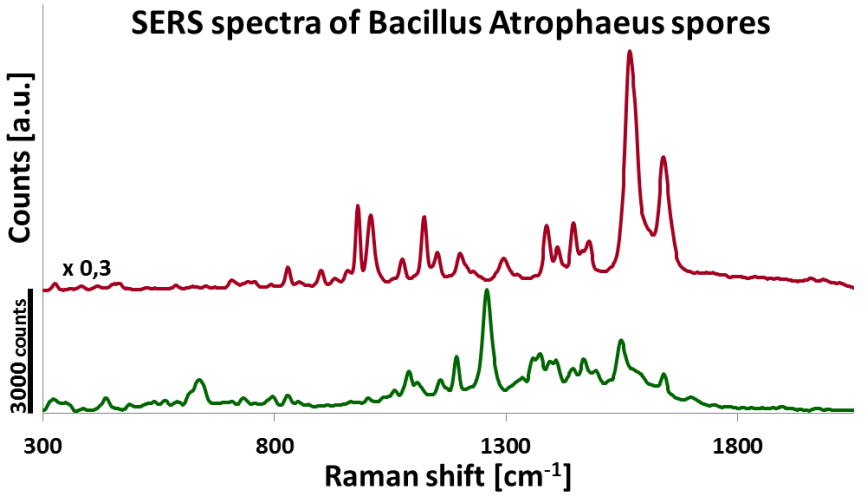


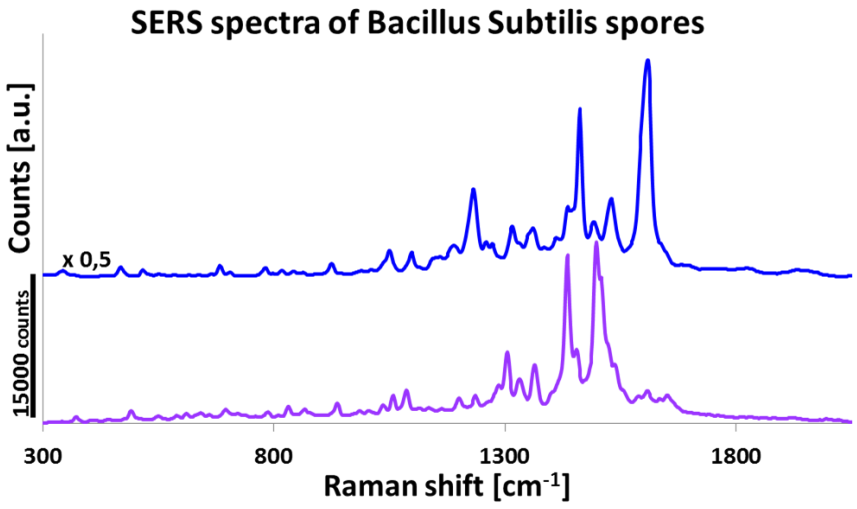


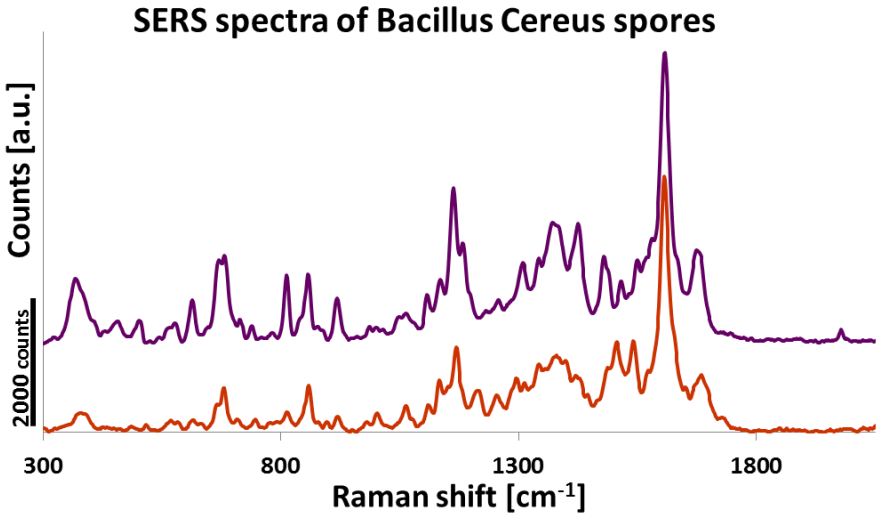


SERS of Bacteria – IOE WAT studies









PROTOCOL

Nature Protocol 2016, 11, 664

Using Raman spectroscopy to characterize biological materials

Holly J Butler[1,2], Lorna Ashton[3], Benjamin Bird[4], Gianfelice Cinque[5], Kelly Curtis[6], Jennifer Dorney[6], Karen Esmonde-White[7], Nigel J Fullwood[8], Benjamin Gardner[6], Pierre L Martin-Hirsch[1,9], Michael J Walsh[10,11], Martin R McAinsh[1], Nicholas Stone[6,12] & Francis L Martin[1]

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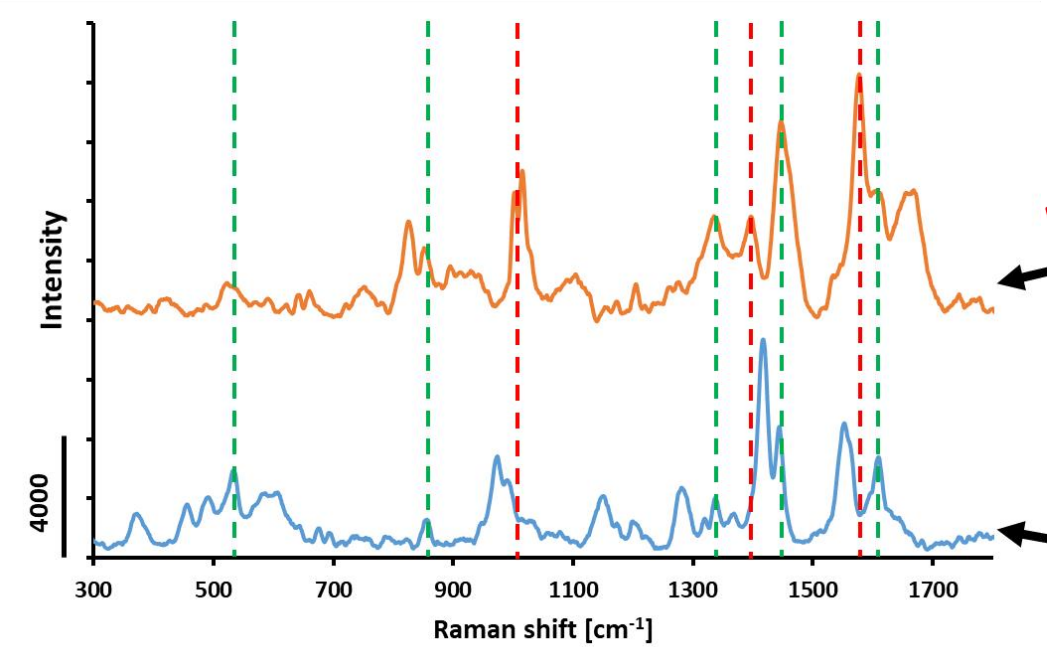
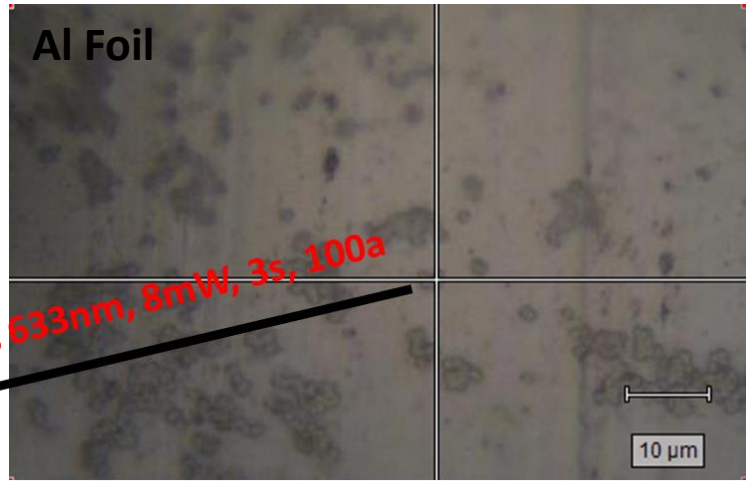
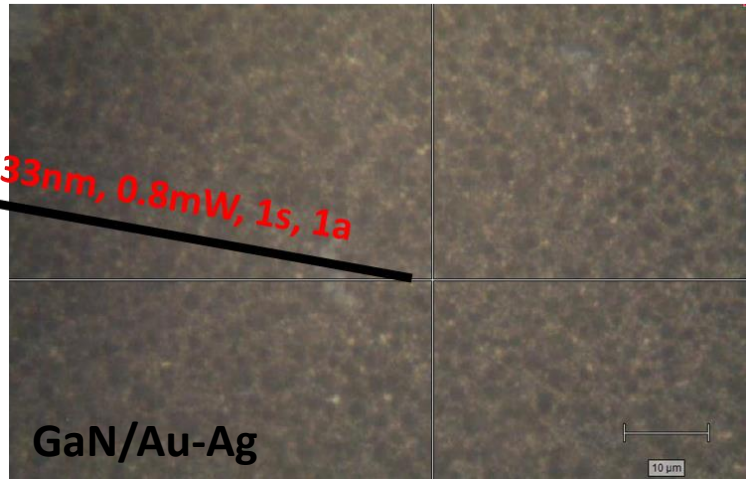
Raman spectroscopy can be used to measure the chemical composition of a sample, which can in turn be used to extract biological information. Many materials have characteristic Raman spectra, which means that Raman spectroscopy has proven to be an effective analytical approach in geology, semiconductor, materials and polymer science fields. The application of Raman spectroscopy and microscopy within biology is rapidly increasing because it can provide chemical and compositional information, but it does not typically suffer from interference from water molecules. Analysis does not conventionally require extensive sample preparation; biochemical and structural information can usually be obtained without labeling. In this protocol, we aim to standardize and bring together multiple experimental approaches from key leaders in the field for obtaining Raman spectra using a microspectrometer. As examples of the range of biological samples that can be analyzed, we provide instructions for acquiring Raman spectra, maps and images for fresh plant tissue, formalin-fixed and fresh frozen mammalian tissue, fixed cells and biofluids. We explore a robust approach for sample preparation, instrumentation, acquisition parameters and data processing. By using this approach, we expect that a typical Raman experiment can be performed by a nonspecialist user to generate high-quality data for biological materials analysis.

SERS of Bacteria – IOE WAT studies

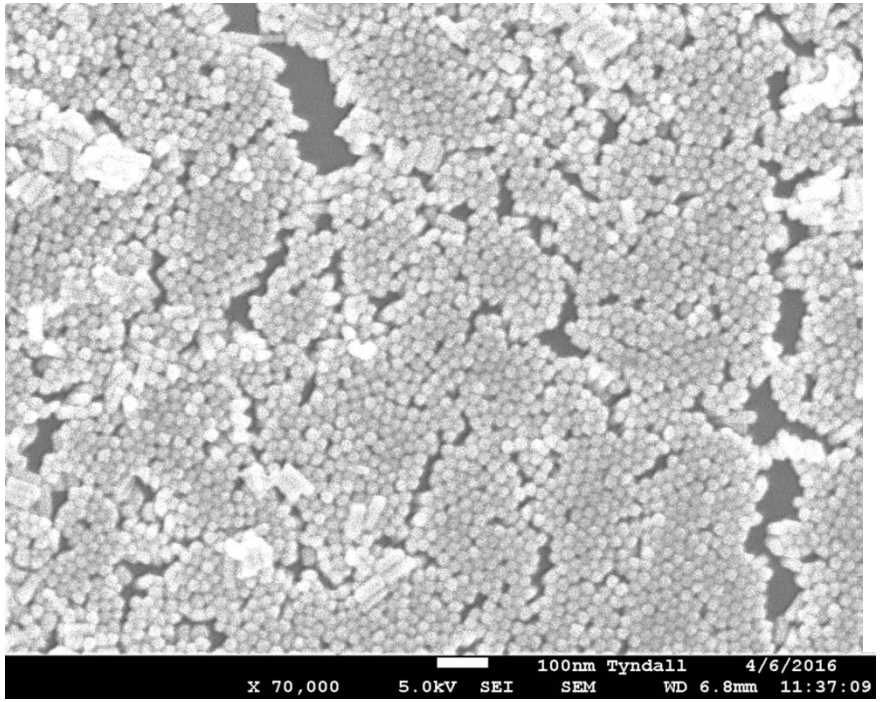
Raman and SERS measurements of Bacillus Atrophaeus spores

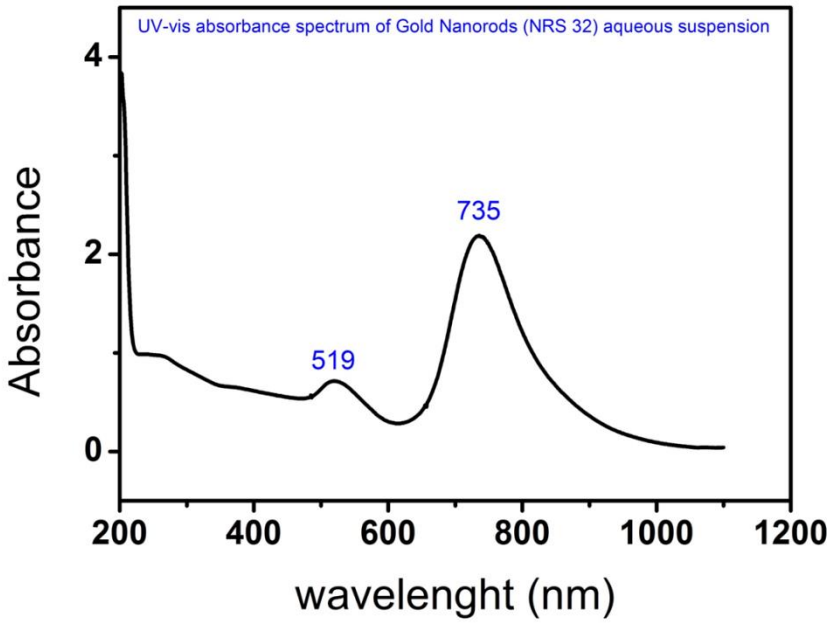






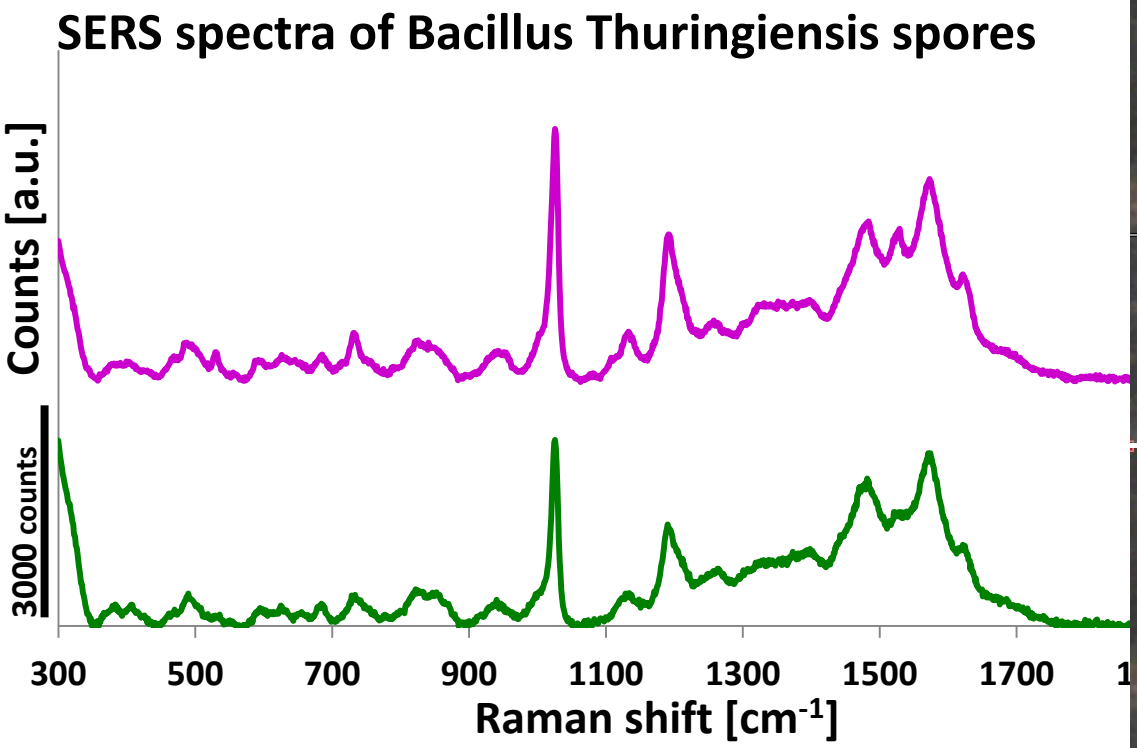
SERS of Bacteria – IOE WAT studies

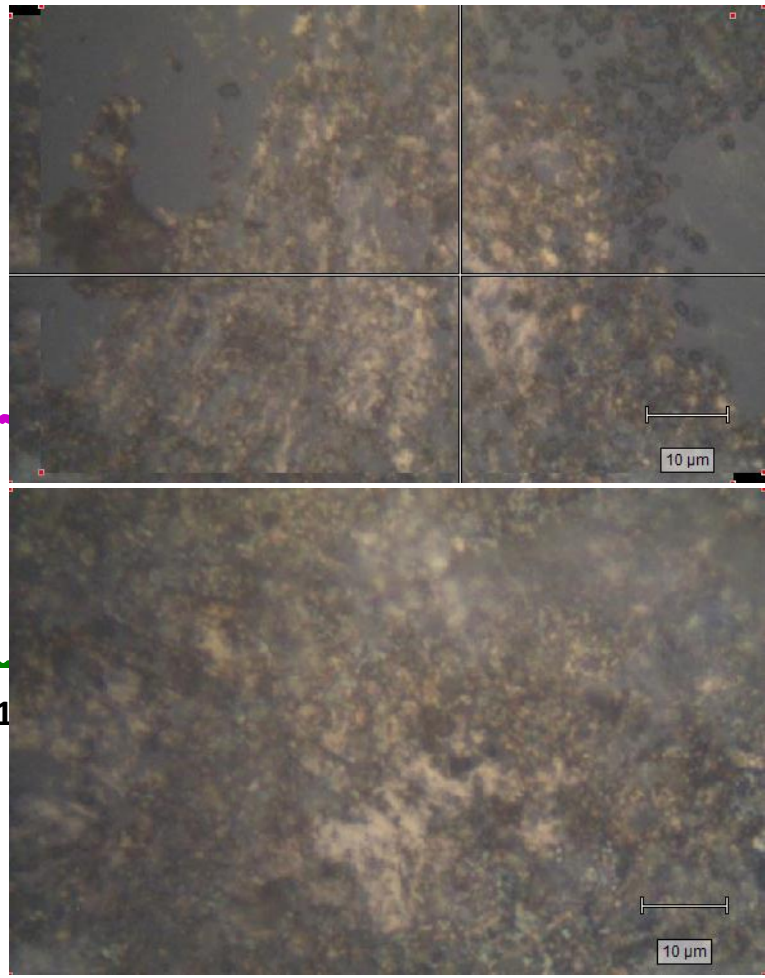




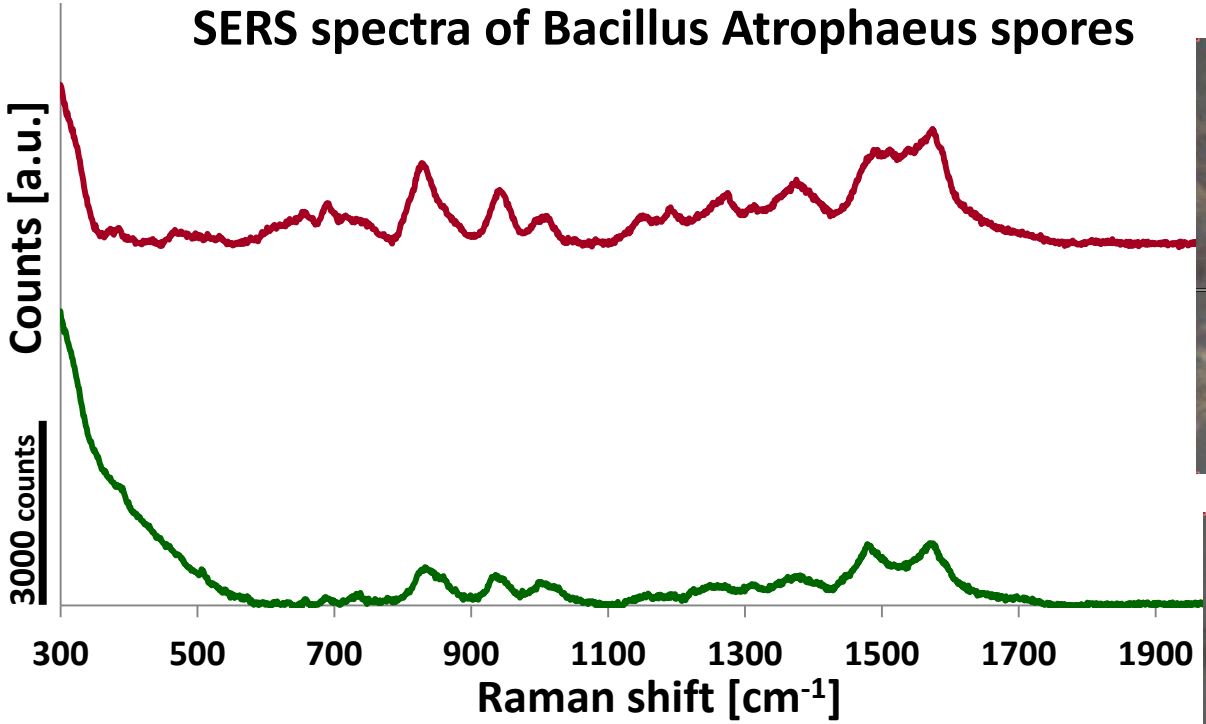
Dr. Daniela Iacopino
Nanotechnology Group,
Tyndall National Institute

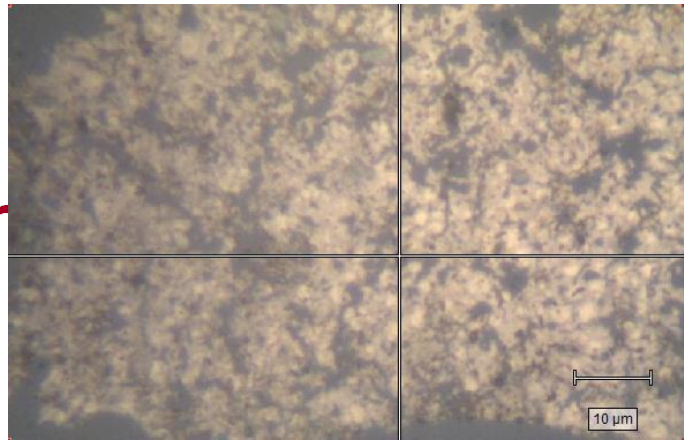
SERS of Bacteria – IOE WAT studies

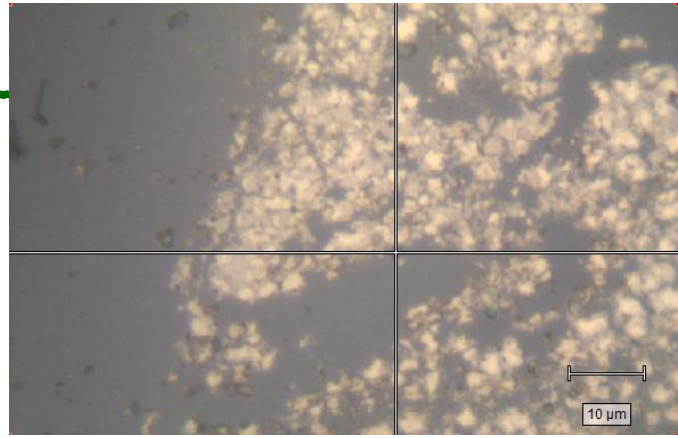




SERS of Bacteria – IOE WAT studies

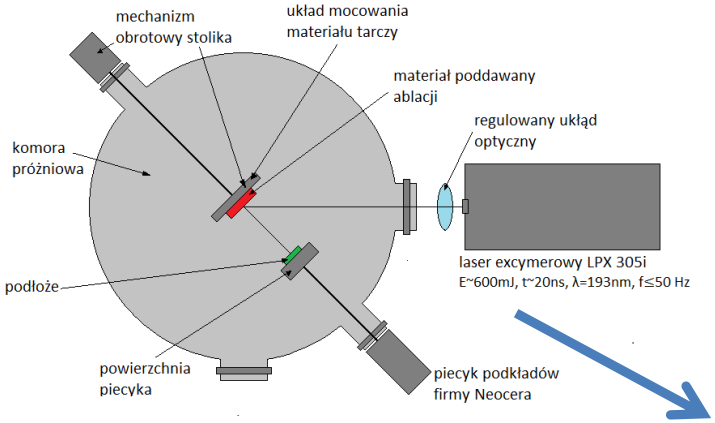




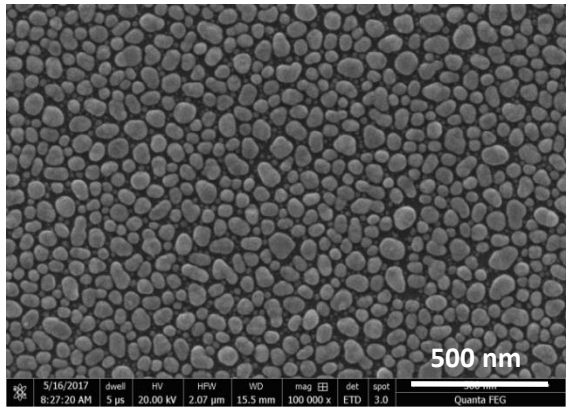


SERS Studies
New SERS substrates developement

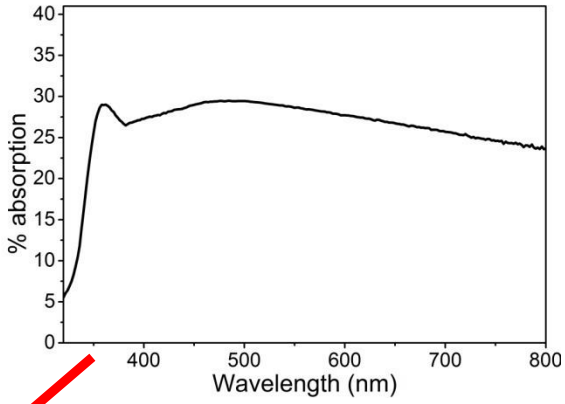
Pulsed laser deposition system



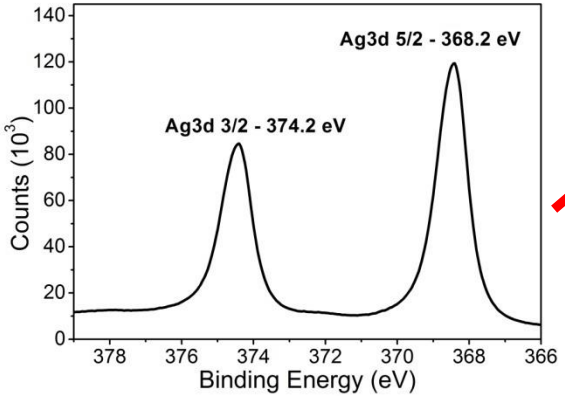
SEM images of the nanostructured silver layer prepared by PLD



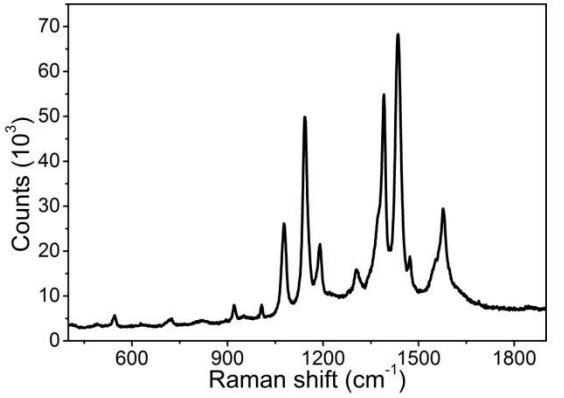
UV-vis spectrum of nanostructured silver layer



XPS spectrum obtained for the silver layer deposited by the PLD technique

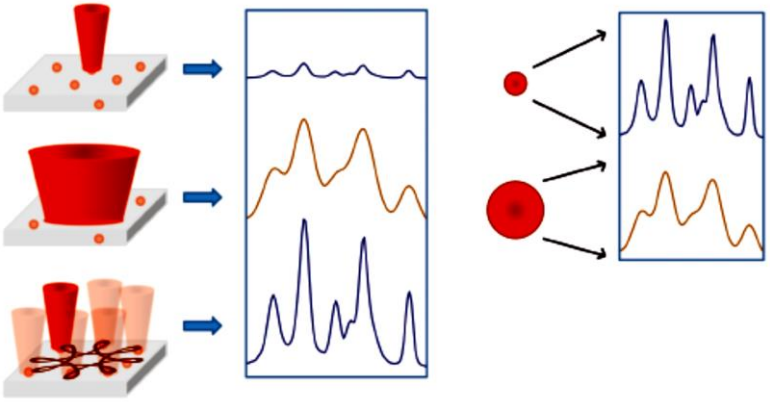


Raman spectrum obtained for monolayer of pMA on prepared silver layer



SERS Studies
Way forward

Orbital Raster Scanning



Orbital Raster Scan (ORS)
Large Area + High Resolution

ORS maintains the small spot-size, high resolution, and samples more material.

M.W. Allen, Y. Mattley Optik&Photonik 2013, 4, 44-47

Summary

- Many literature reports have shown SERS as a very promising technique for detection of bacteria and their spores.
- The main benefits of this technique include high sensitivity (low detection limit), below diffraction limit resolution and fluorescence quenching.
- However, SERS have also some limitations, which include lack of reproducibility and possibility of reduction of band intensity of high-frequency modes.
- Our studies have shown that it is possible to obtain SERS spectra from single bacterial cell using single plasmonic nanostructure.
- Our studies have shown that for biological materials such as vegatative bacteria and bacterial spores the best results are obtained on the SERS substrates with 3D structures , such as nanoparticles, core-shells or GaN/Au-Ag substrate.
- SERS substrate suitable for bacteria detecion exist but in our opinion the main challange is the experimental approach to measurements especially when it comes to portable systems.

Acknowledgements

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