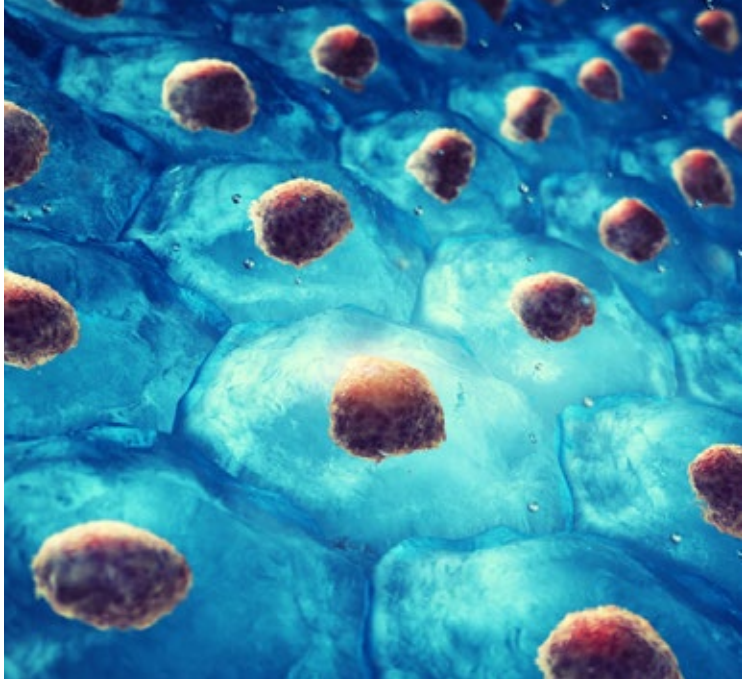
INTRODUCTION

Pittcon 2018 provided a packed academic program incorporating more than 2,000 technical presentations detailing a diverse selection of methodologies and over 90 skill-building short courses. In addition, there was the opportunity to meet over 700 exhibitors and attend interactive presentations and virtual reality demonstrations of the latest products.

A range of great networking events were also incorporated into the Pittcon 2018 itinerary to allow colleagues from across the globe to meet in person and provide the opportunity for new contacts to be made. These included the Universal Island of Adventures networking event at which delegates had exclusive access to rides and attractions at Universal's Islands of Adventure™.

This e-book highlights the latest advances in omics research, nanotechnologies and analytical methodology presented at Pittcon 2018. It includes interviews and symposia presentations from world-renowned experts, including Dr Stefan Hell and Prof. Jeremy Nicholson.



This year's Wallace H Coulter Lecture, entitled "Analytical Science in Precision Medicine: Facing the Challenges of the 21st Century Healthcare" was given by Professor Jeremy Nicholson, Head of the Department of Surgery and Cancer and Director of the MRC-NIHR National Phenome Centre Faculty of Medicine. He discussed how analytical chemistry will become increasingly important in delivering tailored healthcare solutions across the world. For example, the precise combination of microorganisms within an individual can predict health outcomes, including the risk of developing obesity and cancer prognosis.

2014 Nobel Prize winner Dr Stefan Hell, Director at the Max Planck Institute for Biophysical Chemistry in Göttingen and for Medical Research in Heidelberg, delivered the plenary lecture with a last-minute title change to "Far-field fluorescence nanoscopy post-nobel" in which he described the new concept of MINFLUX he developed to enable true molecular resolution with visible light and standard objective lenses in fluorescence microscopy.

01

BIOANALYTICAL IMAGING – WHERE ARE WE NOW?

INTRODUCTION

Bioanalytical imaging technology holds important utility in clinical research, drug development and monitoring, as well as food safety.

Over time, these tools have become more sophisticated in their approach to obtaining qualitative and quantitative data for a wide variety of biomedical applications.

At Pittcon 2018, held this year in Orlando, Fl, Feb 26 - March 1, attendees were able to learn more about bioanalytical imaging platforms and techniques from experts in the field, as well as from exhibiting companies who will be demonstrating their advanced analytical technologies.

Pittcon 2018 heard from speakers discussing ambient ionization techniques for simplifying mass spectrometry (MS) analyses, and using bioanalytical methods, such as liquid chromatography MS (LC-MS), in the cannabis testing industry.

1.1 A GUIDE TO BIOANALYTICAL AND IMAGING

Advancing bioanalytical techniques and platforms have enabled researchers to improve their ability to identify diagnostic biomarkers and detect the effect of certain therapies in living systems. Techniques such as electroparamagnetic resonance (EPR) spectroscopy have been widely applied in the pharmaceutical industry, helping researchers determine the presence of free radicals and transition metals in pharmaceutical products.

Using EPR to detect these species can help identify the stability of the product (eg, shelf life). Bruker, the manufacturer of the EMXnano benchtop EPR spectrometer, were present at Pittcon 2018 to demonstrate this technology. Also, ThermoFisher Scientific were able to demonstrate their workflow solutions for streamlining biopharmaceutical analyses, particularly for protein characterization.

1.2 CURRENT AND FUTURE APPLICATIONS OF BIOANALYTICAL IMAGING

Analytical instruments serve as invaluable tools in a wide range of scientific disciplines, from clinical research and drug development through to environmental monitoring and food safety.

Devoted to the advancement of healthcare and the world we live in, scientists across these fields rely on and routinely use analytical instruments to obtain quantitative and/or qualitative data that will enable accurate and effective analysis of the substances they are studying and testing. The Japan Analytical Instruments

Manufacturers Association (JAIMA), a company dedicated to promoting the advancement of analytical instrumentation defines these instruments as "appliances, tools, or devices that qualitatively and/or quantitatively measure the composition, properties, structure, status, etc., of substances." The instruments are classified based on their application such as in laboratory experiments and research, monitoring of the environment, medical examinations and automation processes.

At Pittcon 2018, attendees were able to listen to experts discuss the wide range of bioanalytical techniques that are currently in use or under development within the diverse scientific community.

Sessions will be held by leading researchers on high throughput and high-resolution techniques used in the fields of nanotechnology, molecular biology, biopharma, clinical medicine, molecular biology and many more.

This includes this years presenter for the Plenary Lecture, Dr Stefan Hell, who will be discussing the science and bioanalytical technology behind breaking the diffraction barrier in fluorescence microscopy.

In the following chapters some of these technologies and their applications will be discussed in more detail, as well as some of the highlights Pittcon attendees can expect to enjoy.

Just some examples of the bioanalytical imaging techniques that will be discussed at Pittcon include ambient ionization methods for the simplification of mass spectrometry (MS) analysis, surface plasmon resonance imaging (SPRI) for real-time molecule detection and electroparamagnetic resonance (EPR) spectroscopy for the detection of free radical and transition metals in pharmaceuticals.

A multitude of companies involved in the manufacture and supply of state-of -the-art, world-leading analytical instrumentation and technologies will be exhibiting at the event and demonstrating their latest products. With major industry players including Thermo Fisher Scientific, Shimadzu, Waters, Bruker, BioRad, Ametek, Zeiss, Metrohm, Wasatch, Hamamatsu, Hitachi and Zeiss, all attending this year's symposium, Pittcon 2018 will provide an outstanding opportunity to hear about the latest advances in analytical instrumentation.

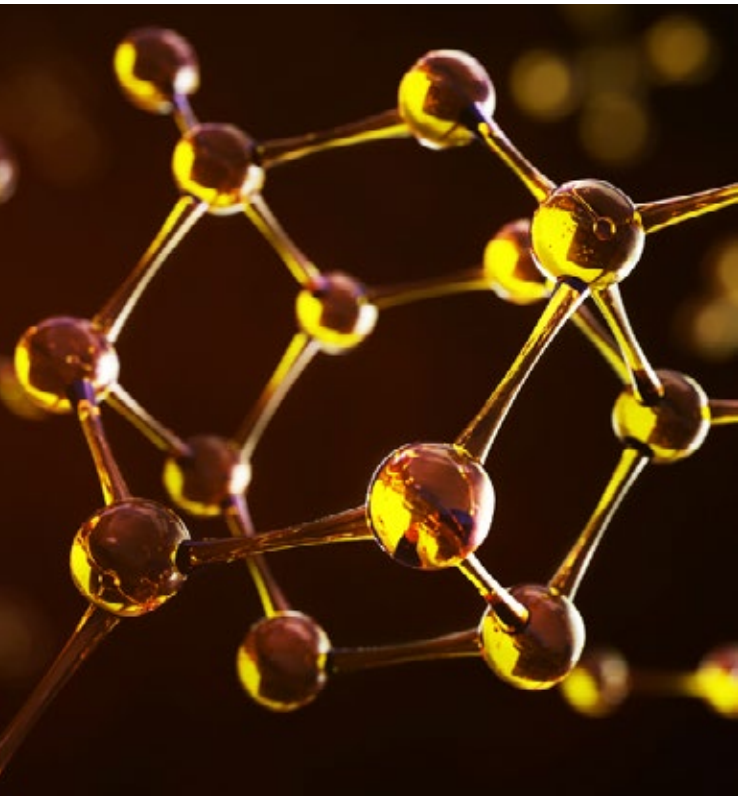
References

- *JAIMA. About JAIME. Available at: https://www.jaima.or.jp/en/about/overview/analytical_instruments/*
- *Ouyang Z, et al. Paper spray ionization devices for direct, biomedical analysis using mass spectrometry. Int J Mass Spectrom. 2012; 312: 201–207.*
- *Horiba Scientific. Surface Plasmon Resonance Imaging. http://www.horiba.com/scientific/products/surface-plasmon-resonance-imaging-spri/*
- *Bruker. Pharmaceutical Applications of EPR. https://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/MagneticResonance/EPR_brochures/emxnana-detecting-and-evaluating-degradation.pdf*
- *Bankert W, et al. A Closer Look at Cannabis Testing. Shimadzu. 2016. https://cdn.technologynetworks.com/tn/Resources/pdf/a-closer-look-at-cannabis-testing.pdf*
- *Hummon A, et al. Evaluation of Therapeutics in Three-Dimensional Cell Culture Systems by MALDI Imaging Mass Spectrometry. Annal Chem. 2013; 85(13): 6295−6302.*
- *University of Pittsburgh. Department of Chemistry. Research Overview. http://www.chem.pitt.edu/person/shigeru-amemiya*

1.2a MOLECULES AND MATERIALS IN BIOIMAGING AND BIOMONITORING

The ever-improving capability of bioanalytical techniques to extract novel and quantitative information, along with advances in fields such as nanotechnology and molecular biology is revolutionizing the analysis of living systems, the detection of diagnostic biomarkers and the evaluation of therapeutic effects in model systems.

The scope of bioanalytical techniques also extends into fields such as forensics, environmental monitoring and food quality control.



In the pharmaceutical industry, characterizing a drug's shelf life is crucial to ensuring its stability and therefore correct dosage and patient safety. The presence of free radicals and transition metals (paramagnetic species) is often what causes degradation of a drug's active pharmaceutical ingredient (API). Electroparamagnetic resonance (EPR) spectroscopy is the only technique that unambiguously and non-invasively detects these species so that they can be quantified and monitored.

Exhibiting at this year's Pittcon was Bruker, developer of the EMXnano benchtop EPR spectrometer. This instrument measures API degradation, determines its cause and predicts its long-term stability characteristics, providing an ideal solution for optimizing product stability.

In the clinical field, one challenge in the area of oncology biomarker research is translating the detection of new oncogenic or tumorigenic biomarkers into treatment approaches. Pittcon exhibitor Thermo Fisher Scientific provides versatile mass spectrometry (MS) solutions that help to detect and validate markers for early cancer detection, aid proteomics studies and monitor treatment response.

In the food industry, increasing demands relating to food safety have led to ever-more rigorous regulations. To address these demands, Pittcon exhibitors Waters, provide sensitive, reproducible and versatile solutions for the screening of pesticides, natural toxins, packaging contaminants and other adulterants.

Bioanalytical instrumentation is also widely used in forensics and toxicology studies for the determination of blood alcohol, abusive inhalants and other volatiles, and Pittcon exhibitor Teledyne Tekmar offers a range of solutions that forensic laboratories and coroner offices can use to ensure their data is precise, accurate and dependable.

Robert Clifford from Shimadzu discussed bioanalytical techniques used in the cannabis testing industry for potency profiling and screening of pesticides, residual solvents, heavy metals, mycotoxins and moisture content.

Cannabis testing was also discussed at last year's Pittcon, where analytical methods were covered, along with the development of laboratory standards, cannabis extraction methods and cannabis detection to meet law enforcement needs.

A critical part of cannabis testing is determining cannabinoid potency, which involves the quantification of at least three major cannabinoids: THC, CBD and CBN. Shimadzu's integrated high-performance liquid chromatography (HPLC) systems are ideal solutions for this, including the new i-Series instruments, which feature intuitive graphical user interfaces that even inexperienced staff can operate.

For pesticide testing, gas chromatograph-mass spectrometry (GC-MS) is the preferred platform. Shimadzu's GCMS-QP2010 Ultra instrument can be used for this, as can the company's GCMS-QP2010 SE. Herbicides can also be tested for on either of these instruments. For residual solvent testing, a headspace GC device can be used such as Shimadzu's GC-2010 Plus with HS-20 Headspace Sampler.

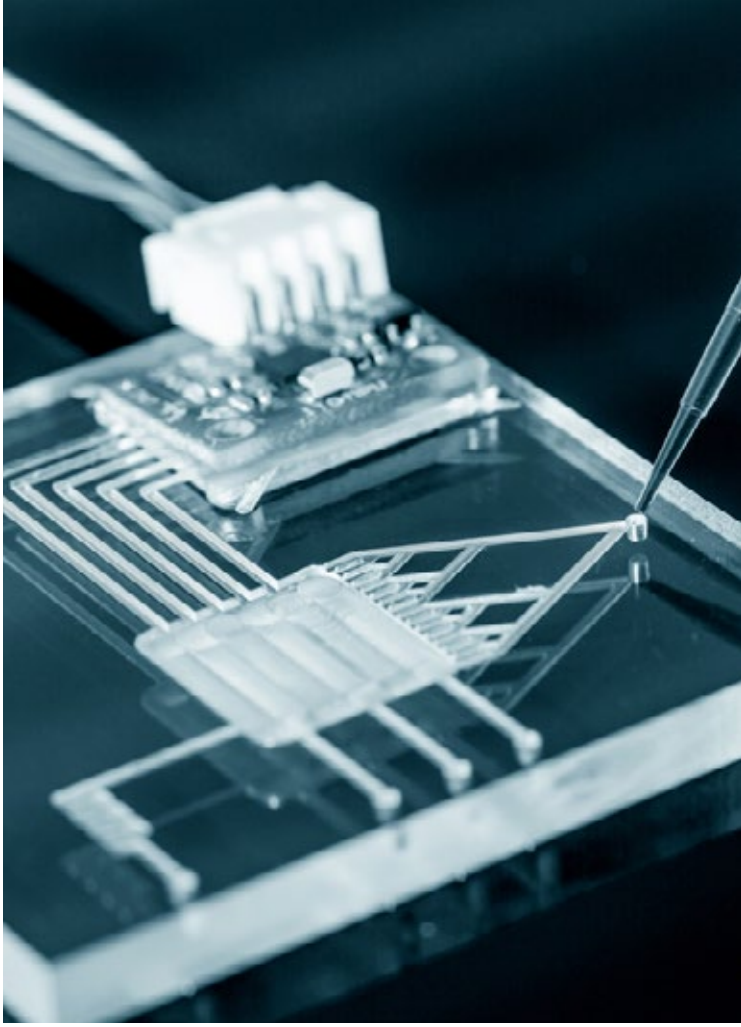
Moisture content can be measured using Shimadzu's MOC63u (and MOC120H), microorganisms can be detected using the company's LC and LCMS systems and heavy metal testing can be performed using the AA-7000 with GFA-7000 or ICPE-9800.

The detection capabilities of bioanalytical techniques will also be discussed in the context of pathogenic outbreaks and the growing interest from farmers, governments and industry in rapid and quantitative in-field testing for the detection of bacteria in food and beverages.

In a 2010 paper, Frederique Deiss (Indiana University) and colleagues report on a simple, electroanalytical system based on the combination of a hand-held glucometer with micro-paper-based analytical devices. While such devices offer easy manufacture, sample collection and disposal, samples with only a few bacteria require time-consuming incubation. At Pittcon, Deiss described a new mechanism he hopes can be implemented into elastomeric microfluidic devices to solve the challenge of detecting single bacterium without culture.

The use of microfluidic devices is also increasing among researchers using MS for proteomic analysis. Thermo Fisher Scientific can demonstrate how combining its mass spectrometers with 908 Devices' ZipChip microfluidics separation technology delivers high-quality separation that revolutionizes MS biomolecule analysis.

In the field of clinical/biomedical diagnostics, the increasing need for portable integrated biosensors for point-of-care (POC) testing has led to the development of innovative, integrated and automated bioanalytical systems. Presenting at Pittcon, Charles Henry (Colorado State University) will discuss disease detection using paper-based analytical devices. Henry and team were the first to demonstrate (in 2009) how the integration of electrochemical detection and paper-based microfluidics provides an inexpensive and portable solution for POC monitoring. Also exhibiting at Pittcon will be Ametek Scientific Instruments, who provides single channel and multichannel potentiostats for the control of current during electroanalytical experiments.



German Gomez-Rios (University of Waterloo) talked about the importance of developing new, high performance bioanalytical methods for drug development and POC diagnosis. Gomez-Rios described his team's work on solid phase microextraction (SPME)-based devices for the extraction/enrichment of analytes from small sample volumes, that can be coupled with MS for rapid analysis. For the study, Thermo Fisher Scientific lent the team a triple quadrupole mass spectrometer, the TSQ-Quantiva. Waters also provide innovative triple quadrupole mass spectrometry, with the Xevo TQ range offering robust and reliable performance.

Facundo Fernandez (Georgia Institute of Technology) discussed the use of ion mobility-mass spectrometry metabolomics for prostate cancer detection.

Prostate-specific antigen (PSA) screening often results in overdiagnosis and overtreatment and rectal exams are limited by low detection rates in non-palpable growths. Here, Fernandez described high-throughput metabolic profiling of serum samples from prostate cancer patients using flow injection (FI) electrospray traveling wave ion mobility spectrometry (TWIMS) time-of-flight (ToF) MS. This enabled rapid cancer detection in serum samples with 90.2% sensitivity, 83.3% specificity and 87.4% accuracy.

Waters say their SNAPT G2-Si High Definition Mass Spectrometry system which is an ultra-performance liquid chromatography ion mobility time-of-flight mass spectrometry (UPLC-IM-TOF MS) system integrated with TWIMS provides the most complete characterization of complex mixtures and molecules.

Amanda Hummon (University of Notre Dame) described an image-based techniques for evaluating drug treatments in 3D tumor models. In a 2016 paper, Hummon looked at the use of matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) for the evaluation of therapeutics in 3D cell culture systems. MALDI IMS enables in situ analysis of tissue sections for the acquisition of data on hundreds of unknown compounds in a single measurement, without prior knowledge of tissue composition. Hummon and team used MALDI IMS to study the distribution of a chemotherapy drug and found the approach was more effective at screening drug efficacy than 2D culture assays, as well as being a lower-cost, higher-throughput alternative to using animal models. Bruker provides MALDI-TOF systems that quickly and efficiently provide results in traditional MALDI applications as well as for cutting edge research in fields such as proteomics.

Lisa Jones (University of Maryland) described an in-cell protein footprinting method coupled with mass spectrometry for the study of protein structure at Pittcon 2018. In a 2014 paper, Jones and team reported that multidimensional protein identification technology (MudPIT) improved hydroxyl radical footprinting for increased identification of quantifiable peptides. At Pittcon, Jones will discussed how the use of fast photochemical oxidation of proteins (FPOP) for the generation of hydroxy radicals in these footprinting experiments can be extended to enable the analysis of proteins inside cells, (in-cell FPOP), demonstrating the technique's potential for studying proteins in their native cellular environment.

For a range of applications including protein identification and metabolite profiling, Thermo Scientific's LTQ Orbitrap XL™ Hybrid Ion Trap-Orbitrap Mass Spectrometer serves as the ideal instrument. It features an HCD collision cell to enhance flexibility of fragmentation experiments for advanced proteomics and small molecule research.

Matthew Lockett (University of North Carolina) discussed the tracking of cellular movement in tumor-like environments. Current cellular migration assays provide limited information on cellular responses to gradients, since they oversimplify the 3D environment of a tissue. In a 2015 paper, Lockett and team described a paper-based invasion assay that enables real-time monitoring of cellular movement with fluorescence microscopy. Using this technique, to study the invasion of cells cultured in the presence and absence of oxygen, Lockett and team were able to demonstrate that oxygen is a chemoattractant for more than one type of cancer cell.

Zeiss, a leading provider of microscopy solutions, were an Pittcon, exhibiting its high-performance microscopy products at the conference. The company's portfolio features the LSM 700 laser scanning confocal microscope, which provides efficient separation of fluorescence signals where crosstalk is prevented and highly overlapping fluorophores are unmixed.

References

- *Bruker. Pharmaceutical Applications of EPR. https://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/MagneticResonance/EPR_brochures/emxnana-detecting-and-evaluating-degradation.pdf*
- *Thermo Fisher Scientific. Cancer Protein Biomarkers. https://www.thermofisher.com/uk/en/home/clinical/clinical-translational-research/protein-analysis/protein-biomarker-discovery-validation/cancer-protein-biomarkers.html*
- *Waters. Food Safety http://www.waters.com/waters/en_GB/Food-Safety/nav.htm?cid=514887&locale=en_GB*
- *Teledyne Tekmar. Forensics and Toxicology Solutions. http://www.teledynetekmar.com/Industry-Applications/forensics*
- *Pittcon Conference & Expo. Cannabis Testing: An Industry Guide https://pittcon.org/cannabis-testing-industry-guide/*
- *Bankert W, et al. A Closer Look at Cannabis Testing. Shimadzu. 2016. https://www.thermofisher.com/blog/proteomics/microfluidics-and-mass-spectrometry-based-proteomics/*
- *Hsing M, et al. MDPI.org. Biosensors for Point-of-Care Applications. Sensors journal. 2004. http://www.mdpi.org/sensors/Biosensors-for-Point-of-Care-Applications.htm*
- *Henry C, et al. Electrochemical Detection for Paper-Based Microfluidics. Anal. Chem. 2009, 81, 5821–5826*
- *Ametek. Potentiostats. http://www.ameteksi.com/products/potentiostats*
- *Gomez-Rios G. Can LC-MS be Replaced by Direct Coupling of SPME to MS in Clinical Applications?*
- *MSACL 2018 US Abstract. https://www.msacl.org/view_abstract/MSACL_2018_US.php?id=176*
- *Thermo Fisher Scientific. iCAP TQ ICP-MS. http://info1.thermoscientific.com/iCAPTQ?gclid=EAIaIQobChMIi7bKmqab 2AIVjpTtCh38bwB6EAAYASAAEgJpiPD_BwE&ce=E.17CMD.TE101.07493.01&cid= E.17CMD.TE101.07493.01&s_kwcid=AL!3652!3!226218193871!e!!g !!triple%20quadrupole%20mass%20spectrometer&ef_id=WD1DVwAAAHofudeq:20171221141934:s#&page=overview*
- *Waters. Tandem Quadrupole (Triple Quadrupole) Mass Spectrometry. http://www.waters.com/waters/en_GB/Tandem-Quadrupole-%28-Triple-Quadrupole-%29-Mass-Spectrometry/nav.htm?cid=134656172&xcid=ext4981&gclid=EAIaIQobChMIi7bKmqab2AIVjpTtCh38bwB6EAAYAyAAEgJVdPD_BwE&locale=en_GB*
- *Waters. SYNAPT G2-Si High Definition Mass Spectrometry. http://www.waters.com/waters/en_GB/SYNAPT-G2-Si-High-Definition-Mass-Spectrometry/nav.htm?cid=134740622&locale=en_GB*
- *Hummon A, et al. Evaluation of Therapeutics in Three-Dimensional Cell Culture Systems by MALDI Imaging Mass Spectrometry. Annal Chem. 2013; 85(13): 6295−6302*
- *Bruker. High-Performance MALDI-TOF Systems. https://www.bruker.com/products/mass-spectrometry-and-separations/maldi-toftof/autoflex/overview.html*
- *Jones L, et al. Fast Photochemical Oxidation of Proteins Coupled to Multidimensional Protein Identification Technology (MudPIT): Expanding Footprinting Strategies to Complex Systems. 2015; 26(4): 540−546*
- *Gross M, et al. "Fast Photochemical Oxidation of Proteins" (FPOP) and Mass Spectrometry Follow Sub-millisecond Protein Folding at the Amino-Acid Level. J Am Chem Soc. 2012; 134(45): 18724−18731*
- *Thermo Fisher Scientific. LTQ Orbitrap XL™ Hybrid Ion Trap-Orbitrap Mass Spectrometer. https://www.thermofisher.com/order/catalog/product/IQLAAEGAAPFADBMAOK*
- *ZEISS. LSM 700 Light Pathways. https://www.zeiss.com/microscopy/us/solutions/reference/all-tutorials/spectral-imaging/lsm700-light-pathways.html*
- *Bruker. EPR in a nutshell. https://www.bruker.com/products/mr/epr/epr-in-a-nutshell.html*
- *Höfler H, et al. MALDI imaging mass spectrometry for direct tissue analysis: a new frontier for molecular histology. 2008; 130(3): 421−434*
- *Heeron R. MALDI Techniques in Mass Spectrometry Imaging. Encyclopedia of Spectroscopy and Spectrometry (Second Edition) 2010: 1443−1451*
- *Hoffman P, et al. MALDI Imaging Mass Spectrometry (MALDI-IMS)Application of Spatial Proteomics for Ovarian Cancer Classification and Diagnosis. Int. J. Mol. Sci 2011; 12(1): 773−794*

1.2b BIOANALYTICAL SOLUTIONS IN BIOPHARMA

Numerous bioanalytical technologies are used as essential tools in many fields that shape our life today, ranging from the diagnosis of disease through to food safety and environmental conservation.

Without such technologies, many of the products we use in everyday life would not have emerged and around one-fifth of Nobel prizes have been awarded to the creators of analytical theories, instruments or technologies in physics, chemistry, physiology and medicine.

One aspect of improving analytical instrumentation is the development of high-throughput screening, a method that employs robotics, automation, high sensitivity detection, data processing software and sample handling devices to enable researchers to quickly perform millions of pharmaceutical, genetic or chemical tests.

At Pittcon, Atis Chakrabarti from Tosoh Bioscience LLC discussed a high-throughput method for analyzing a variety of Immunoglobulin G subclasses from multiple sources including humans, rats, mice and rabbits. Using an analytical protein A affinity chromatography column, rapid separation and robust quantification of antibodies could be carried out, with analysis completed within two minutes. Pittcon exhibitor Thermo Scientific has an instrument portfolio that includes a range of analytical chromatography columns. Amongst them is the POROS™ Prepacked Protein A Affinity Column, which contains an immobilized recombinant Protein A functional group designed for high-throughput purification of antibodies.

In a 2009 article, Chakrabarti reported a successful high throughput method for the analysis of caffeine content in drinks. In 2004, all 25 countries in the EU started to require that any packaged drink containing more than 150 mg/L of caffeine be labelled as “high caffeine content,” leading to a need for a rapid and easy testing method for the quality control of caffeine. Chakrabarti’s article described a high-throughput reversed phase HPLC method that successfully achieved this purpose.

State-of-the-art reversed-phase and HPLC columns are available through Waters, who are also exhibiting at Pittcon.

Imaging technologies used in pharmaceutical applications enable researchers to gain powerful insights into the biological effects of drugs such as their distribution and binding ability. The imaging agents and technologies used in this field serve as important tools in the discovery of biomarkers, the study of patient outcomes in disease progression, and the development of new therapies. Pharmaceutical analyses provide information on the content, quality, stability and purity of pharmaceutical products. Metrohm, a leading provider of instruments and applications for drug quality control, monitoring and improvement, will be exhibiting at Pittcon this year. The company provides solutions for the analysis of active pharmaceutical ingredients (APIs), excipients and impurities, as well as starting materials and finished pharmaceutical products.

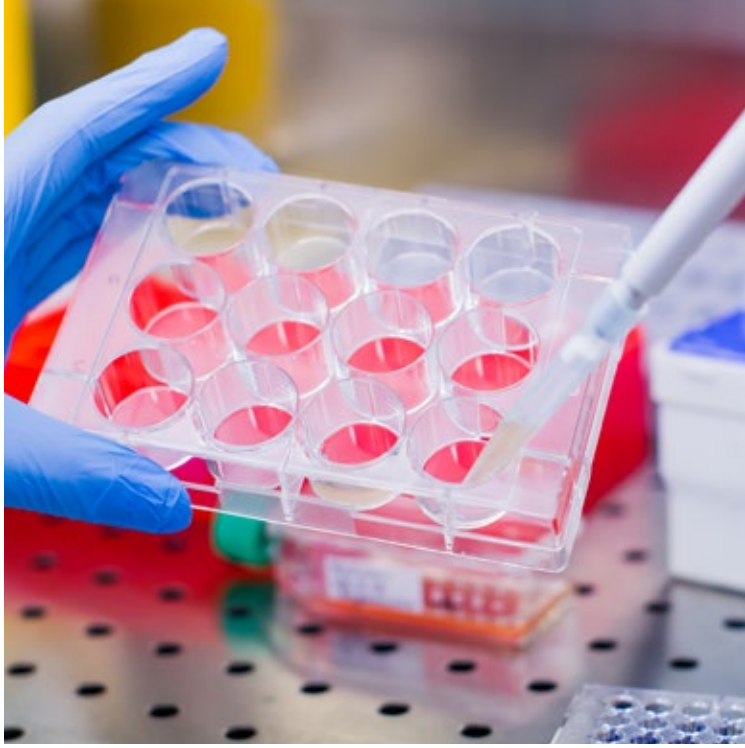
When a pharmaceutical is produced using biotechnology, it is referred to as a biopharmaceutical. These drugs are large, elegant and complex structures such as proteins or nucleic acids, rather than small molecules and they are designed and studied using 3D-modlieng systems. They perform their functions with high efficacy and limited side effects. Furthermore, treatment prototypes are continuously evolving and ongoing research is leading to new types of products.

Thermo Fisher Scientific's instrument range and workflow solutions provide answers to simplifying biopharmaceutical analysis. The company supplies state-of-the-art technology for all aspects of protein characterization, from post translational modifications through to 3-D structures and protein aggregation.

However, the biopharma industry faces various challenges. Reliable reproduction of these large molecules at an industrial scale requires extremely sophisticated manufacturing capabilities and the genetically modified cells that are used to create the molecules need to be frozen for storing and thawed without becoming damaged. The biopharmaceutical also needs to be separated from the cells that were used to make them without their fragile structures being destroyed.

The current market value for biopharmaceuticals is now more than $150 billion and the range of therapeutics is larger than ever before. The market is also expected to explode over the next decade and Thermo Scientific believes there is a need to develop higher-throughput and more in -depth characterization methods.

At Pittcon, Gurmil Gendah (Shimadzu) talked about using liquid chromatography mass spectrometry (LC-MS) platforms to increase the success of biotherapeutic process development and bioanalysis.



The cell culturing involved in the production of biopharmaceuticals requires routine monitoring of multiple medium conditions such as glucose, nitrogen and various other biologically important compounds.

To meet the demands of this multi-component analysis, Gendah and team developed a high throughput LC/MS/MS method that can monitor the relative abundance of 95 compounds. In LC-MS, multiple components within mixtures can be separated by LC and then analyzed by MS to characterize those components with high specificity and detection. The high reliability, productivity and sensitivity of LC-MS make it an increasingly valuable and popular application in the world of pharma.

In a 2014 article, Gendah and colleagues also reported on a silica -based HPLC/UHPLC column (Thermo Scientific ™ GlycanPac ™ AXH -1) they developed for high-throughput and high-resolution separation and characterization of biologically relevant glycans from proteins using LC-MS. At the forefront of instrument sensitivity, stability and ease of operation is Pittcon exhibitor Shimadzu. One of the company' liquid chromatograph mass spectrometers, the LCMS-8040, is equipped with newly improved ion optics and collision cell technology, for even higher sensitivity and enhanced monitoring.

An additional presenter at Pittcon 2018, Amir Liba from Agilent, talked about the use of inorganic for exploring new biopharmaceutical applications. A highlight of the talk was how laser capture microdissection (ICP) MS can be used in new life science applications to analyze the uptake of therapeutic metal-containing drugs into single cells.

References

- *JAIMA. Mission Statement. https://www.jaima.or.jp/en/about/overview/mission/*
- *JAIMA. About JAIMA. Introduction. https://www.jaima.or.jp/en/about/activities/heritage/introduction/*
- *Chakrabarti A. Tosoh Bioscience. High Throughput Reversed Phase HPLC Analysis of Caffeine Using TSK-GEL ODS-140HTP and ODS-100V Columns. The Application Notebook. 2009: 32–33*
- *Waters. HPLC Columns. http://www.waters.com/waters/en_GB/HPLC-Columns-C18-Amide-C4-C8-SEC-HILIC-Phenyl-T3-Shield-RP18-C18%2B/nav.htm?cid=511505&xcid=ext585&gclid=EAIaIQobChMIiJa18J2M2AIVaDPTCh2UqgL3EAAYAiAAEgJw1_D_BwE&locale=en_GB*
- *Thermo Fisher Scientific. Affinity Columns. https://www.thermofisher.com/uk/en/home/life-science/bioproduction/poros-chromatography-resin/analytical-chromatography/affinity-columns.html*
- *Pohl C, et al. Protein A Affinity Column for Monoclonal Antibody (MAb) Titer Analysis. Thermo Fisher Scientific Poster Note.https://tools.thermofisher.com/content/sfs/posters/PN-20806-Protein-Affinity-Column-Monoclonal-Antibody-Titer-Analysis-PN20806-EN.pdf*
- *Metrohm. Select Your Industry. https://www.metrohm.com/en-gb/industries/*
- *https://www.thermofisher.com/uk/en/home/industrial/pharma-biopharma/biopharmaceutical-analytical-testing.html*
- *Thermo Fisher Scientific. Biopharmaceutical Characterization Information. https://www.thermofisher.com/uk/en/home/industrial/pharma-biopharma/pharma-biopharma-learning-center/biopharmaceutical-characterization-information.html*
- *Thermo Fisher Scientific. 'Ultra' Biotherapeutic Characterisation https://tools.thermofisher.com/content/sfs/brochures/AR-UHPLC-Ultra-Biotherapeutic-Characterization-IPT-0416-EN.pdf*
- *Pohl C, et al. Novel Glycan Column Technology for the LC-MS Analysis of Labeled and Native N-Glycans Released from Proteins and Antibodies.Thermo Fisher Scientific. http://www.bioszeparacio.hu/_user/browser/File/Thermo/Applik%C3%A1ci%C3%B3/PN70513_WCBP2014_Glycan.pdf*
- *Shimadzu. Liquid Chromatograph – Mass Spectrometry. https://www.shimadzu.com/an/lcms/index.html*
- *Shimadzu. LCMS-8040. https://www.shimadzu.com/an/lcms/8040/8040_index.html*
- *Agilent. Small Molecules. https://www.agilent.com/en/solutions/pharma-biopharma/small-molecules*
- *10times. Japan Analytical Instruments Manufacturers Association. https://10times.com/organizers/japan-analytical-instruments*
- *U.S. Food & Drug Administration. Medical Imaging and Drug Development. https://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/ucm092895.htm*
- *Science Daily. Biopharmaceutical. https://www.sciencedaily.com/terms/biopharmaceutical.htm*
- *https://www.mckinsey.com/industries/pharmaceuticals-and-medical-products/our-insights/rapid-growth-in-biopharma*

1.2c AN INTRODUCTION TO BIOSENSORS

Over recent years, there has been significant advancement in the development of nanotechnology. Many new nanomaterials have been created and researchers have been busy investigating their novel properties. One area that has seen great progress is the application of nanomaterials in biosensors.

Nanomaterials-based biosensors represent a fusing of expertise in materials science, chemistry, biotechnology and molecular engineering for the enhancement of biological detection.

In particular, the unique physical, chemical, mechanical, magnetic and optical properties of gold nanoparticles, carbon nanotubes, magnetic nanoparticles and quantum dots has meant their application in biosensors has significantly improved the sensitivity of biomolecule detection. This technology could serve as a significant contributor to the enhancement of fields such as molecular recognition, disease diagnosis and environmental monitoring.

At Pittcon, Daniel Chiu (University of Washington) discussed single molecule studies of nanoparticles. Chiu and team have developed microfluidic and nanofluidic systems that have enabled them to make new discoveries about single nanoparticles.

In a 2003 paper by Chiu, the development of techniques for the chemical analysis of nanometer-scale samples isolated from a single cell is discussed. The paper compares two techniques, namely capillary electrophoresis with laser-induced fluorescence (CE-LIF) matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-TOF-MS).

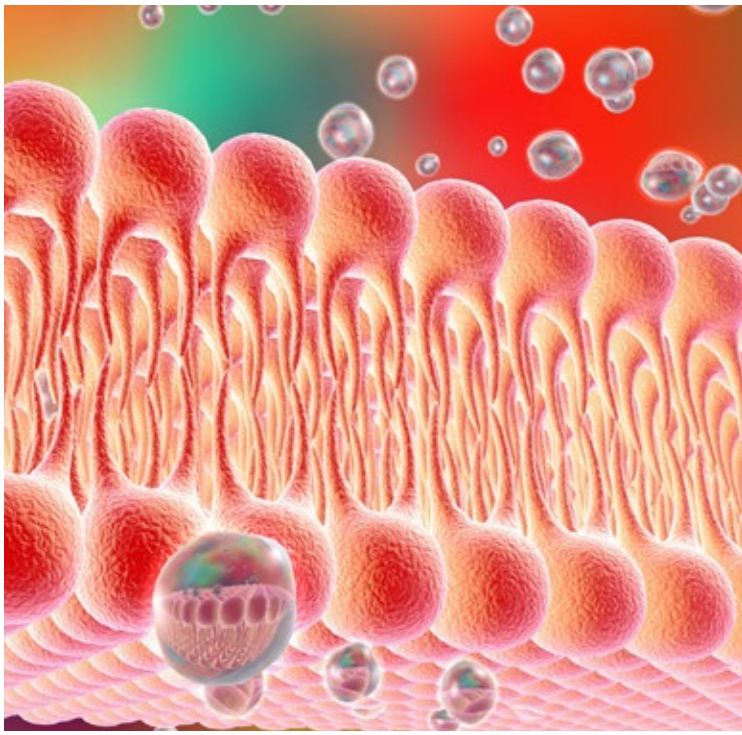
CE-LIF is a powerful analytical technique that can separate small sample volumes and where the use of LIF results in extremely low limits of detection. In MALDI-TOF-MS, molecules are ionized and a time of flight mass spectrometer is used to separate ions with identical kinetic energy based on their mass-to-charge ratio (m/z).

Chiu and team found that CE-LIF revealed the presence of many low molecular weight amino acids and said it may be an ideal technique for the chemical analysis of even the tiniest subcellular structures. Compared to CE-LIF, MALDI-TOF-MS demonstrated reduced sensitivity in detecting low molecular weight molecules. However, it did mainly reveal the presence of peptides and provides a suitable technique for the study of high molecular weight species.

Exhibiting at Pittcon 2018 were leading scientific instrument manufacturers, Shimadzu and Bruker. Both company's product portfolios include a range of outstanding and innovative solutions for quick and reliable MALDI-TOF analysis.

Michael Marty (University of Arizona) presented the advancement of nanodiscs as a platform for analyzing membrane proteins by mass spectrometry. The interactions between membrane proteins and lipids is often essential to their function, but their dynamic and heterogenous nature makes them challenging to define. The advancements Marty will describe open new doors for studying membrane proteins in nanoscale lipid bilayers. In a 2015 study, Marty and colleagues showed that nanodiscs can be used in native mass spectrometry to probe interactions between membrane proteins and the wider lipid environment.

Also at Pittcon were Wasatch Photonics, designer and manufacturer of world-class spectrometers and optical imaging equipment. Among the company's portfolio is the new WP-series of spectrometers, high throughput instruments with an elegant opto-mechanical design.



Amemiya Shigeru (University of Pittsburgh) will discuss the use of scanning electrochemical microscopy (SECM) for high-resolution imaging of single biological nanopores. Shigeru's work is focused on understanding chemically and biologically important membrane transport phenomena and the development of electrochemical sensors based on interfacial transport processes.

SECM is an electroanalytical scanning probe technique that can be used to image substrate topography and surface reactivity at high resolution.

Shigeru, will talk about how SECM imaging of individual nanopores in a physiological buffer shows that the permeability of a nanopore is related to its topography. Shigeru says a "plugged" nanopore is as permeable as an "open" nanopore, thereby indicating that the plug is a part of the transport or a highly permeable substance that is trapped in the pore.

Attendees interested in scanning electron microscopy (SEM) equipment, enjoyed exhibits by SEM solutions experts Hitachi and Zeiss. The ease of use, high quality imaging and compact design of Hitachi's new generation tabletop microscopes redefine the capabilities of tabletop SEM and Zeiss offers a comprehensive portfolio of SEM instruments that provide high resolution surface imaging and excellent materials contrast.

Lane Baker talked about the integration of dual-barrel membrane patch-ion channel probes (MP-ICPs) and scanning ion conductance microscopy (SICM) as a potentially revolutionizing spatially-resolved chemical sensing technique. Baker will explained a series of experiments that fully characterized the analytical performance of this new platform. SICM is a versatile scanning probe microscopy technique for use in nanoscale ion transport studies.

It can measure electrochemical processes in a way that is complementary and sometimes superior to other electrochemical imaging methods.

Previous work by Baker has included the development of chemical and biochemical sensors that use synthetic conical nanopores to detect the presence of analytes. In a 2006 paper, Baker and colleagues describe a sensor that utilized molecular recognition elements bound to the nanopore mouth, blocking its tip, and thereby detecting the analyte. Another sensor made use of conical nanopores in a resistive-pulse type experiment, with the analyte detected via transient blockages in ionic current.

The rapid development of biosensor design is also highly relevant to the food industry, where foodborne pathogens are of increasing concern due to the recent worldwide spread of bacterial and microbial diseases resulting from agricultural trade. There is a growing demand for improved food safety, but conventional methods for detecting and identifying these microbial contaminants take several days to generate results. The advances in biosensor technologies have enabled more rapid detection of foodborne pathogens and are commonly used for the monitoring of food packaging and agricultural processes.

The International Association of Environmental Analytical Chemistry (IAEAC) is an organization that aims to provide scientists with opportunities to exchange cutting edge research in environmental analytical chemistry.

The organization arranges many events including symposia, workshops and short course to serve as platforms for these exchanges. For example, the IAEAC is currently inviting researchers to the 40th International Conference on Environmental & Food Monitoring organised by the Institute for Food Analysis and Research (IIAA).

The Pittcon conference will also saw presenters discuss the development of new bioanalytical technologies for point-of-care testing. John Connelly (Intellectual Ventures Laboratory) discussed some of the technical and market barriers faced in creating and implementing impactful point-of-care diagnostics.

Jian-Hui Jiang (Hunan University) taught us about the use of nanobiotechnologies in the treatment of cancer. He described nanoscale assemblies of nucleic acids and peptides that enable efficient delivery of DNA or peptide probes into tumor cells. In a 2015 study, Jiang reported on the development of a novel electrostatic DNA nanoassembly that may serve as a valuable tool for low-abundance biomarker discovery and regulation in cell biology and theranostics.

Another of Jiang's studies, published in 2013, describes the development of an immunoassay that provided sensitive detection of protein targets as a result of high surface-enhanced Raman spectroscopy (SERS) signal enhancement through controlled assembly of SERS nanoparticles. The SERS immunoassay platform may serve as a tool to aid accurate and early detection of disease biomarkers and to facilitate point-of-care diagnostics.

Also exhibiting at Pittcon were manufacturers of photonics devices, such as Hamamatsu, who offer a surface-enhanced Raman spectroscopy (SERS) substrate that enhances the Raman scattering light from molecules, for highly sensitive Raman spectroscopic analysis.

References

- *Cui D, et al. Recent Advances in Nanotechnology Applied to Biosensors. PMC. 2009; 9(2): 1033–1053*
- *Chiu D. Micro- and nano-scale chemical analysis of individual sub-cellular compartments. Trends in Analytical Chemistry. 2003; 22(9): 528–536*
- *Shimadzu. Principles of MALDI-TOF Mass Spectrometry https://www.shimadzu.com/an/lifescience/maldi/princpl1.html*
- *Shimadzu. MALDI-TOF Mass Spectrometry. https://www.shimadzu.com/an/lifescience/maldi/*
- *Bruker. MALDI-TOF and TOF/TOF MS. https://www.bruker.com/products/mass-spectrometry-and-separations/maldi-toftof.html*
- *Cho B, et al. Biosensors and their Applications in Food Safety: A Review. Journal of Biosystems Engineering 2016; 41(3): 240–254*
- *Robinson C, et al. Probing the Lipid Annular Belt by Gas-Phase Dissociation of Membrane Proteins in Nanodiscs. Angewandte Chemie 2015; 55(2) 550–554*
  Wasatch Photonics. Why Wasatch for Spectroscopy. https://wasatchphotonics.com/product-category/spectrometers/why-wasatch-spectroscopy/
- *University of Pittsburgh. Shigeru Amemiya. http://www.chem.pitt.edu/person/shigeru-amemiya*
- *Amemiya S, et al. Scanning Electrochemical Microscopy of Individual Single-Walled Carbon Nanotube. Anal Chem 2010; 82(5): 1605–1607*
- *Hitachi. Tabletop Microscopes TM4000/TM4000Plus. https://www.hitachi-hightech.com/global/tm/products/tm4000/*
- *Zeiss. SEM. https://www.zeiss.com/microscopy/int/products/scanning-electron-microscopes.html*
- *Martin C, et al. Biosensing with conically shaped nanopores and nanotubes. Phys. Chem. Chem. Phys 2006, 8, 4976-4988*
- *Baker L. Measuring Ions with Scanning Ion Conductance Microscopy. The Electrochemical Society Interface 2014: 47–52*
- *Iaeac. Home. http://www.iaeac.com/*
- *Jian J, et al. Electrostatic Nucleic Acid Nanoassembly Enables Hybridization Chain Reaction in Living Cells for Ultrasensitive mRNA Imaging. J. Am. Chem. Soc. 2015; 137(21): 6829–6836*
- *Jiang J, et al. Surface-Enhanced Raman Spectroscopy-Based, Homogeneous, Multiplexed Immunoassay with Antibody-Fragments-Decorated Gold Nanoparticles. Analytical Chemistry 2013; 85: 9213–9220*
- *Hamamatsu. SERS substrate. http://www.hamamatsu.com/eu/en/product/category/3200/4019/index. html?nfxsid=5a3aa940d4891151379385 6&gclid=EAIaIQobChMIzK26xZmZ2AIVQrHtCh2SAgahEAA YAyAAEgL6uvD_BwE*
- *Wasatch Photonics. Why Wasatch for Raman. http://wasatchphotonics.com/product-category/spectrometers/why-wasatch-spectroscopy/ http:/wasatchphotonics.com/product-category/spectrometers/why-wasatch-raman/?gclid=EAIaIQobChMIzK 26xZmZ2AIVQrHtCh2SAgahEAAYA*
- *Sweedler J, et al. Capillary electrophoresis with wavelength-resolved laser-induced fluorescence detection. Anal Bioanal Chem 2002;373:332–343*
- *Mauzeroll J, et al. Scanning Electrochemical Microscopy: A Comprehensive Review of Experimental Parameters from 1989 to 2015. Chem. Rev 2016; 116(22): 13234–1327*

1.3 FAR-FIELD FLUORESCENCE NANOSCOPY

This section includes the symposia presented by Nobel Laurette Dr. Stefan Hell, who presented the Wallace H. Coulter Lecture at Pittcon 2018.

Dr Hell received his diploma and doctorate in physics from the University of Heidelberg. From 1991 to 1993, he was a post-doc at the European Molecular Biology Laboratory, also in Heidelberg, followed by stays as a senior researcher at the University of Turku in Finland, between 1993 and 1996. Dr Hell was also a visiting scientist at the University of Oxford, England, in 1994.

In 1997, Stefan was appointed to the Mass Planck Institute for biophysical chemistry as a group leader and was promoted to director in 2002, where he also lead the department of nanobiophletonics. Since 2016, he has also been a director at the Mass Planck Institute for medical research in Heidelberg, where he lead the department of optical nanoscopy.

Dr. Stefan Hell is credited with having conceived, validated, and applied the first viable concept for overcoming Abby's diffraction limit and resolution barrier in light focusing fluorescence microscope.

In his technique, called "Stimulated Emission and Depletion" or "Stand Microscopy", one laser beam excites the fluorescent molecules, but another turns off the fluorescence, except for a small area. The laser beams are moved over the specimen, and an amazing image is gradually built up.

For these achievements and their significance for other fields, he has received numerous awards, including the Prize of the International Commission for Optics in 2000, the Hemholdt's Prize in 2001, the Julia Springer Prize for Applied Physics in 2007, and the Corber European Science Prize in 2011. In 2014, Dr Hell shared the Kavli Prize in Nanoscience and the Nobel Prize in Chemistry for the development of single result fluorescence microscopy.

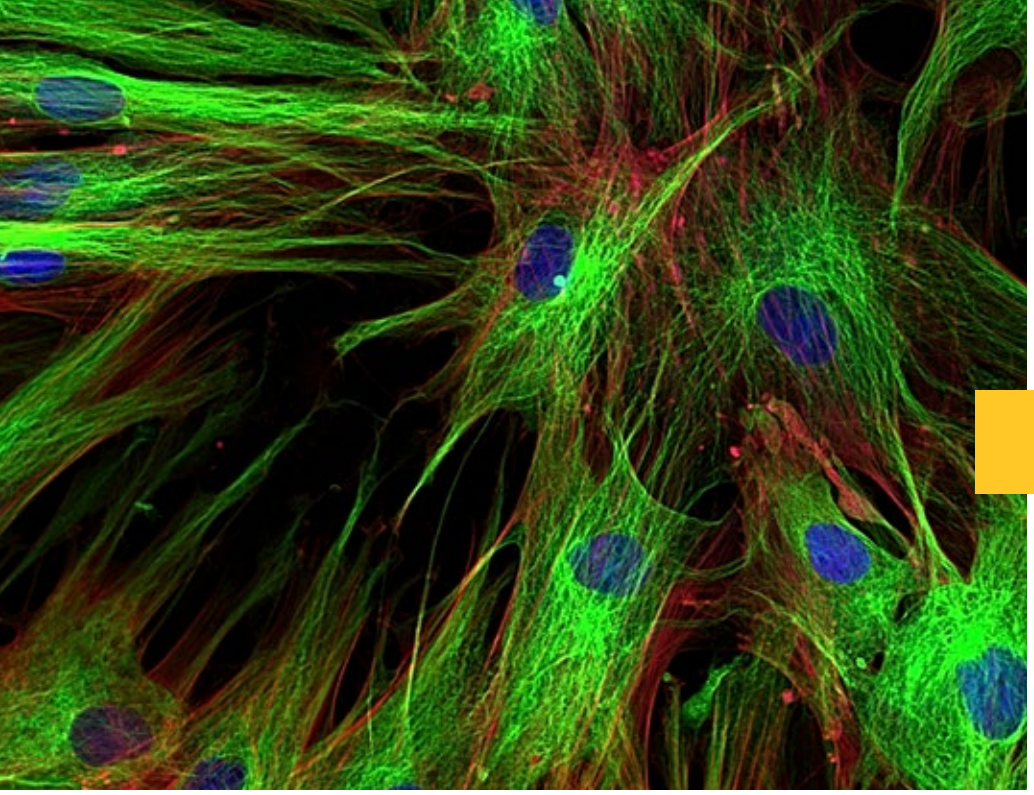
1.3a STED MICROSCOPY AT THE TURN OF THE CENTURY

The 20th Century resolution of any light focusing microscope and fluorescence microscopy was fundamentally limited by diffraction to about 200 nanometers. The best picture you could get with focus-visible light in biological imaging was by confocal microscopy, but that was also diffraction-limited.
The turn of the century witnessed the breaking of the diffraction barrier, meaning that microscopy concepts came up that allowed a much better resolution. The first concept of this kind was STED microscopy.

STED microscopy clearly shows that STED microscopes are capable of attaining much higher spatial resolutions than a confocal microscope or a standard epifluorescence microscope, in this case, by a factor of ten.

This achievement shows that there are physics in the world that allow you to get a much sharper picture than many people had believed possible for a century or more, and has led to the Nobel Prize in Chemistry in 2014.

The above STED picture is taken from the official poster of the Nobel Foundation for the 2014 Nobel Prize in Chemistry. The poster shows a sketch of the STED microscope, and the method that I have developed with my collaborators. It also shows another method, called PALM or STORM microscopy, which shares some fundamental principles but works in different way in very important aspects. It is also very powerful and as such, became very popular.



STED microscopy

STED microscopy has the capability to operate with any fluorophore, whether it's organic fluorphore, GFP or even a quantum dot.

It operates by using a fluorescent excitation beam and a beam that turns molecules off, which is shown in red in the Nobel Foundation sketch below.

The red beam is donut shaped, and by turning molecules off at the outer part of the focus spot, we get the signal just for those molecules at the inner part of the focus spot. Then, by raster scanning those pairs of beams across the specimen, images are much sharper as a result.

At the time, in 1999 or 2000, when the principles of this microscope were demonstrated, STED microscopy looked like this.

This is a picture of an early, laboratory stage STED microscope showing one of my PhD students at that time. At that time, it wasn't known how well the STED microscope worked, and there was still research to be done on the principles. For instance, it wasn't clear what would need to be left out to make it viable.

A modern STED microscope, shown above, could fit into a shoebox, including all of the beams, forming elements, and the scanners, amongst other elements, and can be attached to any epifluorescence microscope. In essence, it's possible to convert any epifluorescent microscope from any manufacturer and turn it into a super resolution STED microscope in three minutes at the push of a button. It's possible to do this for $220,000 in more than 30 units, be it dollars, pounds, euro, or otherwise. With this, the time in which super resolution or STED microscopy was quite complicated and difficult to use is over. Now, it's a push button technique that can be readily applied.

As a matter of fact, STED microscopy has been applied to all kinds of investigations in cell biology. The image below is from a very recent review that was published just a few months ago, and it shows a sketch of the cell and the areas it was applied to such as the cytoskeleton, the mitochondria and the Golgi apparatus, both in fixed cells and living cells.

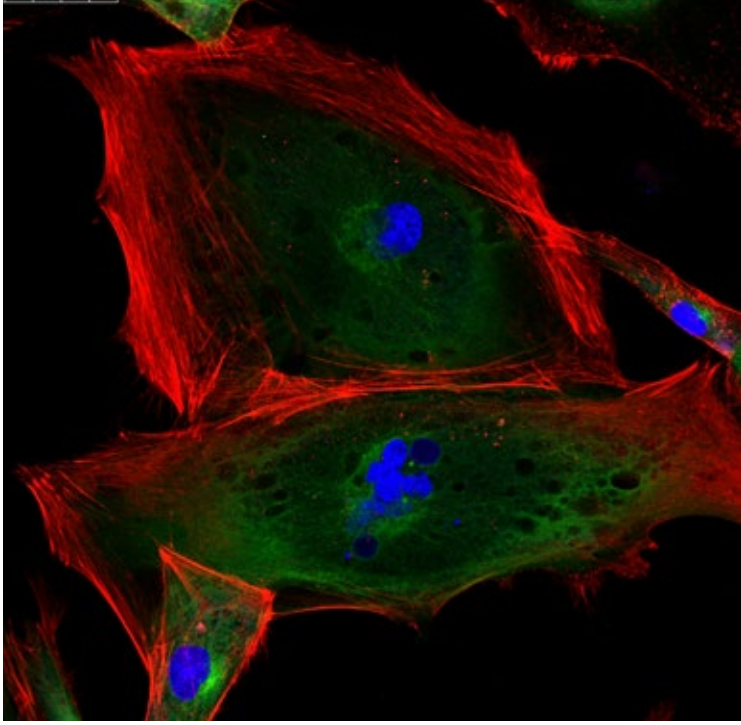
At present, there are some compromises that need to be taken when you apply to living cells, but it does also work for imaging living cells. A new graph, below, shows the application range in neurosciences in particular, where it has also been applied to the imaging of neurons in vivo to the molecular layer and upper layer of living mice, and you can even see them move.

Recent developments and motivations

I'm not going to talk much about STED microscopy, although it's clearly something that was very close to my heart for many, many years, and is still very close to my heart. I'm going to talk about more recent developments and my motivation for these.

The two cornerstones of super resolution fluorescence microscopy, STED microscopy, and PALM or STORM microscopy have many similarities. Of course, there are differences as well. But, one certain similarity that was clear from the outset is that in principle, both methods at the time of conception can obtain a spatial resolution that is the size of a molecule, of one nanometer. In principle, both methods can do this. But in practice, they don't.

In practice, the beginning of spatial resolution is largely by a fact of three, four, five, or ten or so. And only under very rare circumstances will you get a much higher spatial resolution



of below 10 nanometers, for instance. There are special variants of STED microscopy applications where you can get the single digit nanometer resolution, but special fluorophores are necessary. The same applies to PALM or STORM microscopy, with which under special conditions or special variations, it's possible to get down to a few nanometers, but not as a general rule.

When I started out looking into this problem many years back in the early 90s, of course my dream was to get down to a spatial resolution that is really at the size of a nanometer, and so at the time the Nobel Prize was given, it was clear that in principle, you can achieve a spatial resolution of the size of a molecule or a nanometer, but not in practice. So, the way in which the Nobel Foundation put it is very correct.

The microscopes really crossed this threshold but did not attain the ultimate limit. So, after having had the honor of sharing the Nobel Prize, I realised I had to deliver, and I'm going to show that there is indeed a way to get down to one nanometer spatial resolution.

MINFLUX: ATTAINING A MOLECULAR SIZE AT ONE NANOMETER RESOLUTION

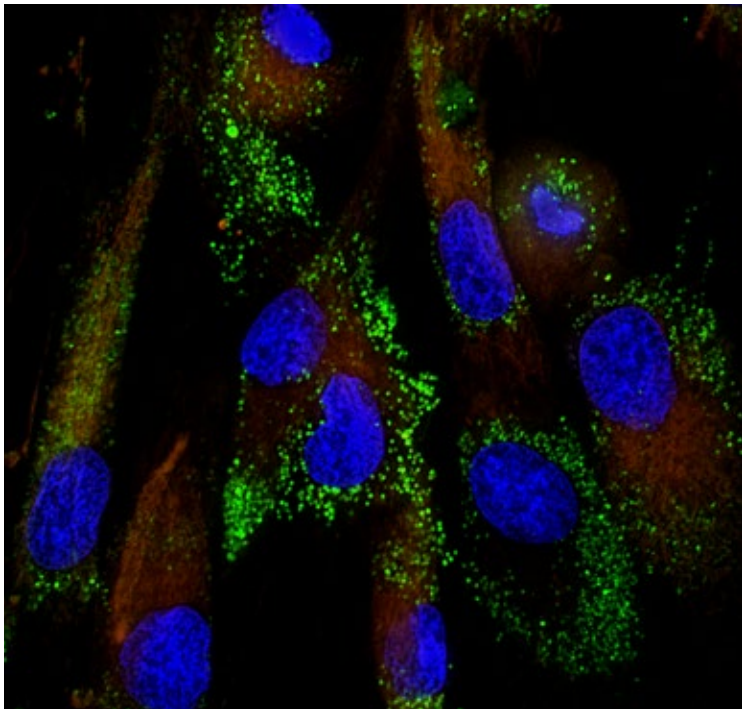
In order to explain to you how we now obtain a resolution that high, you have to understand the basic principles of super resolution or fluorescence microscopy or nanoscopy. In order to do that I'm coming back to the very basics.

Why was the resolution of light microscopy in the 20th Century limited by diffraction? Why did people believe that resolution of a light microscope is fundamentally limited by diffraction? The answer is very simple, because they thought, and this was a misconception, that a separation of the features in a light focusing microscope had to be done by the phenomenon of focusing the light. So, in other words, if you manage to focus a light very, very sharply down to a small spot, then of course, you get high spatial resolution, because you can concentrate that sharp spot of light to a very tiny area, which is shown here.

The same applies if you have epifluorescence imaging. Each feature will produce light that cannot be focused down to a point. So, in the detection plane we've also got a block of light that's limited by diffraction. Again, you're also limited by diffraction on the detector side, meaning that if you have features coming closer than what is given by a basic equation, wavelength divided by twice the numerical aperture, to the objective lens, you cannot tell the features apart, because either the features will be flooded with light at the same time, producing a confused signal, or, in the back focal plane, the diffracted signal of each of the features will be overlapped in the space, and no detector will be able to tell the features apart.

So, the problem was, and I think this is a very, very important insight, that people thought you have to separate by focusing, but if you give up this notion and we separate in a different way, we can overcome the diffraction barrier. The discovery that has actually led to modern super resolution techniques and to the very high resolution was the idea to give up the phenomenon of focusing for the purpose of separation.

Modern super resolution microscopes, whether they're called STED, PALM, STORM or otherwise, in the end, are separate features by molecular states, and once you separate the features by molecular states, the focusing doesn't really matter, because the separation isn't done by focusing anymore.



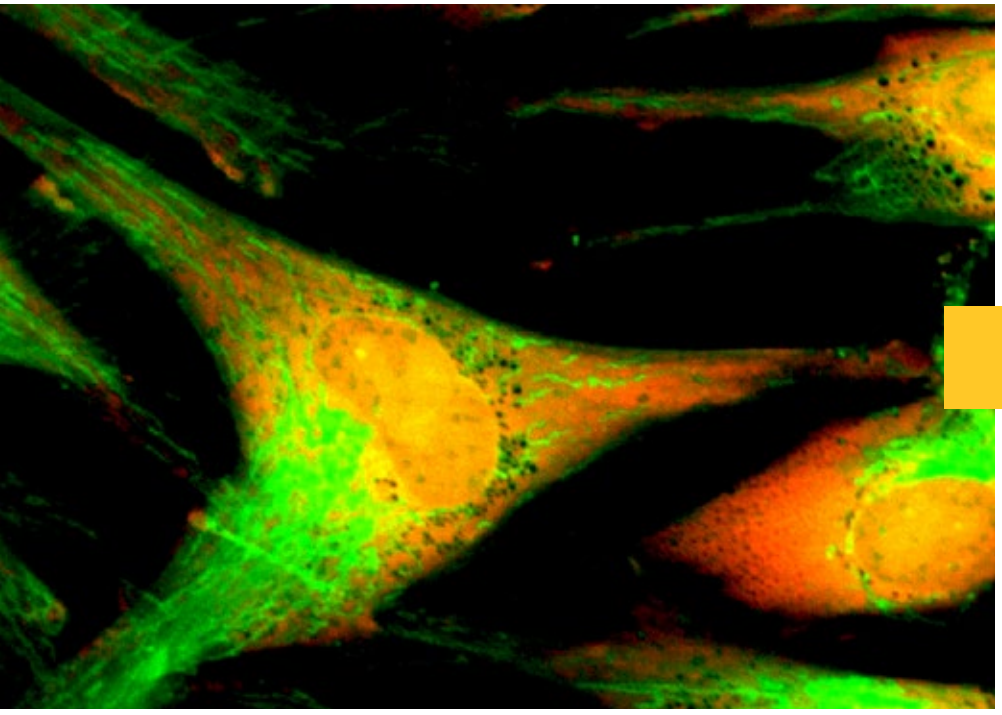
What does this change mean?

If you have a several features falling within this green spot of excitation light, as seen below, and if you manage to keep some of your molecules in a different state, the simplest case in the state which is not capable of emitting fluorescent light, then of course you can separate emitting fluorophores, the yellow ones in the center in the image below, from the black ones, because those cannot emit light. Thus, it's very clear we can separate the feature in the centre of that green spot from those at the outer part because we separate by molecular states.

This has been the basic idea of STED microscopy, and this is how STED microscopy sets itself apart from any other super resolution concept that existed before. It played on and off with the dye in order to make the dyes distinguishable within that green diffracted spot of light.

Similarly, emission pushes molecules from the excited fluorescent state back down to the ground state, and in this way, we can keep the molecules dark. So, not only do we have a beam of light that turns molecules on, pushing them from the ground state to the excited state, we also have a beam that does the opposite and turns them off. By turning them off at the outer part of the spot by using this donut-shaped beam, we can produce two classes of molecules that are in the on state that are emitting light and molecules that are in the off state. Then we go to the next one, and we go to the next one, and so on, so we can get each of the molecules or features emitting by turning them on and off.

Now, a hallmark of STED microscopy is that we use a donut beam that has a zero intensity point at the center to leave an area where the molecules are allowed to assume the on state, and in the rest of the focal area, they have to be off.



For that reason, we always know where the signal comes from. We can say we actually target the coordinate in which the molecules are allowed to be on. So, we always know the x, y, z coordinates of where molecules are on.

As we scan with the donut shaped beam across the focal region, we always know where the signal comes from. We don't have to find out. We know because that donut-shaped beam critically determines where the molecules are on and where the molecules are off, as seen in this graph.

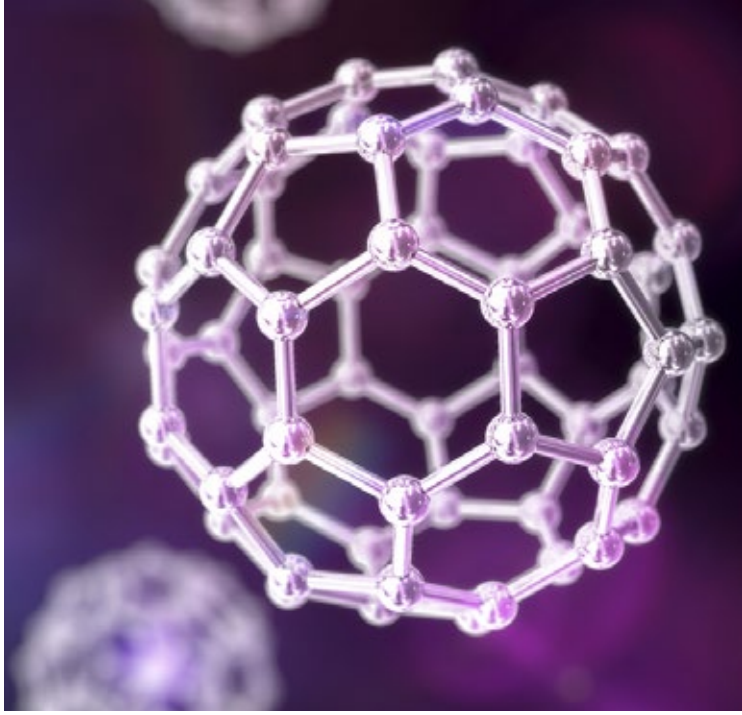
As I scan the donut beam across that green excitation area, we target different features, but we always know where the features are located, because we control the position of that donut with very high precision with a meter scanner, for instance. STED uses the most fundamental way of turning the fluorophore on or off that simulates emission.

If you want to turn off any fluorophore off, say for the physical means, the most general way of looking at this simulated emission. But of course, there are some other states, like dark states, that you can use in order to transitionally turn a molecule off and they have been described in literature. You can push molecules to a long lift dark state or triplet state or electronic transfer. You can have a cis/trans itemization. For example, the cis form is capable of emitting light. This form was fluorophore but a trans form is not, so it doesn't emit light if you put excitation light on it.

As long as you have at least two seperable states, you can use these states in order to make the molecule distinguishable, and because the separation is done by states, but not by the focusing of light, you can break the diffraction barrier. However, you also need the coordinate, and that is done in STED microscopy and related techniques just by having this donut shaped type of beam.

How does PALM/STORM microscopy relate to this technique?

PALM/STORM relates with this completely, and it also uses the same principle of using on and off for separation. But the on/off separation is implemented in a different way.

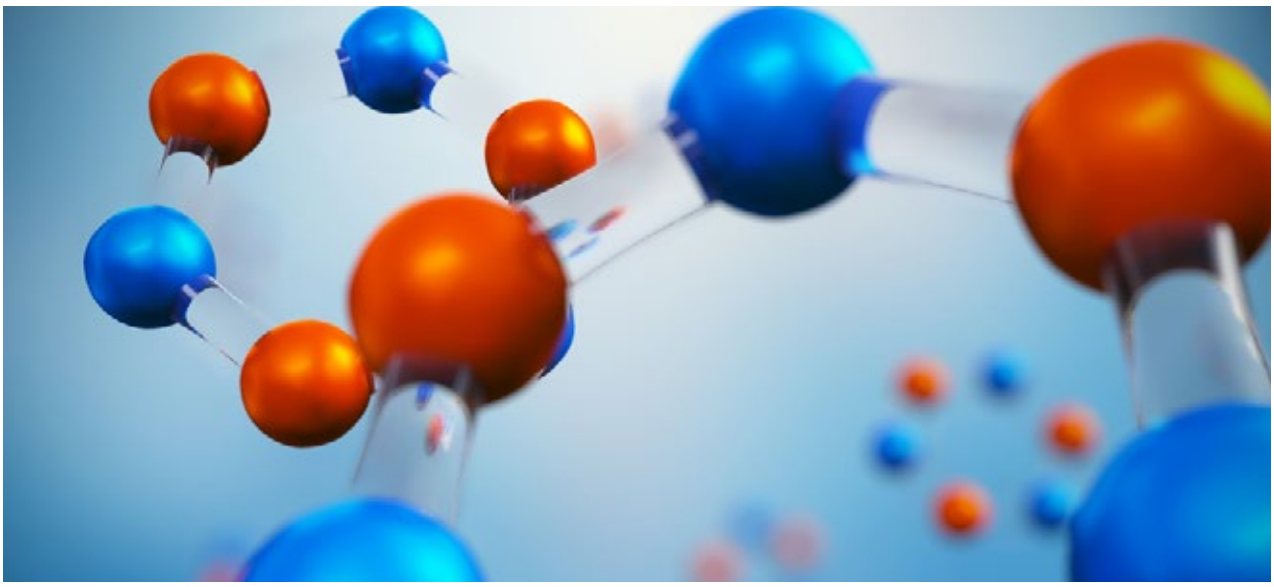


Rather than using a donut or any other type of pattern of light, to determine where the molecules are on and where the molecules off, in the PALM/STORM concept, the molecules are features, and are turned on and off individually on a single molecule basis. So in that feature only one molecule is turned on. Then you can separate the feature from the other feature, because the molecule of that feature is emitting, but the other features of the other molecules are not. Then you turn the next one on, and the next one, and in this way you can separate the features, because only one molecule within that green diffraction area of excitation light is emitted.

Now, if that happens randomly and stochastically, one has to find out where the molecule is located. It's unlike STED where that position is already determined. So you have to find out. This works in the PALM/STORM concept, by making sure that in the on state, there are many photon emissions, so it has to be an on state, which leads to many photon emissions. If you use the cis/trans pair of states, for example, and if the molecule in the on state emits hundreds of photons, it will produce a diffraction block on the camera. It's then possible to anticipate or to calculate the position of the molecule by calculating the centroid, because the centroid or the anticipated maximum of emission must coincide with the position of the molecule, if you back-project it into the object plane.

This has actually been known for a long time. The more photons you have the better it is, and better is the precision you can get regarding the position of the molecule.

Interestingly, it becomes very obvious that there is a fundamental similarity in the relationship between both concepts. It's very obvious that in order to define the position in space with light, with photons, be it on the camera or in the focal region with STED, you need a lot of photons. It's impossible to define a coordinate with just a single photon. But the more photons you have, the better you can define, a donut minimum or emission maximum for example.



So, you always need a high number of photons. While it's clear that there is a similarity between both concepts, in the STED case, the many photons that you need come from the laser. So, the positioning in the STED case is done with those photons that come from the laser because you want to produce a donut minimum, whereas, in PALM/STORM, the photons come from the dye. Once these similarities and differences are clear, so too are the strengths and weaknesses of each concept.

The strengths and weaknesses of the PALM/STORM concept

Well, clear signs of the PALM/STORM concept is that if you want to get down to a high spatial resolution, down to a nanometer or so, it's because you work with single molecules, which is a clear advantage if you want work get molecular resolution as you would already be dealing with those small entities.

But, the downside of PALM/STORM is that you need a high number of photons on the camera. So to get a very high precision of the donut, you will need 10,000 or 100,000 photons, or even more, to get a high precision of the donut. But, that's not easily achieved. The photons that come from the dye are usually limited in number to maybe 100 or 1000, or a molecule breaches or goes to a long lift off state, or it goes on and off and moves away, so you don't get those high numbers of photons in order to get to a high spatial resolution, usually of one nanometer or so.

If you look at STED, photon numbers are not a problem because the positioning is done with the high number of photons that come from the laser. But, a laser has zillions photons. So defining a coordinate instead is, of course, much easier than in PALM/STORM. However, the strength of PALM/STORM remains in the fact that you work on a single molecule basis.

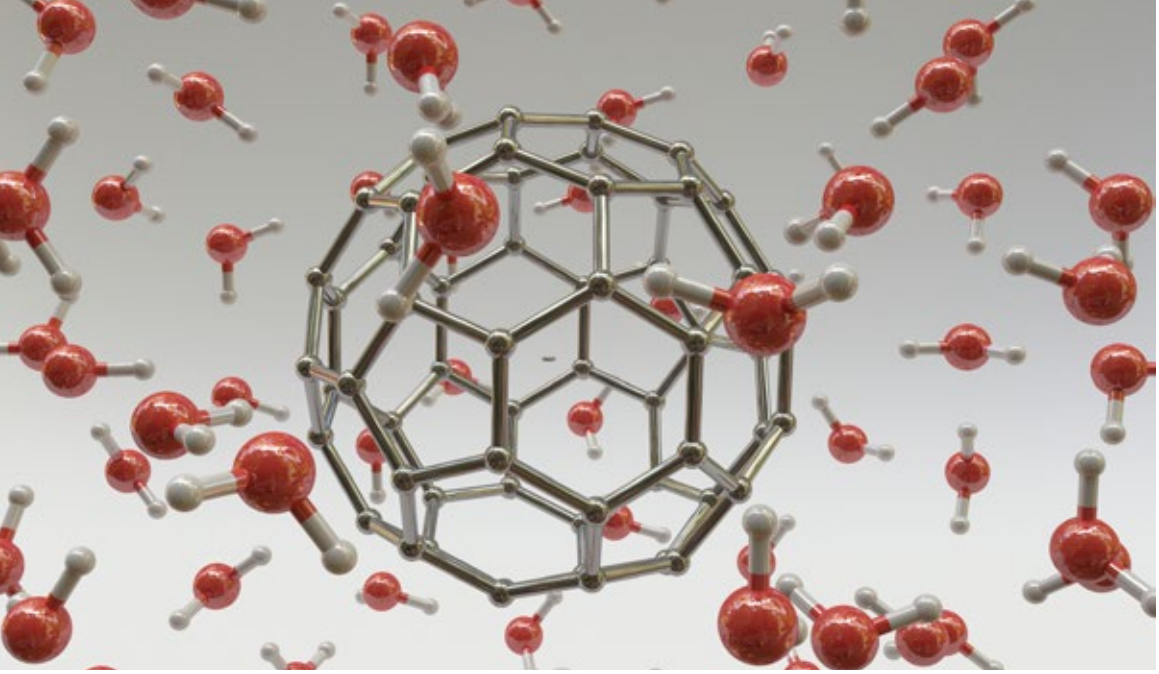
Combining PALM/STORM and STED

So, why not combine the strengths of the two, for instance doing the positioning with the photons that come from the laser but work on a single molecule basis? If you could combine those two, of course you can come up with a new concept. So, I called it MINFLUX, and this should lead in the end to a spatial resolution of a few nanometers that was clearly not anticipated five or six years ago.

The MINFLUX concept In essence, MINFLUX is a new way of localizing individual molecules, which is not using conventional methods with a camera as it's usually done in single molecule tracking or in PALM/STORM.

Usually when people localize individual molecules, they have an epifluorescence microscope. It has a wide field of illumination, which is why I have a green rectangle to show wide field of green excitation light, shown above, and the star in the center is the single molecule. As it is shown here on the diagram, what happens is that someone will have a camera, and that molecule produces a block of diffraction light on the camera, that is displayed on the computer screen. Then you can localalize by calculating the centroid of that diffraction block.

Depending on the number of photons that they have in that diffraction block, they get a certain precision that squares inversely with the square root of the number of photons in that diffraction block. As mentioned previously, the higher the amount of photons, the higher the precision will be. This is the conventional way of localizing molecules, and this is fundamentally limited by the number of photons that are emitted by the dye during the period of measurement. If you do it like this, you fully rely on the number of emissions. There are also some other issues around orientation of the molecules.

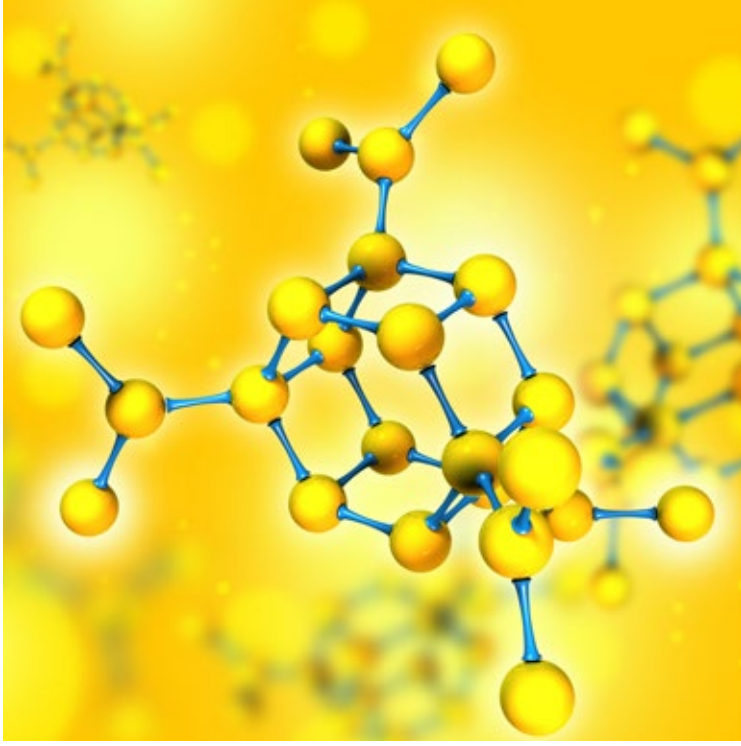


So, we're combining the other strengths of STED with the strengths of using single molecules. So, how do we get the strengths of STED? The strength of the STED concept is that we define the position in space with the donut beam. As a matter of fact, a donut beam is fantastic at defining positions in space. Why? Because a donut beam has a central intensity of zero, and that zero can be defined with very high precision if you have enough photons coming from the laser.

So, donut zero very effectively defines a coordinate inside the sample space. Now imagine we don't do signal emission, we don't de-excite molecules or turn the molecules off. Instead, we use a green beam, and a green donut beam is used for exciting light.

Now, you can imagine, if that molecule, the little yellow star right in the center of the donut in the donut minimum, then there wouldn't be any excitation and no fluorescence light emitted because there is no light and the center of the donut has an intensity of zero, which leads to an absence of signal. We would know, however, that the molecule is in the center of the donut and we would know where the molecule is located because we control the donut position physically with arbitrary precision. We would know exactly where the molecule is located from the absence of fluorescence emission.

If the molecule is slightly off the donut position, or the donut is slightly off the molecule, it doesn't matter and we would still get signal and from the strength of the signal we can actually infer the position of the molecule, and know that the molecule is not coinciding with the donut zero. One can imagine coming up with a concept that measures the position of the molecule by trying to assess the position of molecule with a donut beam. Regarding concerns about not getting any signal if something is in the center and having to wait for a long time to get it, that is actually not the case. We're able to crank up the power of the donut and it won't bleach the molecule because the molecule is in the region where the intensity is very weak. Therefore, without being bleached, we still get signal and there is a way of controlling the signal and the measuring time without bleaching the molecule.



The ideas behind this localization scheme

Now, since my last name is Hell, I thought I have to come up with an anaology of a little demon. So, this is that demon.

Let's imagine the demon knows exactly where the molecule is located. Once the molecule starts moving, of course, the demon will know exactly where the molecule will go, and so the demon controls the position of the donut. So, there's a beam deflector in there, and that beam deflector is operated by the demon, and the demon then pushes the donut in such a way that zero coincides with the position of the molecule.

Now, since the demon knows exactly where the molecule will go, the demon obviously manages to always make the molecule coincide with the donut zero or in other words, the donut zero will always coincide with the molecule because the demon pushes the donut zero exactly to the position of the molecule.

So, why is this side experiment interesting? It's interesting because it shows that in this way the demon could trace the position of the molecule with arbitrary, nanometer precision, without requiring any signal photon emission, because the molecule is in the center of the donut and there is no fluorescence excitation going on there. But from the fact that there are no emissions due to the lack of signal, we know the molecule must be in the center of the donut. And so, we know the position of the molecule at any point in time with arbitrary precision.

What actually happens here inside this experiment is that the positioning is not done with the fluorescence photon of coming out as it's done in conventional localization or in PALM/STORM. Instead, the positioning is largely done by the photons that come from the laser, by the excitation photons. The excitation photons determine the position in space. We then get well-defined coordinates and so you do localization by the photons that come from the light source to a large extent, and you are not limited in the number of photons that you have.

Now, of course, there is no such thing as a demon and even I cannot make one. But, what we can do is approach the demon situation by implementing electronic circuit that measures the fluorescence that is detected when molecule is slightly off the donut position. So, if the molecule is slightly off the donut center, then of course, it would produce some signal

Watch Dr. Hell's demo here:
https://www.news-medical.net/webinar/Light-Microscopy-The-Resolution-Revolution

and would indicated the actual position of the molecule. That signal can be fed into a closed loop which pushes the deflector. Then we would know roughly where the molecule is located.

So, in essence, by operating a closed loop where we try to catch the molecule and bring the donut center close to the position of the molecule, we can trace the position of the molecule with very high precision, but with very few emitted photons.

So, we save a lot of emitted photons and we still get a very high precision. Why? The majority of the localization is done with the photons that come in, so we get a rough position of the photons that come in, and we only need to do fine tuning with a few fluorescence emissions that indicate how off we are with respect to the actual position of the molecule.

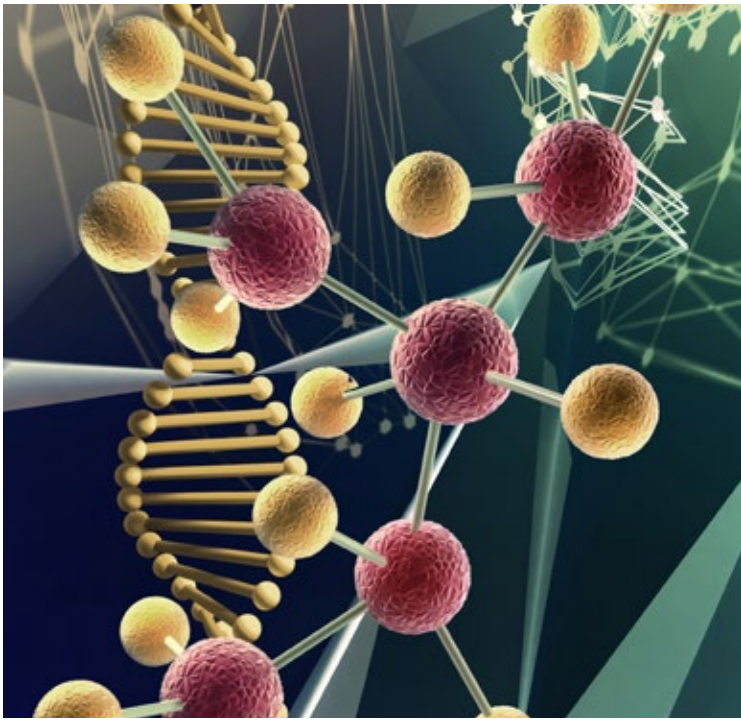
You won't be surprised that I decided to call it MINFLUX. It stems from the fact that we use a minimum for detecting the position of the molecule, and of course it also requires a minimum of fluorescence emission in order to get high molecule precision.

How MINFLUX maximizes information from the signal

How do we maximize the information that we get from the signal? Let's assume the molecule is in between these dots, the purple dot and the yellow dot.

And so the molecule is in between and we anticipate that the molecule is somewhere on that line that has it's length, L.

The donut has a center minimum, and at first approximation, the zero around the minimum is a parabola. So, it's a parabolic approximation. Then we get to the position of the molecule simply by scanning that donut across the molecule, exciting the molecule. The yellow line in the lower panel is the fluorescence signal. As soon as the donut zero overlaps in space with the position of the molecule, fluoroescence is zero. From this, we will instantly know where the molecule is located from the position of the zero emission.

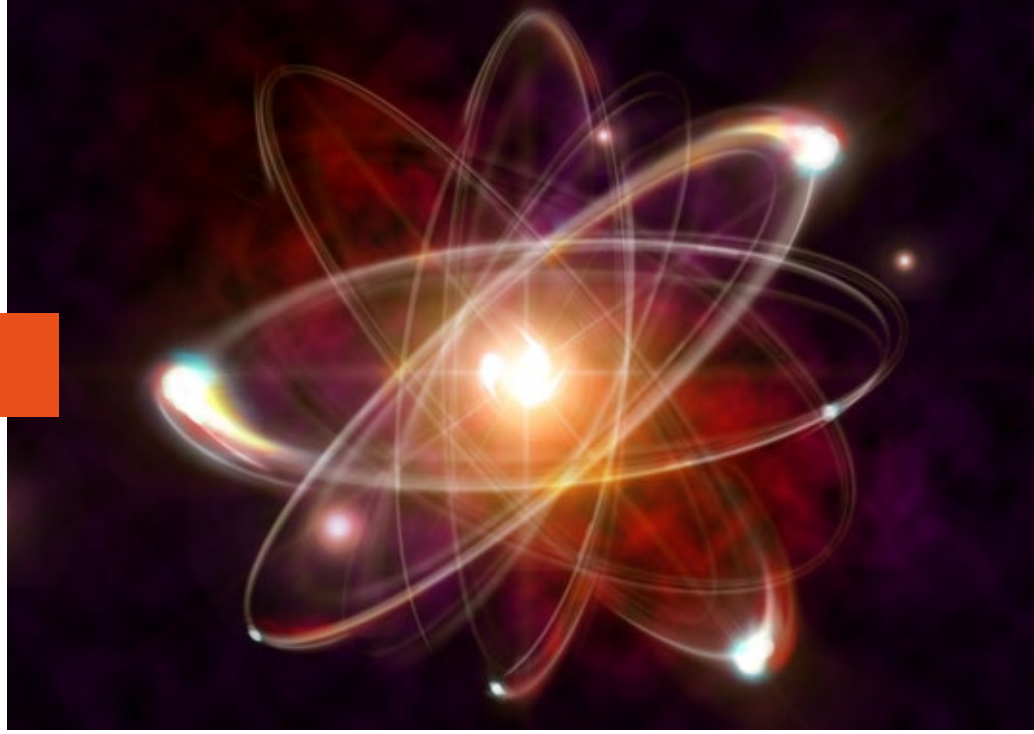


The point I am making is as follows. We don't have to scan that densely across the molecule. If you know the intensity curve of that donut, then it's perfectly enough to measure the end points of the fluorescence signal.

If you know that this is a parabola, so the yellow line is a parabola because the donut intensity on the zero is a parabola, then we can make a simple calculation and find out the position of the maximum Xm by dividing L, the region which we scan, over one plus the square root of the brightness of the two end points. So it comes out as a seventh grade quadratic equation. The simple equation tells us there's no wavelength dependence. If you look at it again, you see the position of the molecule depends on the range L out that is not wavelength dependent and the nominator also doesn't contain the wavelengths.

This may look a little bit strange in the first moment, because we used focused light, and of course, light has a finite wavelength. But it's not that strange. In the end, the molecule, or the singularity, is just a point, and then we check the position of that point with another point, which is a zero of that intensity profile. So, basically, we use two points. And then it doesn't really matter how the zero is graded with the wavelength that is graded. In the end, it's all about the point, the zero intensity point, and a molecule is also a point. This is why our wavelengths don't really matter, and it shows that this concept was very, very powerful.

Now, if you do the math, you will find out that the uncertainty, as there is always an uncertainty of finding that position Xm, and it will depend on the range L, so the range in which we anticipate that molecule and again it squares inversely with the square root of the number of detected photons. So, n is the number of detected photons, the sum of zero and one. The dependence on the number of detected photons remains, one over the square root of n. However, and that's our major difference.



We have a handle on the problem, because it always depends on that range of L, over which we scanned, anticipating the molecule, and we can change that range. We can make that smaller because once we have a course, and an anticipation of where the molecule is located, we can use that range, because we know that it's roughly in that position, and we can make our range smaller. By making it smaller, we make the precision increase. It's very simple.

Making something small in a linear way, since it depends linearly on L is much more effective than waiting for more photons to come, because the number of photons is dampened by this square root dependent. So, it's much more effective to reduce the area than to wait for more photons, because we have a number of photons and we have this square root dependent. In other words, we can increase the precision by iteratively go down with the range where we look out for the molecule. So, this is the essence of it. By the way, there's also no dependence on the molecular orientation, or only to a very, very high order, so it doesn't really matter.

But in 2D, of course, you have to have more points, three will be enough, but four would be better to avoid ambiguities.

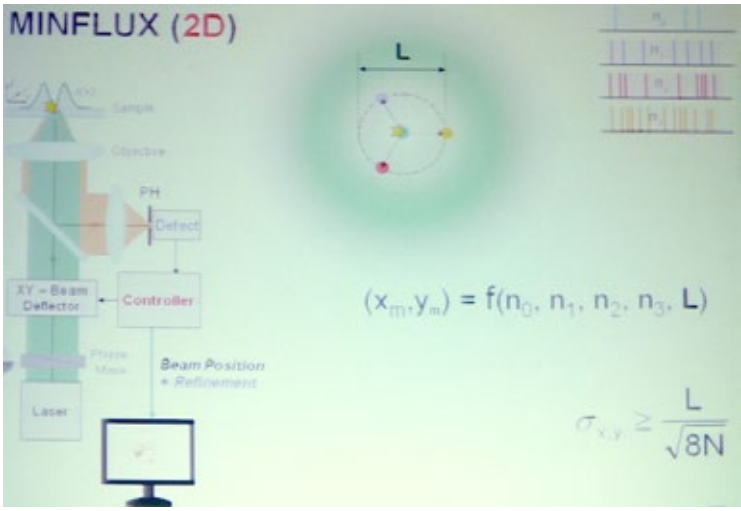
Then, the equation isn't that simple anymore. But, it can be sorted out and you can find the function that gives us a position based on the measurements of the four points where we placed the donut: the red point, violet point, yellow point and the blue one in the center. Then we find out the position of the yellow molecule. Once we know roughly where it is, we don't have to wait to get even more photons. We just squeeze the area in which we look for the position of the molecule and that means that we increase the precision. So, by keeping the number of detected photons the same, and reducing the area, of course, we increase the precision without requiring more emissions.

Above, what we're showing is how many photons you need to get a certain localization precision. At the bottom, you see the total number of detected fluoroescence photons for a camera, which are the two lines on the top for a perfect camera and a realistic camera.

The orange lines that are showing a range of about 50 nanometers are for the new concept, in which we look for a molecule. This is, of course, a theoretical calculation. It shows that the measurement nicely coincides with the theory. Above all, with ten detected photons we get the precision of ten nanometers, which isn't bad. With a camera, you need 290 detected photons for a signal to background ratio of 200. Why do we save fluoroescence photons for localization? It's because, to a large extent, the localization is done with the photons that come from the laser by defining them in that space with the donut-shaped excitation beam.

One has this dependence on the scanning range or on the investigation range, and once we have a coarse approximation of where it is, we make that region smaller and then you need fewer photons. So, the reduction of the investigation range outperforms one over the square root of n dependence. This is an example of where you can really outperform this dependence on the number of emitted photons, and I think this is really important. Otherwise, the only option is to wait for longer, and then in the end you will be limited.

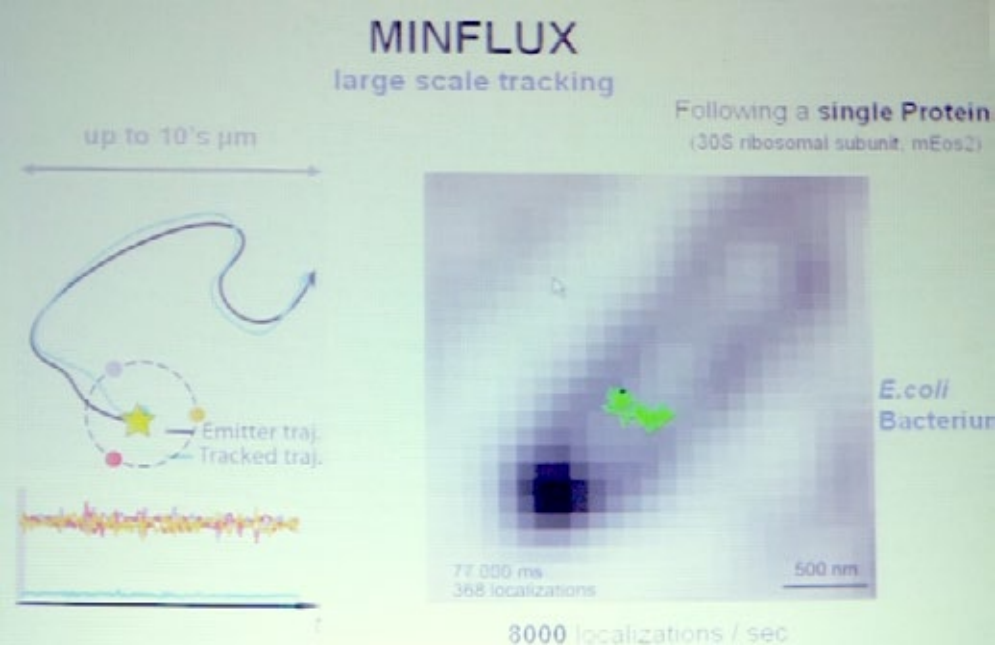
There are several ways of doing imaging now. One method, since it would be single molecule based, would be to turn it on and off as it is done in PALM/STORM. There's no difference in that regard in the sense that you would turn that molecule on with investigative range and keep the rest dark. But, you wouldn't localize the molecules by getting the diffraction pattern



on a camera, but by scanning the position of the molecule with an excitation donut, and making four measurements as is done in here. From those four measurements, you can infer the position of the molecule, or you can track it if a molecule moves around in the focal region.

The blue, yellow, red and violet points above show the measurement points where we place our donuts. We place those donuts and move the zero in quick succession at these points, and then from these four measurements, we instantly can infer the position of the molecule.

We also have the tracking, as above, to move those measurement points along with the molecule. This has actually had two demonstrations, showing that one can track molecules much faster than we have usually done with cameras.



Pictured above is a protein, a ribosomal subunit labelled with mEos2, which is typically used when you see a molecule localization technique. Here we get about 8,000 localizations per second, with a precision in this case of about 40 nanometers in a living e-coli. It's a substantially higher speed that can be 100 times, 200 times faster than what is usually done. I think this has a great deal of potential for investigating movements of molecules, especially of macromolecules on very small scales. Why? If you look just at single molecules, macromolecular entities, then you can confine the investigation range, the L, to 10, 20 nanometers, meaning that you need only a few photons resolution to get a very high spatial resolution or to get down to a nanometer or so.

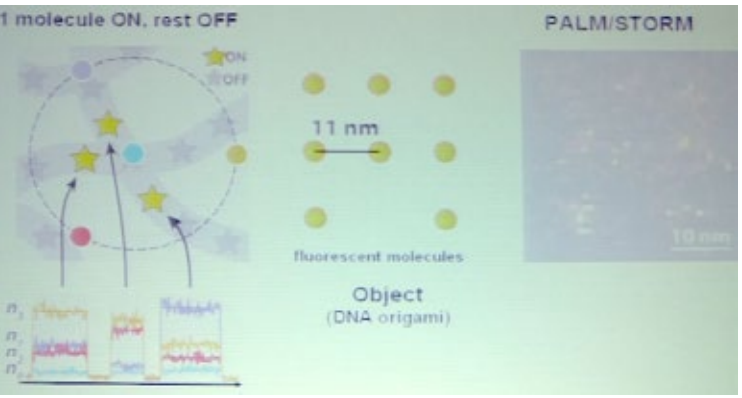
As a demonstration, we labelled a piece of DNA with a fluorophore, but in this case, the fluorophore was arrested, in a way. So, we sent a sigma of about 2.4 nanometers, which is not bad, or for a sigma of 2.4 nanometers localization precision required only 400 microseconds. The measurement was done on the four points, the violet, yellow, blue, and the green points. And the cloud actually shows the distribution of localizations.

The next thing that we did was to allow the molecule to move by putting it on a DNA bridge. This is seen in the distribution of the localization that has now spread out. But, that isn't a certainty. The precision of the position of each of the molecules at any given instant was very high, 2.5 nanometers, and localized in 400 microseconds.

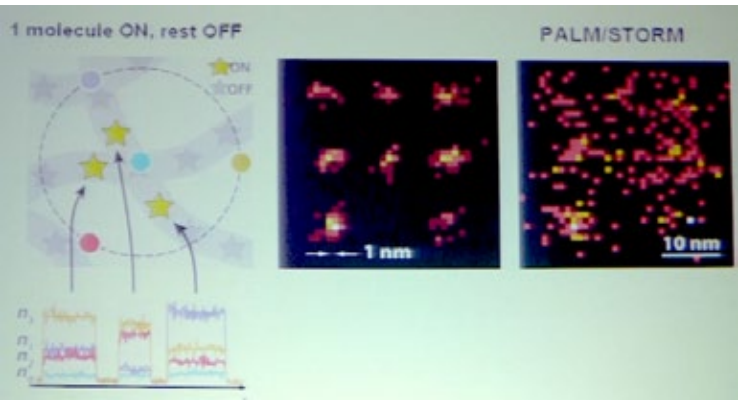
In this experiment there were seven times fewer photons needed than with a camera. This has allowed us to take a reconstructed movie of the movement of the molecule, showing how the molecule moved during the measurement. This gave us that distribution of localizations over time.

I think there's a lot of potential for a kind of dynamic structure biology, and I think this has implications across various fields including pharmacology.

The true molecular revolution was not attained with standard fluorophores at the time that the Nobel Prize was given, with only a few exceptions. So, molecular arrangements like this, where the molecules were just 11 nanometers apart, as shown in the image of a specific molecular arrangement below, could not be readily imaged.



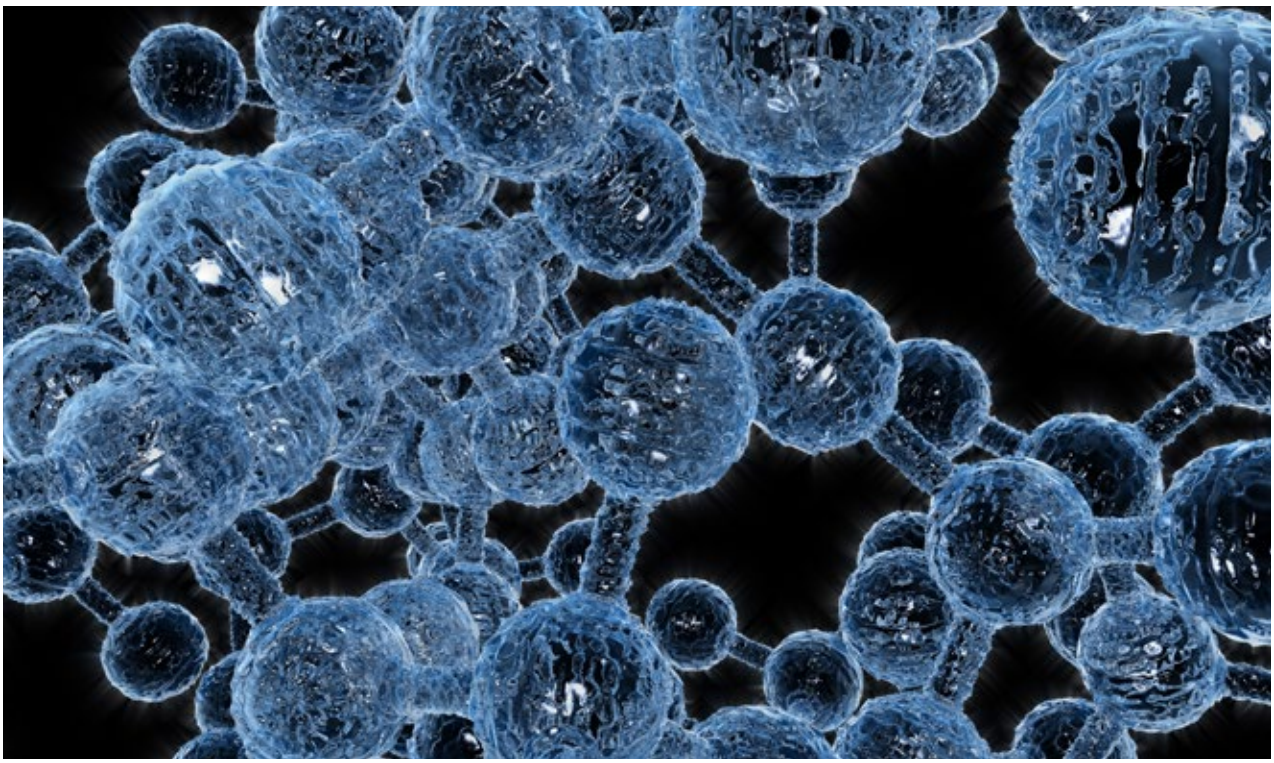
So, we have simulated a PALM/STORM image under ideal conditions.



In this simulation, there is perfect single and no background, and this is the comparison above. It's clearly because of the fact that we require fewer photons to locate our molecules that we could resolve those molecules very easily with the same number of photons.

I like these pictures very much, because although not visually very arresting, these molecules are only about six nanometers apart, which are molecular distances. This is six nanometers, so the diffraction barrier is 250 nanometers. 20 years back, people would have said, "What? You want results from molecules that are six nanometers apart with focus visible light and there's no dependence on the wavelengths? Come on. You must be crazy." But this is reality. It is like that.

We can image at a resolution that is down to the size of a molecule and with no wavelength dependence. That's it. And don't forget, it's done with regular, objective lenses. It's focused visible light, and the key to this achievement is not resolve by the phenomenon of focusing, but to resolve by molecular states.



Conclusion

To sum up, resolution at a true molecular scale can indeed be achieved. So this is where the first experiment showed this is possible in a very general way. The localization speed, if it comes down to localization can be increased 10 fold, 100 fold, and this is just the beginning. I think it will even be 1000 fold and even more in the future because the conception speed and the photons saving limits have not been reached.

Approaching the molecule position, iteratively, which I have not done in these measurements, will lead to further photon savings because the one over square root of n dependence is fundamentally outperformed. So, this is very effective method. Of course, just calculating the centroid is not a central element of breaking diffraction barriers. It is one way of getting the coordinate, but it's not the most impressive or efficient one. Applications can be made in many ways, including imaging and applying high resolution to all kinds of areas, but I can imagine complementing FRET for instance, energy transfer, and applications structure or biology, like protein folding and many others.

1.4 MULTIPLEXED AND SENSITIVE BIOANALYSIS USING SERS

This section of the ebook includes a presentation from Karen Faulds at Pittcon 2018, titled "Multiplexed and Sensitive Bioanalysis using SERS".

Karen's research involves using Raman spectroscopy specifically for surface enhancement. The area she's interested in is bioanalysis and with a particular emphasis on its sensitivity and multiplexing. She wants it to be able to save low concentrations as well as multiple analytes in one sample:

We've carried out a lot of work previously in molecular diagnostics and detection of DNA sequences that I'll discuss here, as well as developing different protein assays used in functionalized nanoparticles.

We've done some work in nanopatterning particularly right down to molecules or nanoparticles and surfaces, for example, to look at stem cell differentiation and surfaces based on the stiffness and the spacing of the molecules in between. Surface-enhanced Raman spectroscopy (SERS) is about absorption of molecules onto roughened metal surfaces and we focus on nanoparticles. This includes silver and gold, and historically silver is more commonly used because we get strong enhancements for silver surfaces, but now that we're starting to do more work for in vivo and animal models, we have been using more red-shifted nanoparticles of gold.

Surface Enhanced Resonance Raman Scattering

- Adsorption of analyte onto a roughened metal surface greatly enhances Raman scattering (10^6)
- If analyte is coloured can further enhance the signal due to resonance (10^{14})
- Our preferred metal surface is silver nanoparticles
- Can use commercially available fluorophores as Raman reporters

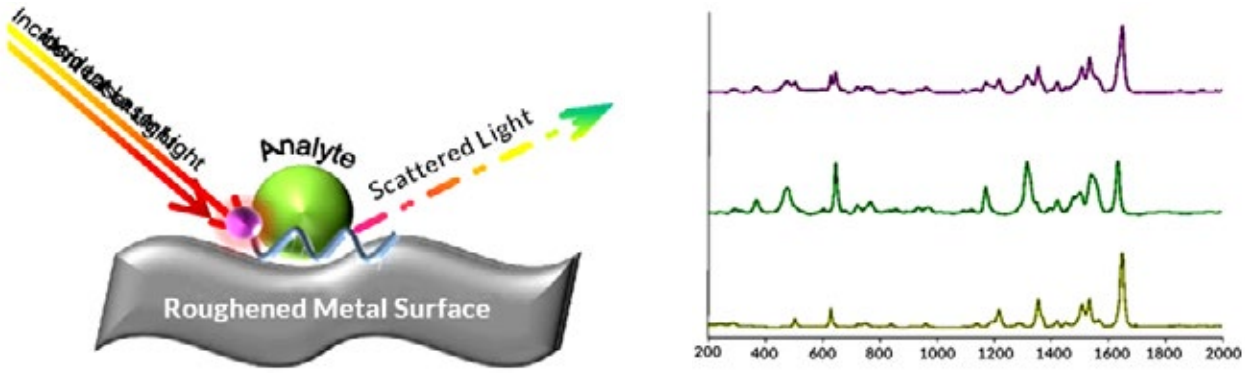


Figure 1: *Slide from Karen Faulds presentation on 'Multiplexed and Sensitive Bioanalysis Using SERS' at Pittcon 2018.*

Where possible, we like to use the resonance enhancement as well. If we tune our excitation wavelength to an analyte, we get greater enhancements in the Raman scattering back.

We've carried out a lot of work using commercially available fluorophores because there's a lot of choice available, we can tune our fluorophore absorbances to our excitation wavelengths. It means we can also tune to existing labels in chemistry and this has led us to completing a lot of work in direct detection of DNA sequence with commercially available fluorophores. As well as getting the fluorescence spectrum, if you absorb it onto the surface you also get the SERS spectrum and our nanoparticles can absorb some of the fluorescence.

Multiplexing

Multiplexing (figure 2) can be either: having multiple individual analytes that we can absorb onto the surface of one nanoparticle, or using different coatings of different labels on the surface of different nanoparticles that are then coated with a biorecognition molecule. An example of a biorecognition molecule is an Optima, which is an antibody DNA sequence that are used to target something within the system.

An individual analyte spectrum from within the mixture is obtained if we have these all in one system, when we get a multiplex spectrum, we're interested in how to work out what individual analytes were present and how do we use that data in the system.

Multiplexing (detecting multiple analytes in the same sample)

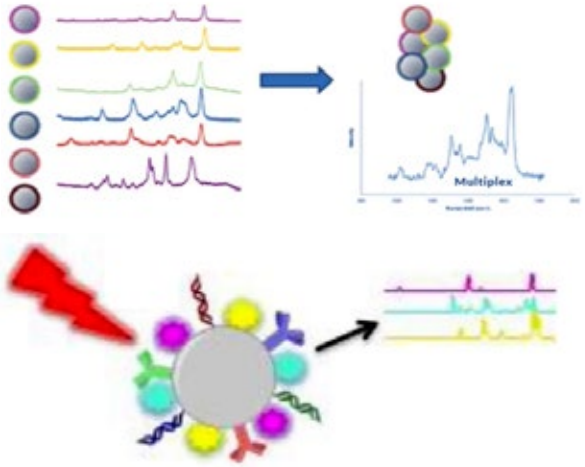


Figure 2: *Slide from Karen Faulds presentation on 'Multiplexed and Sensitive Bioanalysis Using SERS' at Pittcon 2018.*

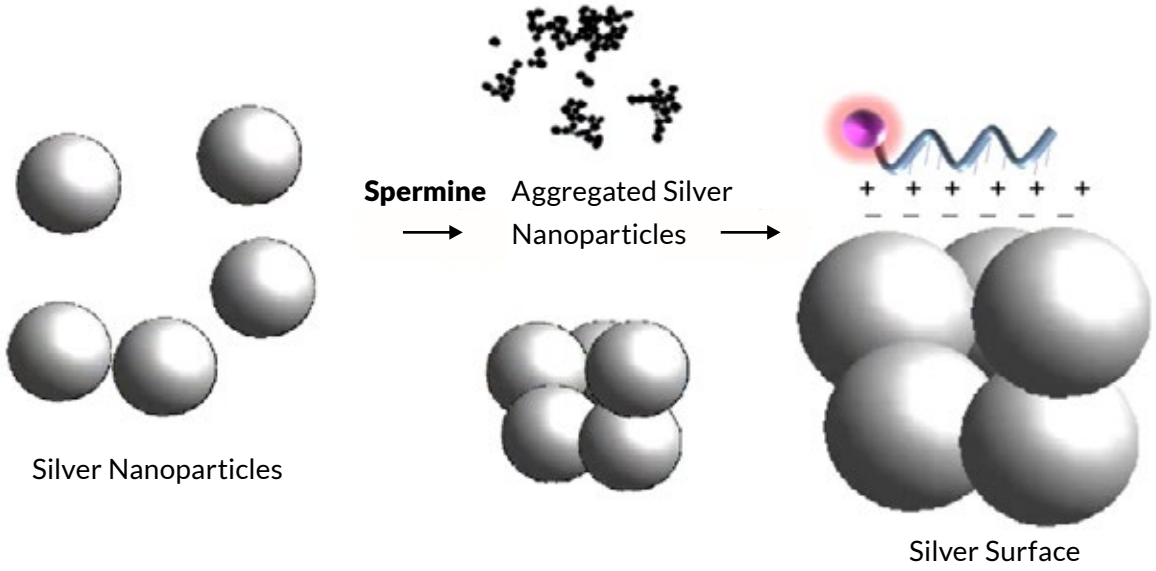
There are two main approaches to doing SERS using nanoparticles, there's direct adsorption which involves taking your nanoparticles and putting your analyte onto the surface and detecting it directly. A second approach is the use of a nanoparticle as a label, where you functionalize the surface and use the entire nanoparticle as a label.

Fundamental to either of these approaches is that you need to control and understand your nanoparticle surface in order to get what you want onto that surface. We've done a lot of work on the detection of DNA sequences (figure 3). When you make nanoparticles, they are typically highly negatively charged on their surface. If you have a positively charged analyte, you can electrostatically attach them to the surface, but if they're negatively charged you need to think more carefully about your surface chemistry and how you're going to get them onto the surface.

Therefore, for DNA we used spermidine, which is a polyamine that coats the nanoparticles with a positive charge, as well as aggregating the nanoparticles. This aggregation is important because when you go from isolated nanoparticles to aggregated nanoparticles you also get an increase in the SERS response. This allows us to use DNA which is negative to be put onto the surface.

There is not much SERS from the DNA sequence itself, because we've got a big resonant label attached, therefore we're looking for the response from the label rather than the DNA itself.

Detection of Modified Oligonucleotides



*K. Faulds, W. E. Smith, D. Graham, Analytical Chemistry, **2004**, 7, 412*
*K. Gracie, W. E. Smith, P. Yip, J.U. Sutter, D.J.S. Birch, D. Graham, K. Faulds, Analyst, **2014**, 139 (15), 3735-3743*
*K. Gracie, M. Moores, W. E. Smith, K. Harding, M. Girolami, D. Graham, K. Faulds, Analytical Chemistry, **2016**, 88 (2), 1147*

***Figure 3:** Slide from Karen Faulds presentation on 'Multiplexed and Sensitive Bioanalysis Using SERS' at Pittcon 2018.*

Different DNA sequences have different labels attached with different absorbances of these labels. We use the resonance effect where we tune our excitation wavelength to the absorbance of the molecule and when we do this, we can get extremely sensitive detection.

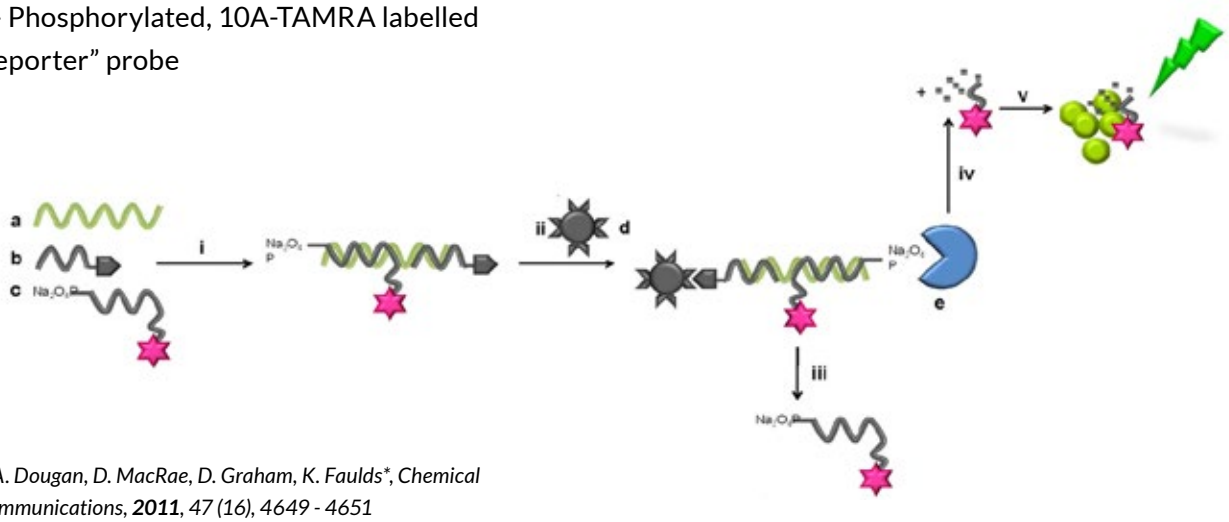
DNA assay - λ-exonuclease

DNA is not naturally labelled, therefore we must create acids to detect it. One acid that we developed is using λ-exonuclease, which is a toroidal structure where end with the hole of the enzyme is bigger than the other end, this allows double-stranded DNA to enter which is then digested and single strand DNA out. It also requires a highly phosphorylated group for recognition. A schematic diagram of the assay we developed can be seen in figure 4, we design one probe (b-) that is complementary to half of the target DNA (a-) and which has a biotin label attached, and this is our SERS probe. So, it is complementary to the other half of the DNA sequence, so it's a sandwich probe, it has a SERS label on it and it has a five prime phosphate group. The five prime phosphate group is a recognition site for the enzyme so that has to be pleasant for the enzyme to digest.

When a target is present this will all hybridize, and the reason we attach a biotin group is so that we can add magnetically coated streptavidin beads that allow us to wash away any excess probe. This is important because if we have any excess signal, this will create a background response. Once all this occurs we add our enzyme, we then digest and then we get our SERS response (figure 4).

DNA assay - λ-exonuclease

a - Unlabelled target
b - Biotinylated "capture" probe
c - Phosphorylated, 10A-TAMRA labelled "reporter" probe
d - Streptavidin coated magnetic bead
e - λ-Exonuclease



J. A. Dougan, D. MacRae, D. Graham, K. Faulds, Chemical Communications,* ***2011****, 47 (16), 4649 - 4651*

Figure 4: *Slide from Karen Faulds presentation on 'Multiplexed and Sensitive Bioanalysis Using SERS' at Pittcon 2018.*

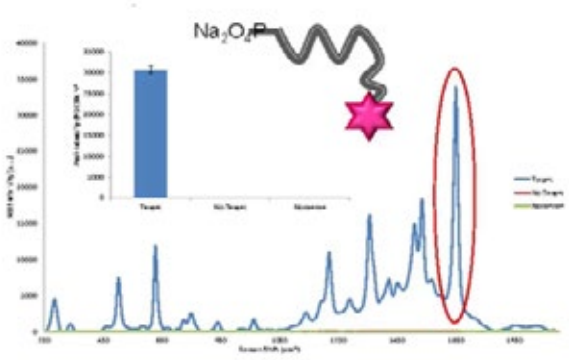
Cerebrospinal Fluid (CSF) multiplex bacterial meningitis pathogens

We're interested in using multiplex detection because we want to be able to take more things in one sample. An example that we're using for this is for bacterial meningitis pathogens, which are normally detected in CFS. Most people that have meningitis are newborn children who have not yet been vaccinated against the disease and so taking the CFS sample is highly traumatic, as well as the sample being very small. So, it's a good example of where we have a very small sample and want to get a lot of information back from it.

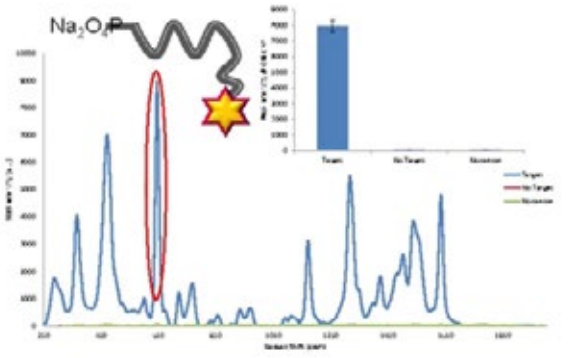
We began by looking at three different pathogens, clinically these three pathogens wouldn't be present at the same time. Using this assay, we used three probes, each of which were complementary to each of the different pathogens. Looking at figure 5, you can see the three different labels we used, TAMRA, FAM and Cy 3, and that they've all got unique SERES spectrums. And in our controls, where there's no target present, there's no complementary DNA to bind to and so we don't get a signal.

Single Pathogen Detection

S. pneumoniae - TAMRA



N. meningtidis - FAM



H. influenzae - Cy 3

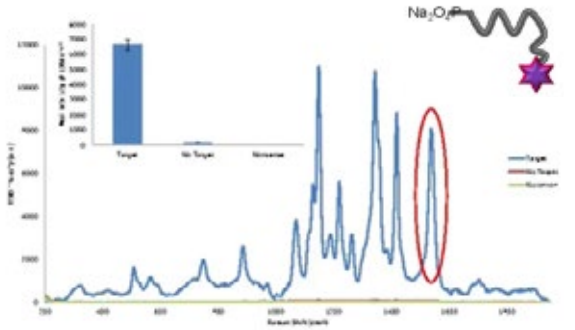


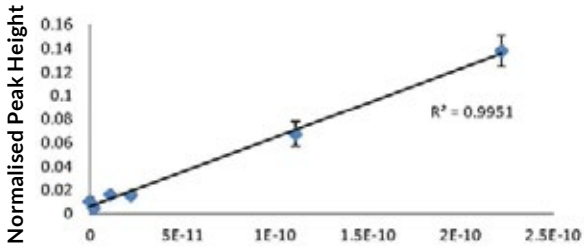
Figure 5: *Slide from Karen Faulds presentation on 'Multiplexed and Sensitive Bioanalysis Using SERS' at Pittcon 2018.*

In addition, we got good quantities of detection, and looking at figure 6, you can see that we got a linear quantitative response and very sensitive detection and so we can do this for each of the single pathogen species.
In terms of multiplex, in this case all three pathogens would not be present, but in other conditions you might have multiple bacteria present for example fungal infections or when multiple infections are present.

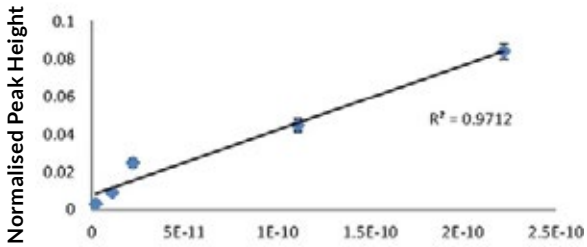
However, not only do we want to be able to detect that there's three to one of them present, but we're also interested in quantifying how much of each pathogen is present. Therefore, for example if you have multiple bacterial infection one might be present at a much higher level than the other one and be causing the issues in the individual.

Limits of Detection

S. pneumoniae



N. meningtidis



H. influenzae

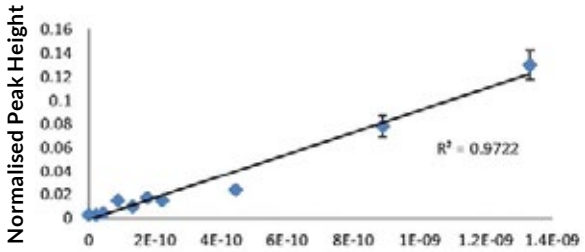
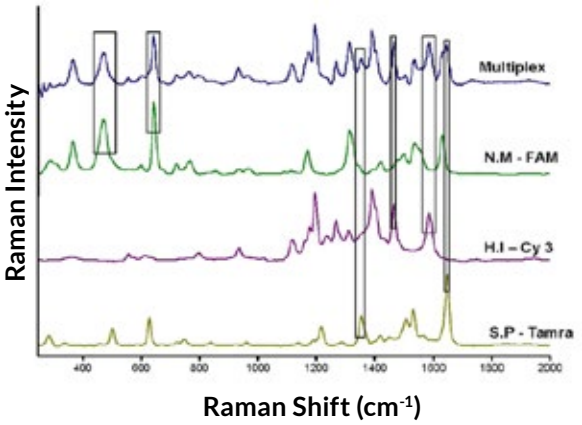


Figure 6: *Slide from Karen Faulds presentation on 'Multiplexed and Sensitive Bioanalysis Using SERS' at Pittcon 2018.*

Pathogen	Calculated Limit of Detection
S. pneumoniae	17.4 ρM
N. meningtidis	15.0 ρM
H. influenzae	21.7 ρM

To achieve this, we've designed other probes that could be easily seen within the multiplex. Looking at figure 7, you can see that there's a multiplex for all three are present and the three unique peaks for each of the species. However, that allows us to see it visually but as we move to lower levels and different concentrations of each, it becomes harder.

Cerebrospinal Fluid (CSF) Multiplex - PCR Product

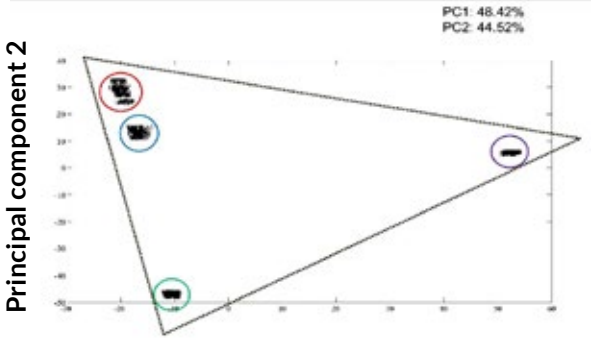


Streptococcus Pnuemoniae (S.P)
Neisseria Meningtidis (N.M)
Haemophilus Influenzae (H.I)

Figure 7: *Slide from Karen Faulds presentation on 'Multiplexed and Sensitive Bioanalysis Using SERS' at Pittcon 2018.*

We can see that using multivariate analysis we can separate using a multiplex spectra and figure 8 shows you the simplex. The multiplex is closer to the FAM spectrum and as this is a surface based technique, if one of the species has a stronger affinity to the surface then it's going to dominate that multiplex. As you can see (figure 8) this is occurring with FAM, but they can still be separated easily.

Multiplex - Multivariate Analysis



Red : N. meningtidis - FAM
Green : H. Influenzae - Cy 3
Purple : S. Pnuemoniae - TAMRA
Blue : Multiplex

- 5 replicates and 5 scans of each replicate
- Overlap of each scan shows assay is reproducible
- All three dyes are separated, multiplex is quite close to the centre (closer to FAM)

Figure 8: *Slide from Karen Faulds presentation on 'Multiplexed and Sensitive Bioanalysis Using SERS' at Pittcon 2018.*

In order to quantify this within the multiplex, in collaboration with Roy Goodacre from University of Manchester, we set up a system where we have all three of them present but they're all present at varying concentrations within that multiplex.

This creates a matrix of 66 samples with ten different concentrations of each of the labels within the multiplex all at varying amounts at different times. This meant that 66 samples emitted five replicates and we produced these PCA plots (figure 9). They're separated into each of the different components and the size of the spot is related to the concentration of the sample, therefore proving that we can quantify it.

Quantification
Multivariate Analysis - PCA score plots

- Replicates of 66 different labelled DNA combinations and concentrations were averaged
- Three plots are the same, data points are labelled according to each dye
- Size of the dot is proportional to the concentration
- Triangular shape suggests concentrations can be quantified

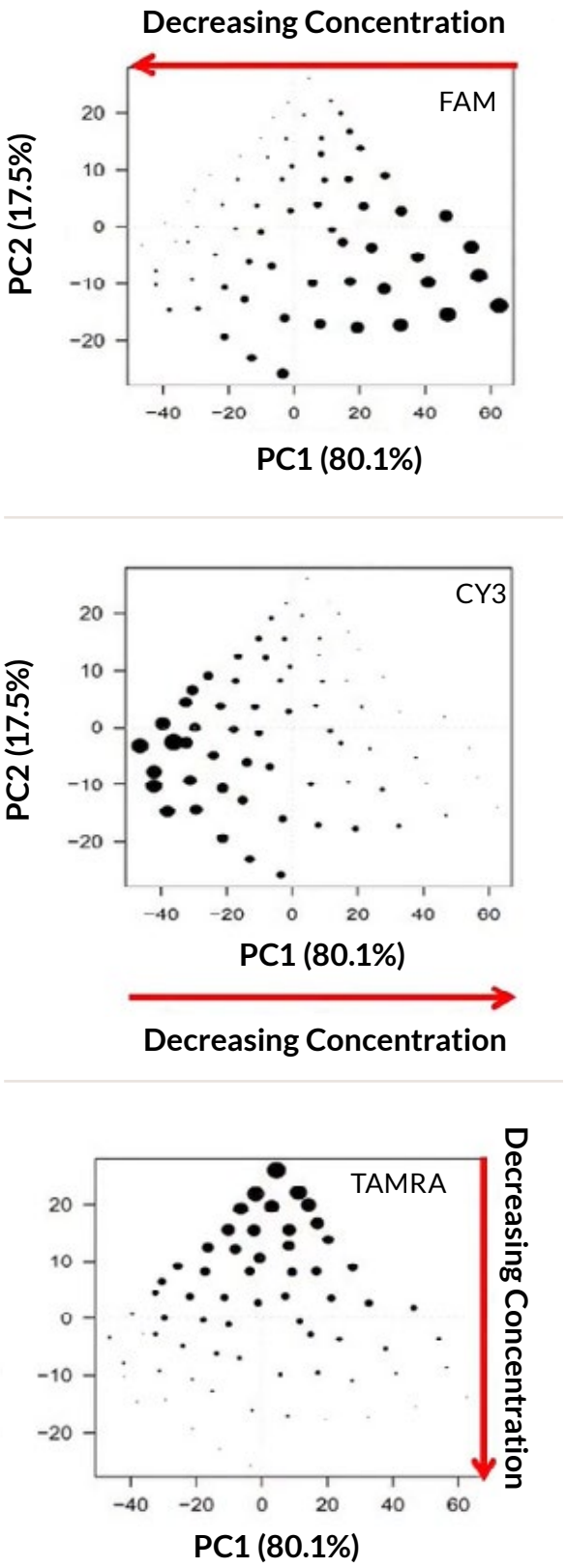
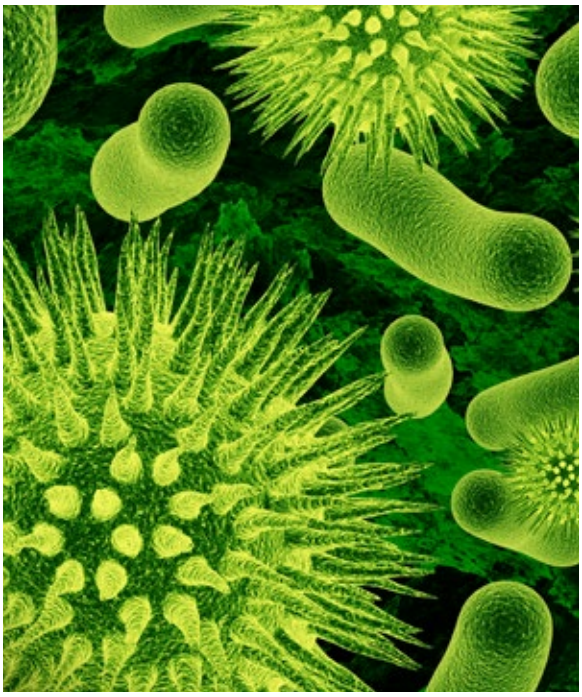


Figure 9: *Slide from Karen Faulds presentation on 'Multiplexed and Sensitive Bioanalysis Using SERS' at Pittcon 2018.*

Using half of the data for training and the other half for testing, we were able to produce this really nice linear concentration dependence, which can be seen in figure 10. This allows to be able to use the concentration curve for each of the analytes, but it is important to remember that these are all within the mixtures and so independently of other concentrations of the other analytes that are present, we can quantify individual analytes within that.

To test this, we then analysed some 'blind' samples and we sent the data then to Manchester and got them to report what they found the concentrations to be. We then compared their results to the actual values and found that the correlation was quite good. We did see however a bit of an issue with the FAM probe, which is being caused by the dominating nature it has on the spectrum.



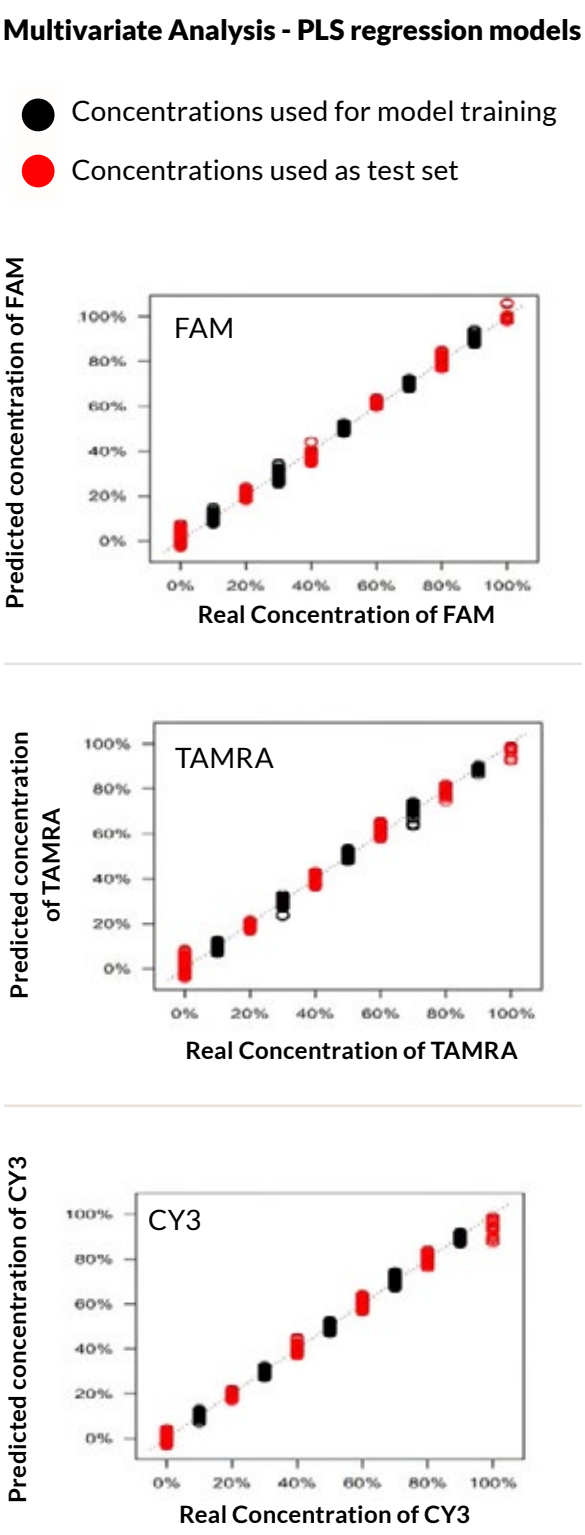


Figure 10: *Slide from Karen Faulds presentation on 'Multiplexed and Sensitive Bioanalysis Using SERS' at Pittcon 2018.*

HRP/TMB substrate system

TMB is a substrate that's commonly used for enzyme bases assays, such as ELISA (enzyme-linked immunosorbent assay). In the presence of HRP and peroxide digests the substrate to produce ablue colored one electron oxidation product that's a mixture of the radical cation and the charge transfer complex and it absorbs 650 nm.

Normally in ELISAs acid is added to push it to the yellow product which absorbs at 450 nm and it completes the reaction. We were attracted to this because of the yellow product has an absorbance of 650 nm (figure 11) and so it could be resonance for the 63 laser excitation wavelength but we also thought that the 450 peak would also get favourable resinous contributions. What we found was that we get good jamming signals, so the yellow product at 514, which is in resonance, and then the 633 where it's off resonance produces a good spectrum. Then we move to 514 which is off resonance for the charge transfer and then 633 which is on resonance for the transfer. What we liked seeing was that the resonance Raman peaks start to appear that are not present in the Raman spectra.

Looking at figure 11, you can see that these are much more intense, so we'll get good Raman spectra but then we also these peaks that start to dominate, and you don't see any of the Raman anymore at lower concentrations making a good Raman system.

HRP/TMB substrate system

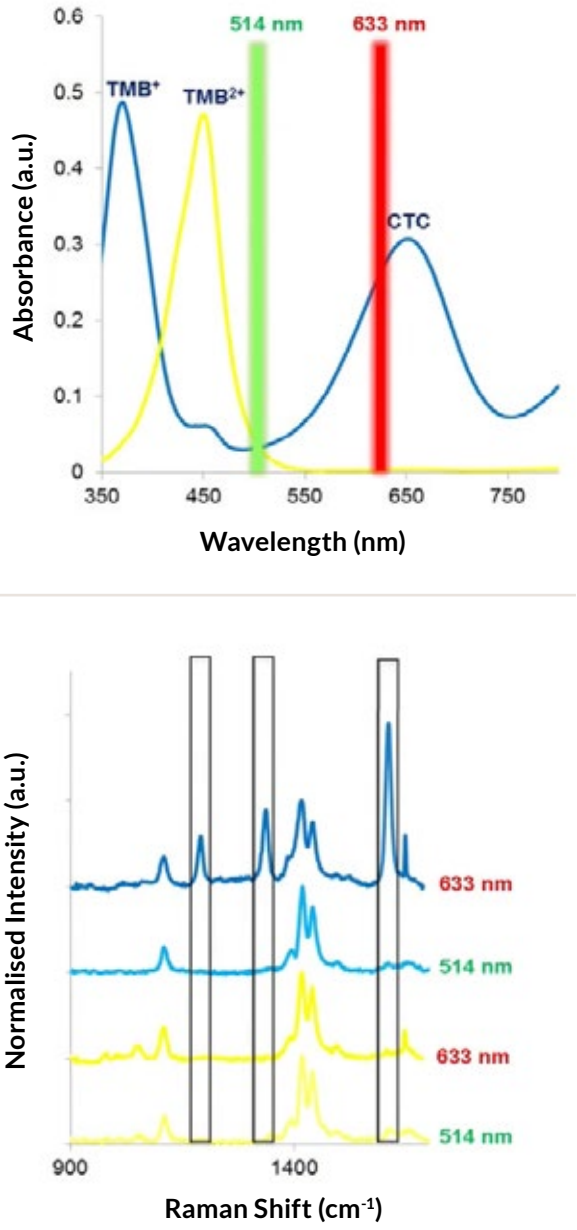
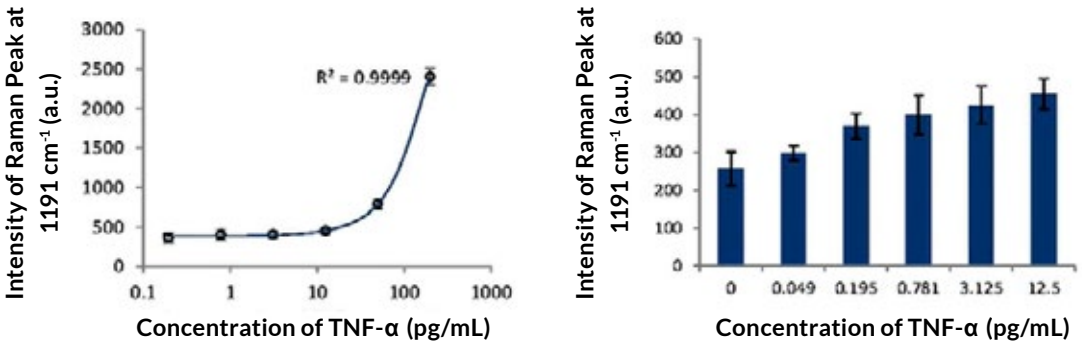


Figure 10: *Slide from Karen Faulds presentation on 'Multiplexed and Sensitive Bioanalysis Using SERS' at Pittcon 2018.*

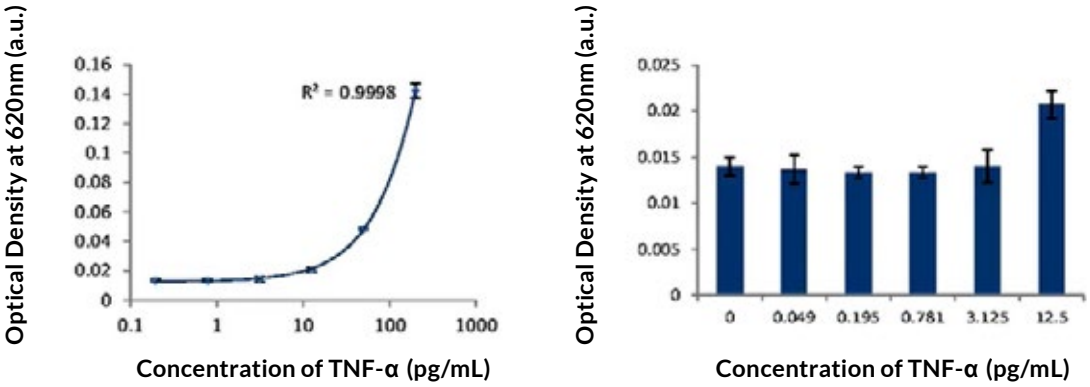
Figure 12 shows the colorimetric standard response and the Resonance Ramen, we found that we're getting good orders of magnitude and sensitivity. As illustrated in the graphs it starts to tail off quite quickly with colorimetric and the resonance.

ELISAs for Detection of TNF-α Standard Curves

Resonance Raman Based on Peak Intensity at 1191 cm^{-1} (Blue complex) 633nm **LOWER DETECTION LIMIT:** 90 fg/mL



Colorimetric Method Based on Absorbance at 620 nm (Blue complex) **LOWER DETECTION LIMIT:** 4.50 pg/mL



S. Laing, A. Hernandez-Santana, J. Saβmannshausen, D. L. Asquith, I. B. McInnes, K. Faulds, D. Graham* Anal. Chem.*, **2011**, *83(1), 297-302*
K. Gracie, S. Pang, G.M. Jones, K. Faulds, J. Braybrook, D. Graham, Anal. Method., **2017**, *9, 1589-1594*

Figure 12: *Slide from Karen Faulds presentation on 'Multiplexed and Sensitive Bioanalysis Using SERS' at Pittcon 2018.*

We got these sorts of results before evening moving on to SERS. The benefit of just using normal Raman like we did here is that we can use portable instruments. An example of its application is in cortisol detection and developing a test for athletes at track side.

We wanted to know whether it could be done more sensitively as well as easier and we so we tried swapping the TMB end for the assay in the portable detector. We found that we were still getting the same detection limit. We then wanted to try SERS, and thought we're going to have even better sensitivity.

Referring to figure 13, the Resonance Ramen data shows a linear response. This is the SERS data and when we add nanoparticles, which have been plotted on the same scale, the signal is enhanced and there is much higher intensity.

However our linearity response is no longer reproducible (bottom right graph), but what you notice here at this point is our blank is not blank, which is the issue, that is skewing the data.

ELISAs for Detection of TNF-α Standard Curves

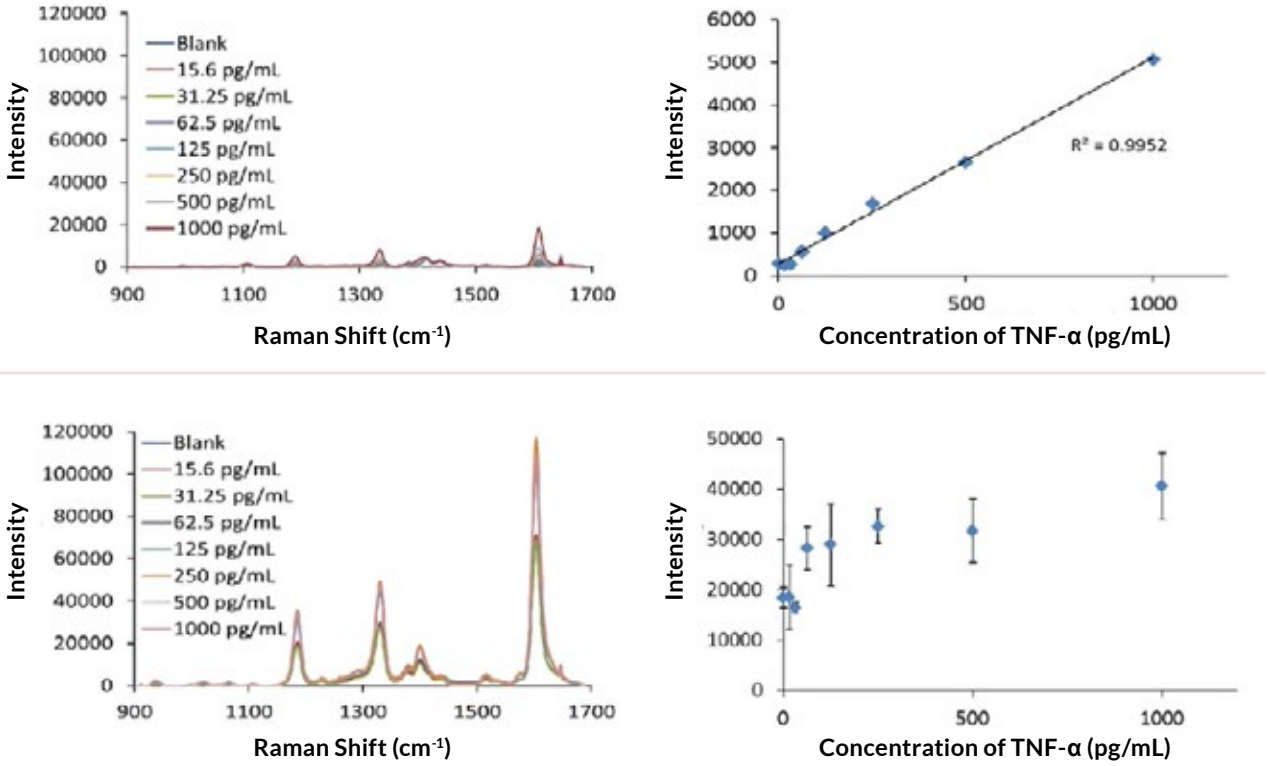


Figure 13: *Slide from Karen Faulds presentation on 'Multiplexed and Sensitive Bioanalysis Using SERS' at Pittcon 2018.*

A few years later, when working in a similar system, we found that the same thing happened again and that the nanoparticles themselves have a catalytic effect. The peroxide is broken down by the catalytic effect of the nanoparticle, which then turns over that substrate and so you get a color as well so none of our blanks were blank because the nanoparticles were turning over substrate.

If we remove anything from it we don't get a signal, but it's when the nanoparticles, TMB and peroxide are present, we get a strong surge response. If you vary the peroxide concentration you can get a linear response, and you can use it as a peroxide detector. But we wanted to use this in an assay format, and so wanted to use this to our advantage rather than it being very bad interference in a reaction. To develop a protein ISA assay based on this, and what we refer to as SLISA, instead of ELISA using an enzyme, it's a silver-linked immunoassay.

We start by selecting an antibody for capture,

for example CRP used for the detection for cardiovascular disease or sepsis where we're interested in inflammatory markers. You have a nanoparticle, we functionalize it with the antibodies so that the antibodies act as a recognition element, but we no longer need an HRP enzyme attached, because a nanoparticle is going to act as our insight. When we add our TMB substrate, if it's bound to the antibody then the nanoparticle turns over that substrate and we get color. The substrate then goes down onto the surface and gives us a SERS response.

As with the development of all surface-based assays, there was a lot of issues with blocking and nonspecific adsorption, but this method produces quite discriminative results making it easy to determine if the antibody is present or not. However, the Raman is more sensitive and by changing the blocking conditions we can eliminate any off-spot signal background, and we can get it so that we only see a signal when the target is present and this can be exploited for different biomarkers other than antibodies.

Bacterial Food Poisoning

Another approach we we're looking at is functionalized nanoparticles in bacteria detection. Bacteria detection is of interest both clinically, but also for food and processing applications as well.

The food industry is particularly interested in bacteria in the production lines and for their cleaning verification to ensure bacteria in food production areas is being eradicated.

In this approach, we take a magnetic nanoparticle that we functionalized with gold. So, we've got a magnetic core with a silver coating on the surface and in this case we functionalize the surface with a lectin. We use PEG surface layer and then EDC couple the lectin onto the surface. The reason we're interested in lectins is because they are carbohydrate binding proteins and there are carbohydrates present on bacterial cell walls and also in cells. This step is a nonspecific step, so the lectin will bind to multiple bacterial species, and so it is a nonspecific test.

We can use this to bind to bacteria, and then we can use a SERS nanoparticle that's specific to the bacteria, in this case it's got an antibody that is specific to the bacteria as well as a Raman label that will give us our SERS response. It's simpler to have that nonspecific capture step so that if we can put in multiple bacteria, for example food samples have lots of different types of bacteria in them, even if they're not at harmful levels.

Multiple bacteria can be magnetically pulled down, we then add a mixture of functionalized nanoparticles, so that we have a specific antibody to one bacteria and its specific SERS code and again these will bind specifically to the bacteria in this to produce a multiplex spectrum and we can to work out which ones were present.

CONCLUSION

We are trying to use multiplexing to get us more information back from tests when these are biologically significant samples and also may have a low volume by exploiting the benefits of SERS. We are also keen to make these assays, and a challenge here is the magnetic from an engineering perspective.

1.5 ULTRAFAST TWO-DIMENSIONAL INFRARED SPECTROSCOPY

The last presentation in this section will include Michael Fayer's talk on Fast spectroscopic measurements of the dynamics and interactions in complex chemical system: My group has done a tremendous amount of work on water and here I am going to introduce some of the methods and techniques that we use in 2D IR spectroscopy.

2D infrared (IR) spectroscopy is what you get when you have a vibrational mode of a molecule.

You take a fourier transform infrared (FTIR) spectrum, you're going to get some line shift, and that line shift is broad. What does that mean? You've found a molecule and it's got some vibrational mode and the frequency of that mode is core sensitive to the intermolecular interactions of the whole surrounding environment.

Since you have different structures in a medium, it doesn't matter if it's a liquid, glass or protein, it won't be uniform. So, you have a range of intermolecular interaction and that range of intermolecular interactions results in a range of vibrational transition frequencies. If you take a spectrum, you see some line width and that may tell you that you have a range of environments, but it doesn't tell you anything about the dynamics e.g. how those environments are changing from one another over time.

If you look under the line, what it's composed of is a lot of individual, homogeneously broaden lines, and if you looked at one of those, it shows a molecule which has a particular frequency at time, T = zero. The structure of the medium can be seen to evolve, it could be a liquid or a protein, but as the structure of the medium evolves, the inner molecular interactions change until the frequency of this molecule evolves in time, and this is called spectral diffusion.

Knowing how the frequencies of molecules are changing with time, as a result of changes in their structure, the time dependence of the frequency evolution is the time dependence of the structural evolution. In an example of two experiments, there's a pulse sequence that involves four pulses. These three pulses interact with the sample to produce a polarization, which gives rise to a fourth pulse.

This fourth pulse is known as a vibrational echo, in analogy you can add a large spin-echo. This pulse is combined with what's called a local oscillator, so that you can heterodyne detect it and you can get phase information. I've got all these frequencies, I've got this inhomogeneous line and I've got these molecules with different frequencies, and what happens is the first two pulses (frequencies at time T = 0). Then the different frequencies are recorded and you wait a length of time and label this TW.

During this waiting period, the structure of the system is evolving and as the structure evolves, the frequencies evolve. Therefore, when you carry out a third pulse, they no longer have the same frequencies that were recorded at time zero and you have a vibrational echo, which comes out of the sample and that tells you what the final frequencies are.

So, to summarise, you label the frequencies, you wait, then you read the frequencies out. Then you do 248 transforms and you get a spectrum. Then you increase the waiting time, you label the frequencies, you wait longer, you read the frequencies out and the frequencies, that will have evolved even more as you wait for a longer time.

The frequencies will change more and more, and so by looking at a series of 2D spectra and analyzing them correctly, you pull out the time evolution of the frequencies. Which means you pull out the time evolution of the structure.

When you do this, for example with a water molecule, when you scan the time between these two pulses, echo pulses go into a spectrograph with high rate of ray detectors like a IRCCD, and you measure a whole bunch of frequencies, which produces an interferogram in each frequency, and you can do 48 to 248 transforms and you end up with a spectrum.

Once you have interferogram, you label, wait, read, label, wait, read, and so on to create a series of spectrum and information on the changing shape of the spectra with time. You can use various methods to convert this change of shape known as the frequency correlation function. This is your mathematical connection between the spectra you're taking and underlying physical models, this is something that can be simulated and you can compare your data and use the data in the simulation to get an even more detailed molecular level understanding of what's going on.

Water

Water always has hydrogen bonding, liquid water which typically has four hydrogen bonds, principally in a tetrahedral arrangement, but those hydrogen bonds aren't static, they're constantly evolving in time.

Hydrogen bonds are being broken and formed, and the whole hydrogen bonding network is evolving in time. This is one of the characteristics that makes water so important in chemistry, biology and geology, is that this hydrogen bond rearrangement allows it to solvate ions and do many other things.

Methane has a similar molecular rate, is about the same size, but it's a gas. It's the hydrogen bonding's that makes water a liquid and gives it it's properties. When we do experiments, this is the OD hydroxyl stretch of HOD and H2O. We don't look at the OH stretch of water, because pure water has vibrational extirpation transfer. This is like electronic force or extirpation transfers, except it's vibrations. And that causes the extirpation to jump from one water molecule to another and messes everything up.

However, I to only watch extirpation transfers and so you add a small amount of HOD and H2O and you look at the OD stretch, that doesn't change the water dynamics, it doesn't change the hydrogen bond rearrangements that you're watching around it and so that's what we look at. There's a very broad line for water with some with weaker, longer, fewer hydrogen bonds and others that are the stronger, shorter hydrogen bonds, but these are constantly inter converting.

Advancement of technology

The very first significant 2DIR data on water was taken in 2004 by John Asbury and his friends. It took us literally several months, so you see the shape evolution here, see how the spectrum's changing, we didn't have great techniques for analyzing it but we did it. And we got six points, and that took several months to do. But that was the first detailed measurements of the dynamics of water, really at the molecular level. Now, we use water to just tune-up our instrument. Everything's gotten so much better and so much faster that we can do this and we do this in the morning to make sure that everything's working right and we can take many, many, many more points.

We also have new, much, much better ways of analyzing the data that we did initially. Something we developed called the center line slope method, which directly gives you the frequency, frequency corelation function. It doesn't involve, it eliminates a lot of experimental artifacts. Okay, so what do you get out of this thing for just bulk water?

You find two time scales, one is point four picoseconds, and the other is one point seven picoseconds. Simulations with Jim Skinners group, who was at the University of Wisconsin, now he's at the University of Chicago, and his people and my people, they've looked at this and with really good simulations, you can assign what these things are.

So you've got water hydrogen bonds, here's an oxygen, here's a water and here's a hydroxyl. The very short time scale, for very local hydrogen bond fluctuation's, mainly length scale fluctuations. Some angular, but the major part of what's causing frequency change is variations in the hydrogen bonds lengths.

But on a longer time scale, the whole hydrogen bond network [inaudible 00:10:30], that's what the one point seven picoseconds is, so one point seven picoseconds gives you the time scale for the complete hydrogen bond network rearrangement. That's a concerted process, it involves all these hydrogen bonds and many water molecules flipping around.

The first thing we looked at is what happens when you have ions, when you have concentrated salt solution, and our example was heavy towards hydronium. When adding 10 mol of lithium chloride to water, the spectrum of this methyl SCN has two peaks. A peak that is almost the same as when water is hydrogen bonds to the lone pair of the nitrogen and the other peak is lithium ion interacting strongly with the lone pair. That shifts the spectrum, because it's got different intermolecular interactions and you both these things are occurring in equilibrium. And we can measure this using 2D IR chemical exchange, which is like chemical exchange spectroscopy and NMR, except 10 orders of magnitude faster.

So, you get two peaks on the diagonal on the 2D spectrum, but since you have exchange, which means there are two species that are inter converting, you must label them and then switch to this frequency, which gives you off diagonal peaks. The slightly smaller diagonal peak is due to the negative going one to two feature each to out, but as the time goes on, the chemical exchange peaks go in. This happens in a 60 picosecond period. We're never going to see something like that by looking at water with a 1.8 picosecond lifetime.

From this, as well as using kinetic equations, the equilibrium constant, the ratio transition dipoles and other measurements we can get very detailed information. As it's in equilibrium there are reactions going in both directions and so the two off diagonal peaks grow together. You have one direction of the equilibrium where the methyl thiocyanate hydrogen is bonded to water and then the water moves away and the lithium comes in, that's 60 picoseconds. Then you also have the reverse reactions, water coming in and lithium moving out of the way, that's 92 picoseconds. They're different, because it's in equilibrium, the rate is concentration times the rate constant. And if we know the equilibrium constants, we can get the rate constants. Therefore, we can measure the solvation process where the exchange of species that are interacting with the solute is occurring.

The problem with trying to understand what a hydronium ion does in water using spectroscopic techniques, is that when you add a lot of acid to water, the water spectrum goes crazy. You get peaks in the water spectrum from the near IR to the far IR, that don't give us any information.

We did the same thing but looking at hydronium, and instead of lithium chloride in water, this is now HCl, a very high concentration acid solution.

Our idea was to put a probe molecule in with it so that we had something we knew what it was and that we could read. The splitting of hydronium ion creates a smaller peak because it has lower charge density and its interaction with the nitrogen is weaker, and you get these two overlapping bands, but can be taken apart. Then you can do the same chemical exchange experiment and again obtain two diagonal peaks, these are decaying because of the chemical exchange and over their lifetime you see the growth and then the decay of the off diagonal peaks. Then from this, we can work out the rates.

What you find it that the reaction where hydronium is being bonded to water, that's about 3.9 picoseconds, and for the reverse, water to go to hydronium, that is about 7.1 picoseconds. Notice that's 10 times or more, faster than what we saw with the lithium chloride, even though lithium is a much smaller ion.

Proton shuffling

So, there are two mechanisms that could make this happen. It could be the exact same mechanism as lithium chloride, with a water hydrogen bonded and a hydronium, coming in, moving the water away etc. But unlike lithium chloride, there's another possible mechanism. A hydronium ion is going to be hydrogen bounded to a water molecule. This can change by proton shuffling where the proton moves and effects the fundamental step in proton dynamics, or proton diffusion in water, and a hydronium is now a water. When measuring hydronium to water, water to hydronium that's what we see, we did the isotope effect.

So, instead of H20 and HCL, we did D20 and DCL. So again, we can measure this chemical exchange and we found was things slowed down, as a result of the reaction hydronium to water caused by the proton moving. That takes it from 3.9 to 5.7 picoseconds. The ratio is 1.46 and this helps us understand what happens to proton diffusion in water. The results suggest that when you go from H20 to D20, bulk proton diffusion slows down by route 2, as a result of a proton shuffling mechanism.

Monolayers

Another thing we've done recently is started looking at the air water interface and what does water do when it's interacting right at that interface with a monolayer sitting on the interface. Now, the thing about this is that there are very few molecules in a monolayer. So, we came up with a new method of being able to drastically increase the signal. Normally, pulses one and two come in, then pulse three comes in and then out of the sample comes a signal co-linear with pulse three that propagates in the forward direction and we detect this. Then you scan the time between pulse one and two and you get the interferogram, because the pulse three acts as the local oscillator, the heptagon detects a signal and gives you phase information.

However, there's a problem with this, the signal is actually the modulation of the local oscillator. So you've got this echo signal as you scan the time you get this interferogram. But what you've got this is local oscillator and what you're measuring is the modulation of the local oscillator. So, you've got this big flat constant signal and you're looking at this modulation. When the signal is fairly strong, this is not a problem, but when you've got a monolayer and there are very few molecules, the signal is very weak. The modulation becomes almost negligible. So here's the trick, when you have a monolayer or a very thin film, you get a polarization in the film. You not only get a signal emitted in one direction, you also get a signal emitted in the reflection direction.

Now, this signal is the same size going in these two directions, but the reflection of pulse three, which is the local oscillator is much weaker in reflection. In fact, if you do this with polarization, you get Brewster's angle, and you

can adjust the angle so that as you approach Brewster's angle, you can make the reflection of pulse three go towards zero. The signal doesn't change, but now you can bring the local oscillator down as small as you want and what that does is allows you to increase the percent modulation.

We did a time evolution off a monolayer, we dissolved it in water, and the water hydrogen bonded to the carbonyls. We measured 1.7 in just bulk water for hydrogen bonding arrangement, like my methyl thiocyanate, the CO's vibration acts as a probe of the water dynamics. And so, while this in bulk water, we also have the water at the interface, and you see it slows down. So, we think this is the hydrogen bond rearrangement of water at the interface hydrogen bonding to the COs.

When we're looking with HOD or we're looking at methyl thiocyanate, which only has one place to bond hydrogen, here we have three carbonyls and so it is quite different. The first thing is, like with this Methyl SCN, we think that this is giving us the water-hydrogen bind rearrangement, and this is the water-hydrogen bond rearrangement found at the interface.

The three carbonyls, and we did simulations and then grabbed little hunks in simulation via electronic structure calculations on it, and this allowed us to see that you could have three carbonyls, where each could have one water-hydrogen bonded to it. But, you could have two waters hydrogen-bonded to each other or anywhere from six hydrogen bonds to zero hydrogen bonds. The peak is around two, three and four.

So, this is another mechanism for getting spectral diffusion. The calculation showed that each of these configurations has a different frequency, for example, if you go from three hydrogen bonds to four hydrogen bonds, you're changing the frequency. So, that's also a source of spectral diffusion, and we believe that's the slow component in that data.

And, it's much slower for this reason: Water-hydrogen bond rearrangements, are normally concerted.

You preserve the number of hydrogen bonds. Everybody flips around, this one goes here, another one comes in, and the number of hydrogen bonds is maintained. If I'm moving from three hydrogen bonds to four hydrogen bonds to the carbonyls, I have to pull a hydrogen bond out of the water ensemble, leaving a hole there. Water doesn't like to give up a hydrogen bond and now there are only three. Now, it is moving from three hydrogen bonds to two hydrogen bonds, so I have to place a hydroxyl into this already well hydrogen-bonded network and find a place for it. As you can imagine this takes a lot longer and is much slower.

CONCLUSION

At the core of all scientific disciplines dedicated to improving human health and the world we live in, is the use of analytical instrumentation.

Over recent years, advances in the development of these crucial technologies has revolutionized the analysis of living systems, the diagnosis of disease and the monitoring of treatment. The scope of bioanalytical techniques has expanded into high impact areas such as gene expression, metabolomics and proteomics, with further applications ranging from environmental, pharmaceutical and forensic analyses through to the monitoring of food safety.

With the increasing demands placed on researchers in the analysis of biomolecules, single living cells, proteomics and genomics, new bioanalytical platforms are emerging as invaluable tools in the advancement of many fields including biology, biomedicine, biomaterials science and engineering.

Pittcon serves as a hub where experts in the field of analytical instrumentation can gather to hear all about the exciting new advances that have been made in this field, as well as those that are planned for the future.

The latest developments in analytical tools and techniques applying to a range of different technologies will be covered, including mass spectroscopy, mass spectrometry, chromatography, electroanalytical systems, micro- and nanofluidic devices and microscopy. The number of areas where these technologies can be applied is vast and include biomarker detection; point-of-care diagnostics; pesticide, toxin and contaminant screening in the food and agriculture industries; the evaluation and monitoring of therapeutics; drug quality control, and single nanoparticle studies.

Researchers have developed instrumentation that can perform at a higher-throughput and greater efficacy than ever before, but as realized by organizations such as The Japan Analytical Instruments Manufacturers Association (JAIMA) and The International Association of Environmental Analytical Chemistry (IAEAC), there is still a need to push for further development of analytical technologies and the analytical instruments industry.

1.7 RISE OF THE "OMICS"

'Omics' research is the non-targeted and non-biased analysis of a specific biological sample, the findings of which may give rise to hypotheses that can then be tested by further investigations.

It incorporates a range of disciplines and sophisticated analytical technologies. Together, they provide a holistic view of the molecules that make up a cell, tissue or organism and how they respond to environmental stimuli.

Genomics is the systematic study of an organism's genome, which contains the DNA that dictates how a cell develops and regulates cell function. When a gene receives the excitatory signal, a template for a specific protein will be produced in the form of mRNA. The study of all mRNA in a cell is called transcriptomics and provides a picture of the genes that are actively being expressed at a given moment.

Proteomics is the study of the resultant proteins, and metabolomics is the study of global metabolite profiles, which can detail how protein expression and cellular pathways are affected by different stressors, such as toxins and disease states.

Although all areas of 'omics' research are closely related, each provides different information, which can be pieced together to give a more complete picture of cell function. For example, although an organism has one genome and the proteome is the direct product of a genome, the proteome is ever-changing in response to current environmental demands. Furthermore, the number of proteins can exceed the number of genes. This is possible through alternative gene splicing and post-translational modifications. Without the study of both gene sequences and the complement and structure of protein, the full story could not be unraveled.

'Omics' research is continually evolving as enhancement of analytical technologies and the development of novel methodologies make more and more in-depth investigations possible. Mass spectrometry and nuclear magnetic resonance have revolutionized the detection and quantification of analytes in proteomic and metabolomic research.

Similarly, advances in microarray technology have allowed DNA analyses to become widespread and enabled large-scale sequencing to be conducted more rapidly and with greater accuracy. However, requirements are continually changing, and collaboration between researchers and the manufacturers of analytical equipment allow analytical methodologies and instrumentation capabilities to be refined to meet specific needs, be they in the laboratory of a research team, hospital, or forensic department.

Sensitivity and resolution are being steadily enhanced to reduce the impact of limits of detection. However, it remains important that the increasing sophistication of analytical technologies does not breed complacency. Due attention must still be given to proper sample collection and preparation and the interpretation of data obtained.

The increasing capabilities of analytical technologies mean that a huge amount of data is now obtained in 'omics' research, necessitating powerful hardware and software to undertake the complex data analysis. In addition, extremely rare events are now being captured for which numerous measurements may not be possible, reducing the robustness of any conclusions drawn.

This chapter will provide an overview of recent research in genomics, proteomics, metabolomics, and metallomics, which studies the role of metals within a cell. It will also highlight some of the latest technologies that have made the research possible.

1.7a THE GROWTH OF PROTEOMICS

The study of protein structure, its relationship to function and the effect of external stimuli on protein structure has massively furthered the understanding of multiple systems in the body and the etiology of many diseases.

With advances in biotechnology and the compilation of databases defining DNA and protein sequences, proteins can now be investigated in greater detail than ever before.

Consequently, there is an entire branch of science dedicated to the large-scale analysis of proteins—proteomics.

The vast array of molecular biology and imaging techniques now available are applied to analyzing and defining the structure, function, and interactions of proteins expressed by a particular cell or tissue.

It is possible to evaluate changes in protein content and protein structure in response to external cues, giving an insight into how cells modify the way they function to meet current requirements dictated by their immediate environment.

The findings are correlated with existing databases and potential applications of the new-found knowledge are explored. Proteomics has proved particularly valuable in elucidating the mechanisms of disease and identifying potential targets for novel therapies.

Sessions at Pittcon 2018 provided details of the latest in proteomics research, the development of novel methodologies and new uses for existing technologies in the analysis of proteins, and how proteomics research can be applied to address ongoing biological conundrums. Proteomics has shown significant growth over the last decade and it is predicted that this will continue for several years to come; the global proteomics market is projected to be worth USD 21.87 billion by 2021.

Numerous innovative technologies have been used to provide a wide range of sophisticated techniques for use in the study of proteins. The analysis of highly complex mixtures is now commonplace, but researchers continue to adapt, combine and enhance the available technologies in their quest to answer more and more complex biological questions.

The speed with which these new technologies have been developed and utilized to address crucial questions and support hypotheses has led to guidelines being developed to ensure a high level of stringency and accuracy in all proteomic research methodologies.

The proteomics rush, however, has recently been quelled in several countries as a result of the economic crises, which has meant that there are fewer funds available for the purchase of high cost equipment and research grants. Such economic restraints, however, have not hit Brazil.

As Daniel Martins-de-Souza explained in his presentation at Pittcon 2018 entitled "Proteomics in Brazil: Current Status and Perspectives", proteomics in Brazil continues to grow both quantitatively and qualitatively. Brazil is home to over one hundred mass spectrometers dedicated to proteomics and several of the leading names in the field, making it a powerful center for proteomics investigation.

The role of mass spectrometry

Mass spectrometry is a sophisticated tool used across a broad range of disciplines and applications. Its combination with stable isotopic labeling has been particularly valuable in proteomics. Mass spectrometry studies have provided the basis for numerous hypotheses relating to biochemical mechanisms and their involvement in disease states.

Indeed, mass spectrometry is one of the most important developments in protein identification and quantification.

In the last decade, the sensitivity of analyses and accuracy of results for protein analyses by mass spectrometry have increased by several orders of magnitude. Mass spectrometry can simultaneously analyze hundreds of peptides, allowing investigation of changes in expression and modification of proteins involved in several pathways and networks which can then be related to function.

At Pittcon 2018, in a presentation entitled "Structural Proteomics: Mass Spectrometry as a Tool for Structural Biology", Daniel Martins-de-Souza explained how mass spectrometry has been pivotal in gaining knowledge of protein structure, function and modification. Various modifications and adaptations have been applied to the basic concept of the mass spectrometry technology tailoring it to specific proteomic studies.

Emerging mass spectrometry methodologies

In his seminar at Pittcon 2018 "Structural Mass Spectrometry and Top Down Proteomics of Proteoforms and Their Complexes: Mass Spectrometry", Professor Neil Kelleher of Northwestern University will be exploring the latest cutting-edge methodologies using mass spectrometry to study whole proteins.

Broadly speaking, mass spectrometry studies of proteins follow either a bottom up or top-down strategy. Typically, bottom-up proteomics have been used in which proteases are used to break the protein and the resultant mixture of short peptides is analyzed by mass spectrometry. However, it became apparent that a single gene can produce a range of different proteins due to polymorphisms. Furthermore, expressed proteins can be modified by a range of intracellular processes that change the molecular weight of the protein. Consequently, analysis and interpretations of such a complex heterogeneous mixture of peptides is not straightforward.

Mass spectrometry of intact proteins, without previous proteolytic digestion—top-down proteomics—thus became increasingly common in the characterization of protein primary structures. The top-down approach shows which protein forms are present and their relative quantities before the protein is broken down for complete characterization. However, it is not suitable for the study of entire proteomes.

A top-down/bottom-up hybrid methodology has been developed to take advantage of the best features from the two strategies. This hybrid strategy is known as middle-down proteomics and analyses larger peptide fragments (>3 kDa) that contain multiple post-translational modifications. It is used to facilitate the identification of biomarkers and to characterize recombinant proteins.

New technologies are being developed to facilitate the successful implementation of a top-down proteomics.

Native electrospray mass spectrometry is a particularly promising technology for the identification and characterization of whole protein complexes. Although it can currently assess proteins of 100 kDa to 1 MDa, it is likely to soon be capable of complete proteome compositional analysis.

Mass spectrometry hardware

A range of different, high specification mass spectrometers, each designed to optimize the results of particular types of analyses, are needed to undertake quality proteomics studies. Representatives from world-leading producers of mass spectrometry equipment will be at Pittcon 2018, showcasing the latest technologies that are advancing the capabilities of proteomics research.

Thermo Fisher Scientific, the worldwide leader in liquid chromatography mass spectrometer solutions for protein research, demonstrated their high-resolution accurate mass spectrometers, such as Orbitrap Fusion™ and Q Exactive™, which are designed for the identification of potential biomarkers.

Bruker, another provider of high-performance systems for proteomics research, continues to build on its innovative and extensive range of state-of-the-art technologies and solutions. Their latest addition, Impact II™, an ultra-high-resolution benchtop Q-TOF platform will be on display at Pittcon 2018.

This mass spectrometer provides market-leading full-sensitivity resolution for analysis of multiple molecules in a single process.

The highest proteome coverage reported to date was achieved with Impact II™ (11,257 proteins were identified in single measurements of cerebellum).

Bruker have also been working to facilitate the advance of top-down proteomics and have developed a solution to help address some of the challenges of achieving a stable and robust nanoflow during electrospray mass spectrometry.

Glass Expansion manufactures high-precision components for mass spectrometers capable of achieving high accuracy even within the narrowest of analytical specifications. At Pittcon 2018, they presented the most recent addition to their achievements, the glass expansion helix spray chamber™ that improves transport efficiency, precision, and washout to optimize sample introduction.

References

- *Graves PR, Haystead TAJ. Molecular Biologist's Guide to Proteomics. Microbiol Mol Biol Rev. 2002; 66(1): 39–63*
- *Liebler DC, Zimmermann LJ. Targeted Quantitation of Proteins by Mass Spectrometry. Biochemistry 2013;52:3797–3806*
- *Wilkins MR, et al. Guidelines for the Next 10 Years of Proteomics. Proteomics 2006;6 Issue 1:4–8*
- *Kelleher NL. Top-down Proteomics. Analytical Chemistry 2004; June1:199 203*
- *Siuti N, Kelleher NL. Decoding protein modifications using top-down mass spectrometry. Nat Methods 2007;4(10):817–821*
- *Catherman AD, et al. Top Down proteomics: Facts and perspectives. Biochemical and Biophysical Research Communications 2104;445(4):683 693*
- *Beck S, et al. The impact II, a very high resolution quadrupole time-of-flight instrument for deep shotgun proteomics. Mol Cell Proteomics. 2015;14(7):2014 2029.Chapter 2 – Mass Spectrometry Dominates the "Omics"*

1.7b WHY SHOULD WE WEIGH EVERY PROTEIN IN THE HUMAN BODY?

In this interview conducted at Pittcon 2018, Prof Neil Kelleher describers the work he has done as part of The Human Proteome Project:

Our mission is to advance the measurement of human proteins with greater precision, to bring to the world the benefits of absolute molecular specificity when it comes to interrogating proteins at the molecular level. Hence, the full expression of this vision is to sequence the human proteome, and that's what the Cell-Based Human Proteome Project is about. It brings us into a really interesting, open, and provocative conversation about science and technology.

The Cell-Based Human Proteome Project is something I proposed in 2012 and have been advancing since then with support from the Consortium for Top Down Proteomics and the Paul G. Allen Frontiers Program. The proposal was to map 250,000 proteoforms in 4,000 different cell types.

We have already determined the human genome. What is the importance in understanding proteins at the same level?

The genome is the blueprint. Now 20 years later, we know there's about 20,000 human genes. They create millions of different molecules in all the different cell types.

When you start talking about disease mechanisms, the precision with which we understand the biology driving disease is related to the precision with which we analysed the proteins involved. So to is our ability to detect and treat diverse disease types and sub-types.

Biologists would agree that proteins are the mediators of much of what we call a disease phenotype. For example, looking at the outward expression of cancer cells growing in someone's organs - that phenotype is a combination of the genes and the oncogenes driving the cancer, what type of cancer it is, how to defeat it and shrink the tumor – all of these things involve proteins which make up a specific disease phenotype for a particular individual. One must fully understand the proteins in order to understand and treat the disease in attempts save that person.

What is a proteoform and how are they used in the field of proteomics?

A proteoform is the exact molecular composition of a protein molecule, and it is the unit of currency that the proteomic community is beginning to measure and share. It can be composed of several sources of variation that make biology so enigmatic and difficult to pin down.

Proteins vary due to the number of processes that can occur to them, including polymorphisms, mutations, alternative splicing, isoforms and post-translational modifications. One simple way to describe all of this variation is the term "proteoform". Everybody in proteomics is picking up the word – it's very much becoming less of just a "word" and more of a "movement" in proteomics.

How can a protein be measured to determine its proteoforms?

The measurement strategy goes right to the heart of measurement science, and we are advocating for a new platform to improve proteomics: call it "Proteomics 2.0".

We embrace the idea that instead of inferring proteoforms from the bottom-up, we measure them directly, using top-down mass spectrometry. This is the idea of weighing the whole protein first and then degrading it once you have mapped what proteoforms exist directly at the proteoform level.

The measurement approach that you use really impacts how you view protein diversity. The field has largely been driven by bottom-up proteomics. Therefore it's been enlightening to use a new approach in order to investigate what's happening in the regulation of protein-based biology.

How is science being held back by not knowing all forms of a protein?

Basic biomedical research is all about increasing precision, about the molecules of life. As you have hypotheses about some mechanistic aspects of cell biology, by knowing the proteins precisely and having the reference catalogue of human proteoforms, this reference would be as enabling in the long run, as the human genome has been from genetics to clinical medicine.

The other thing that you would get is more measurements per dollar. If you have the reference list of known proteoforms and cell types, you can expanding the types of reagents that you could create and buy. Economies of scale, volume and cost drops, would definitely follow this kind of project.

> "Overall, it's about elevating the efficiency of biomedical research now and finding higher value protein-based markers of disease."
>
> *Prof. Neil Kelleher*

Current processes for biomedical research, such as drug development, are highly inefficient, and would become more efficient if we knew all of the possible proteoforms.

Please outline the information you will be presenting in your session titled "Mass Spectrometry" at Pittcon 2018.

It will be to review the Cell-Based Human Proteome Project, to frame it for those who don't know about it, highlighting the vision of "A billion proteoforms at $1 each". I will then go deeper into the nature of the project and the motivations for it.

"I will also discuss the kind of measurement science used in the project, top-down proteomics, with the art and science of measuring proteoforms becoming a major focal point."

Prof. Neil Kelleher

Something that has happened this year that has greatly de-risked the Cell-Based Human Proteome Project and made it much more feasible, is the Human Cell Atlas – a group funded by the Chan Zuckerberg Biohub, whose mission is to regularize and categorize all the types of human cells.

With this, I feel like the tide might be turning in favor of this idea that we should map and sequence all the different proteins in all the different cell types. The problem was we didn't have a map of what all the cell types were but that's now being addressed. This will be my story to tell at Pittcon 2018.

At Pittcon 2018, there will be all the best vendors producing equipment for proteomics research. That includes not only mass spectrometry and chromatography, but also antibodies and other complementary technology that would be stimulated by the project.

How is Mass Spectrometry involved with weighing proteins in the Cell-Based Proteome project?

It's hard to envision another technology besides mass spectrometry that can precisely measure the atom composition of protein molecules before they are known. To directly map and sequence proteoforms, you have to directly analyze the whole protein – which is only possible using whole protein, or top-down, mass spectrometry.

However, it is important to note that it is the style of performing mass spec and the way that samples are handled which makes it possible to sequence proteoforms. This method is called top-down mass spectrometry or top-down proteomics. That's the only way we currently have to discover proteoforms. Once they're known, you have all sorts of single-cell, single-molecule technology that could be used.

Please outline the top-down strategy for analyzing proteins.

It's in the name – first you weigh the whole protein at what we call the MS1 level, then from there you measure its components, at the MS2 level.

I sometimes think of the word protein as fiction - if you have 10 different proteoforms that make it up, then what do you mean by the protein? That's why we want to catalogue those proteoforms precisely, so we know exactly what that protein is.

For a given protein, you must imagine spreading the components out. Say there are 10 signals, which all weigh differently or have different atom compositions. Then you would isolate one of them in the gas state, in the mass spectrometer. In addition, you can separate them in the condensed phase using chromatography or electrophoresis before mass spectrometry. There's room for a lot of innovation there.

Once you have the proteoform in its pure form, even if only for a microsecond inside of a mass spectrometer, you then it fragment it into all of the pieces that serve as a fingerprint - the MS2 level. You can have hundreds of fragment ions that are produced from a proteoform, and that allows you to identify, which of the 20,300 human genes produced that proteoform and exactly what it is.

There are two modes of top-down, there's the denatured mode and the native mode. The native, newer mode, allows good coverage of very high mass proteins and even whole protein complexes. Most of proteomics right now is the denatured mode top-down, but I will argue at Pittcon that native mode has a lot of upsides, and that we should be developing more technologies for top-down proteomics discovery in native mode.

How many proteoforms have been discovered so far? Please provide an example of how these have advanced science.

Everyone thinks there are more proteoforms than there are. It is easy to think that, because of how many modifications can be possible on proteins, the variety in mass, how it's scaled (exponentially), and more. You create all these potential proteoforms in a computer but how many does biology actually make?

"What I'm trying to do is to catalogue them and show that they can be mapped, even high-mass, highly-complicated proteins, and that they exist in a limited number of proteoforms."

Prof. Neil Kelleher

There are a growing number of cases, but one particular example was found in heart disease and the protein ApoC-III. In this protein there were four proteoforms. One of which was glycosylated and correlated very closely to peoples HDL-C levels (the good cholesterol). It is from here that we are able to investigate further into whether specific proteoforms indicate risk – for example, someone's risk of heart attack? Although more research is needed here to take the next step, the basic assertion that proteoform mapping and sequencing will lead to deep functional insight, is proving to be true.

It is in microbiology, where bacteria is easy to grow and experiment with, where top-down proteoforms have excelled in creating clarity. There's a case of mapping of 25 proteoforms in a bacteria, and only a few of them had a certain post-translational modification, meaning that those proteoforms were in the membrane of the bacteria.

In another example, researchers investigated the bacteria that causes meningitis. The authors mapped 20-30 forms of proteins called pilin, and they had an unusual post-translational modification on them. The more of that post-translational modification they had, the more infectious, and the more pathogenic, the more virulent the organism was. This gives us a huge insight into better understanding disease and therefore developing treatments.

What does the future hold for protein-based drugs?

Protein-based drugs are one of the major driving markets that's creating more interest in top-down methods. For example, there's a drug that treats multiple sclerosis and it's a protein-based drug. They mapped 138 proteoforms of that drug as it aged on the shelf.

During drug development, it seems inefficient and even illogical to me to digest it into hundreds of pieces to then do your analysis, as in the bottom-up approach. I understand that was the only way we could do things previously, but there are many uncertainties which arise from this method, due to the effects of oxidation or deamidation. So then it makes you question, "Was it my method or was it the drug?"

For these reasons, I think that top-down has a huge potential for protein-based drugs. If you want to know the precise molecular composition of a protein, the way to do it is top-down.

What is making researchers reluctant to adopting this method?

In the past five years, the industry and the state of technology has really changed. Previously, you had to have a custom solution for each individual research goal; however now there are suitably available commercial solutions.

Currently, the main reluctance towards top-down is due to the ease of bottom-up, as this is the method that people are used to performing. However, if only a minority of people are doing top-down, others don't pay attention to it, and therefore it becomes difficult to reduce it to practice and make it easier, so that other people adopt it.

Sometimes, I find advances in technology are like a see-saw. All the weight is on one side but as soon as you can see it lifting, at some point, there'll be a change that's more swift. At some point, there'll be a critical mass on the other side of the see-saw and things will change more rapidly in favour of top-down methods.

It is part of my goal to boost the awareness of top-down proteomics and the benefits it brings. I want to tell people that if you have the individual drugs that you want to characterize, individual proteins, it's achievable through top-down proteomics.

How do you see the top-down approach developing the world of proteomics?

You have two sides to this. One extreme says that all proteomics by 2030, will be top-down. On the other side, you have people that don't think top-down will ever be capable of performing full, deep proteomics discovery and so bottom-up will just be the dominant approach.

"I fall in the middle. The see-saw is currently at an equilibrium for me. Bottom-up is useful if you just want to profile the proteome. But if you want to get into regulatory switches, and you really want to be precise about which proteoform you're dealing with, top-down has to be involved."

Prof. Neil Kelleher

If you want to do bottom-up proteomics but you already know what proteoforms are there, and because you have a reference list, you can make much better use of bottom-up data. That's why, with the Cell-Based Human Proteome Project, I see a much more complementary role than some. However, the top-down still has to yet get that moment where it's value is recognized widely and therefore naturally elevated in the proteomics community. That will take a bit more time, no doubt.

Living cells comprise a complex, ever-changing solution of small-molecule endogenous metabolites, intermediates and metabolism products. These are the substrates and products of numerous inter-related pathways that are activated or inactivated according to requirements dictated by their immediate environment.

The unique chemical fingerprint of a cell consequently changes as the result of specific cellular processes caused by external stressors. The study of the relative quantities of endogenous metabolites is called metabolomics. It provides a comprehensive snapshot of the biochemistry of a biological system and gives an insight into time-dependent metabolic responses to changes in the environment.

These changes in metabolism can lead to changes in gene transcription and protein expression or changes in protein structure and function. Metabolomics is therefore commonly combined with genomic, transcriptomic and proteomic studies to fully elucidate the mechanisms of action through which drugs or other contaminants cause toxicity.

The latest metabolomics research, including novel analytical techniques to improve selectivity and sensitivity whilst minimising bias, will be presented at Pittcon 2018.

1.7c RESOLVING INTERFACIAL PROTEIN DYNAMICS BY STREM

In this interview, Christy F. Landes, Professor in the Departments of Chemistry and Electrical and Computer Engineering at Rice University in Houston, describes her work in single-molecule spectroscopy:

I've been working in single-molecule spectroscopy since my postdoc at UT Austin in 2004. My postdoc training threw me straight into the deep end. I showed up at UT and my boss said, "You're going to work on this protein that is important for HIV's virulence", and I had to learn and see what a virus was, and then I had to learn what single-molecule spectroscopy was.

The moment that I really got into it, I understood, first of all, how different biomolecules and biopolymers are from normal molecules.

For any material that exhibits intrinsic structural or dynamic heterogeneity, only single molecule methods make it possible to directly correlate conformational changes with dynamic information like reaction rate constants or product specificity. Ensemble methods would average out all of those properties.

The clearest example is with an enzyme that switches between bound (active) and unbound (inactive) conformations in the presence of its target. If we measured using standard methods, we could extract, at best, the average conformation (which would be something in between the bound/unbound forms and neither useful nor accurate) and the equilibrium binding coefficient. With single molecule methods, in the best case we could extract each conformation and the equilibrium rate constants for the forward and reverse process.

As a spectroscopist, if I'm studying say acetone, every molecule of acetone is exactly the same. If you're studying vibrations, there's obviously a thermal distribution of vibrational energies in all the acetone molecules in a bottle or a vial. If you're studying biopolymers or biomolecules like proteins or DNAs, their structure is constantly changing depending on their targets. You can't capture the intricacies of the structural changes as a function of the reaction happening with standard spectroscopy. All you ever get is an average.

It's very similar to if you want to understand the structure of a protein. Protein crystallography is incredibly powerful, but it involves freezing the protein structure into what is usually considered the most probably confirmation, which is one of the low energy structures that protein can form. If you want to actually understand how the protein is performing its function, its function is exactly related to its structure and how the structure changes. To be able to see that dynamically, the only way to do it really is with single-molecule spectroscopy.

What first interested you about single-molecule spectroscopy, and how did you come to work in the field?

I became fascinated with the challenges associated with the method, but also its strengths and how it fundamentally links statistical mechanics to experimental observables. With ensemble methods, you measure average values and extract statistical relevance, but with single molecule methods, it is possible to design an experiment in which the probability distribution is your direct observable.

What are the main challenges in measuring the fluorescence signals from a single molecule, or a single chemical reaction event?

I'm a spectroscopist at heart and so of course learning something new and unprecedented about a biomolecule is important and interesting.

"But the thing that really gets me excited is tackling the challenges that are associated with doing these types of measurements and figuring out new and interesting ways to solve those problems."

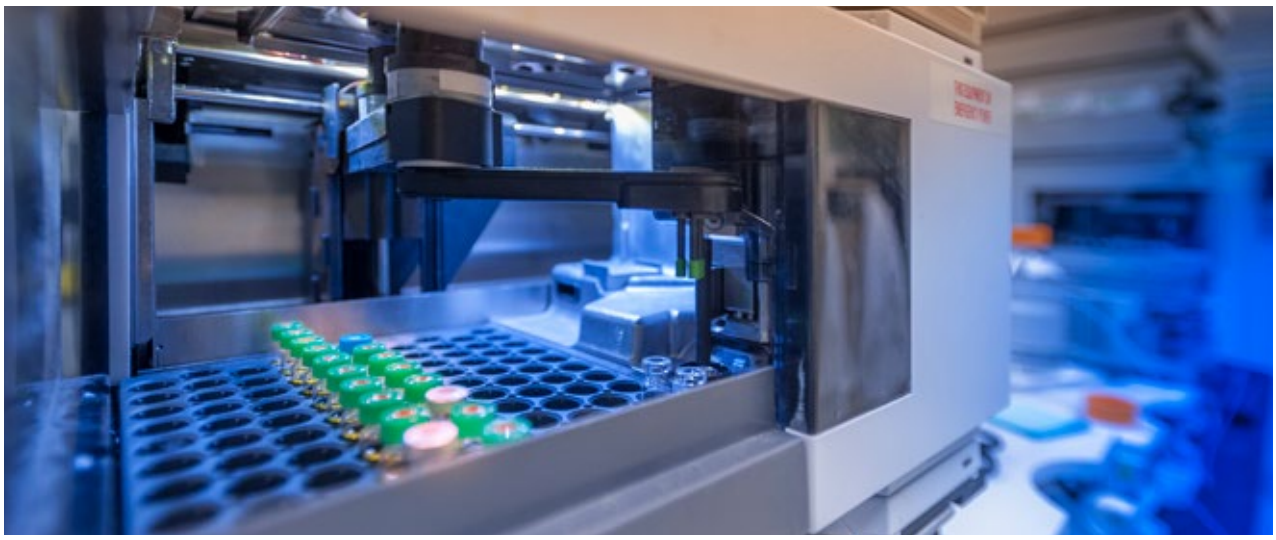
Prof. Christy F. Landes

There are three main challenges. In single-molecule fluorescence spectroscopy or microscopy, the quantum yield of a fluorescent molecule can approach 100%, but still that means if you're only measuring the fluorescence from a single molecule, that's very few photon counts per unit of time and space. Collecting those photons accurately and precisely and being able to distinguish emitted photons from the noise that's associated with every type of measurement and background that's associated with every type of measurement is a very interesting challenging problem that single-molecule spectroscopists are working on.

The second is data processing: most measurements involve high volumes of data that is almost empty of information. Twenty, even ten years ago, if you're a spectroscopist you would work all day and all night to align the laser so that you could collect a few data points at 3:00 in the morning and be thankful for your 10 data points that you would then try to then fit to an exponential function or a Gaussian function. Single-molecule spectroscopy has advanced so quickly that we're able collect terabytes of data in just a couple of hours, which weren't even possible to store just a few years ago.

Almost by definition, most of your sample has to be nothing but background in order to get single molecule resolution. Processing a terabyte of data in which most of the bits of data are background, but hidden within are small amounts of incredibly rich details a very interesting information theory problem. We're active in developing new data analysis methods based on information theoretic principles.

The third is that labelling and handling single biomolecules introduces the possibility for the biomolecule to alter the label's quantum yield and for the tagging molecule to alter the biofunctionality. You can't totally solve this problem, but we must minimize it and quantify it, which involves a lot of tedious but important control measurements.



How has the hardware and/or software influenced the ability to process these large data sets?

I really think it's both, at least that's my opinion. One of the reasons this is possible is that data storage is cheap now. I remember how much I prized my floppy disk. I kept rewriting it and reformatting it to make sure that I would have my 1.44 megabytes worth of information. A lot of this is incredible advances in hardware, but then also the data piece that I'm super excited about.

"Everyone throws around this term big data, but it really is true that we're drowning in our ability to collect and save such huge volumes of data that we need to come up with new ways for how to process that data."

Prof. Christy F. Landes

Actually, I think for the future we need to come up with new ways for how to actually collect it. I think the ideal spectroscopy experiment is adaptive in that you use smart algorithms while you're collecting the data.

If you can get to a point where the software and algorithms are thinking intelligently enough to do what you want it to do without you even asking to do it, then that processing of data becomes even quicker.

I think in the future collection software will be smart enough to know when it has a good molecule and needs better spatial resolution and better time resolution and will adapt to collect better, finer grained information when you need it, and coarser grained information when you don't.

We're working on using some machine learning to teach our collection software when things look good and when things don't look good, when we need better resolution and when we don't.

Can you explain what STReM is, and how it allows you to observe dynamics of single molecules?

"STReM stands for Super Time-Resolved Microscopy, and as STORM, PALM, and other methods are designed to improve spatial resolution of optical microscopy, we desire to improve the time resolution."

Prof. Christy F. Landes

STReM makes use of point spread function engineering to encode fast events into each camera frame.

To some extent, it's actually very related to some of the data science principles that underlie what I mentioned above. The double-helix point spread function with 3D super-resolution microscopy method was developed by a group at the University of Colorado and now lots of other people are designing their own phase masks, and point spread functions. The thing we have in common is signal processing principles at the center.

One of the things that happens in signal processing is all the time you go back and forth between real space and Fourier space because all sorts of say noise filtering happens in Fourier space because you can separate noise from signals in Fourier space that you can't in signal space. That's why, for example, FT IR or FTN NMR is so much better than regular IR and regular NMR.

What these guys at Colorado recognized is that if they manipulated the phase of the light from their microscope, they could compress three-dimensional information about their sample into the two-dimensional point spread function that they collected on their camera at the image plane. It's super smart. You're doing exactly the same measurements with exactly the same laser or lamp on exactly the same sample in the microscope.

The only difference is that you allow yourself some room at the detection part of your microscope to manipulate the phase of a light such that your point spread function is no longer an Airy disk but this two-lobed double-helix point spread function, and the angle that point spread function tells you the three-dimensional position of your sample in your sample plane even though you're only doing a 2D image. It's genius. It's super cool.

STReM is built on that principle. I'm interested in dynamics. I want to see how proteins interact with interfaces, and lots of times that happens faster than my camera frame. Cameras are great and they're getting better all the time, but a camera is very expensive, and so you can't buy a new camera every six months to get slightly better time resolution. What we realized we could do is manipulate the phase of the fluorescence light to get better time resolution.

In other words, very similar to the double-helix 3D method, we are encoding faster information into a slower image. The point spread function is no longer a Gaussian or airy disk. It's these two lobes, and now the two lobes rotate around the center, and the angle tells you the arrival time of your molecule faster than the camera frame. You can get 20 times better time resolution with the same old camera that everybody uses.

What molecules/reactions have you been able to observe so far using this technique?

We are interested in understanding protein dynamics at polymer interfaces for the purpose of driving predictive protein separations. The improved time resolution of STReM is revealing intermediates that are suggestive of adsorption-mediated conformational rearrangements.

"Protein separations are getting increasingly important as the pharmaceutical industry moves away from organic molecules as therapeutics."

Prof. Christy F. Landes

The industry is moving towards therapeutics that are often proteins or peptides or sometimes they're DNA or RNA aptamer-based. Separating a protein therapeutic from all the other stuff in the growth medium is hugely time-intensive because at the moment there's no way to optimize a separation predicatively. It's done empirically. There's a bunch of empirical parameters that are just constantly messed with to try to optimize it.

Our long-term goal is to find a way to be predictive about protein separations. What that means underneath is being able to exactly quantify exactly what a protein does at that membrane interface in terms of its structure, its chemistry, its physics, how it interacts with other proteins in a competitive manner. Some of those things are faster than our camera can measure. Ideally we can catch everything, and that's why we need faster time resolution.

How does STReM compare to other single-molecule techniques you've employed before, such as FRET or super-resolution microscopy?

STReM is compatible with a wide range of super-resolution methods in that it is possible to combine 2D or 3D super-localization but with the faster time resolution of STReM. There remains the challenge of fitting or localizing more complicated point spread functions.

FRET is a way to measure distance. You would like to understand how protein structures change on the 1 to 10 nanometer scale, but we still don't have a microscope to measure 1 to 10 nanometers. Essentially, you take advantage of this dipole-induced dipole interaction that just happens to be over that length scale, and the math of that energy transfer process actually gets you the distance so you don't have to resolve it. FRET is also a math trick, to me anyway.

"The STReM technique, because it involves fluorescence, is very compatible with FRET or any other type of super resolution microscopy."

Prof. Christy F. Landes

The only challenge is that traditional microscopy approximates that Airy point spread function that you get at the image plane as a Gaussian and it's easy to fit a Gaussian.

Any undergrad with Excel can fit a Gaussian and identify the center and width of the peak, which is what you need for super resolution microscopy, and it's also what you need for FRET for that matter. The point spread functions that you get with these more complicated techniques like STReM or the 3D double-helix are more complicated to fit. Again, the math is more interesting and challenging.

You could do STReM with FRET. You could do STReM with PALM or STORM. People are using that double-helix with STORM, I think, to get 3D images with STORM, but your point spread function fitting and localization is more complicated.

What are the next steps for your own research? And how do you think your findings will contribute to a better understanding in the wider field of biochemistry?

Our big picture goal is to achieve enough mechanistic understanding of protein dynamics at polymer interfaces to transition to a predictive theory of protein chromatography. Protein separation and purification accounts for billions of dollars every year in industrial and bench scale efforts due to the fact that each separation must be optimized empirically.

How important is Pittcon as an event for people like yourself to share your research and collaborate on the new ideas?

In the US for general science and I would say medical science, Pittcon is the place to go to get information about the newest ways to measure things. It's the only meeting that's all about measurement. If I have something I need to measure, I go to Pittcon to learn the newest ways to measure it. If I have a new way to measure, I go to Pittcon to make sure that everybody knows that I have a new instrument or method to measure something. It's the only place.

1.8 MASS SPECTROMETRY AND METABOLOMICS

Metabolomic studies aim to identify and quantify a huge variety of different metabolites in complex mixtures, which is a huge challenge to standard analytical techniques.

High-frequency nuclear magnetic resonance (1H NMR) and mass spectrometry are the techniques most suited to metabolomics, as they can simultaneously measure numerous endogenous metabolites.

Mass spectrometry has the added benefit of also allowing quantitative assessments. The capabilities of mass spectrometry are further increased by using it in combination with other separation and analytical technologies. A selection of these are discussed below.

Ultra performance liquid chromatography – mass spectrometry

Ultra Performance Liquid Chromatography (UPLC) is one of the most significant developments in separations science in recent years. The re-development of high-specification equipment, including the column, injectors, pumps, and detectors has reduced dispersion, allowing sharper and more concentrated peaks to be achieved.

Using UPLC in conjunction with mass spectrometry combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. UPLC-mass spectrometry provides vastly improved sensitivity and spectral quality in the analyses of complex solutions.

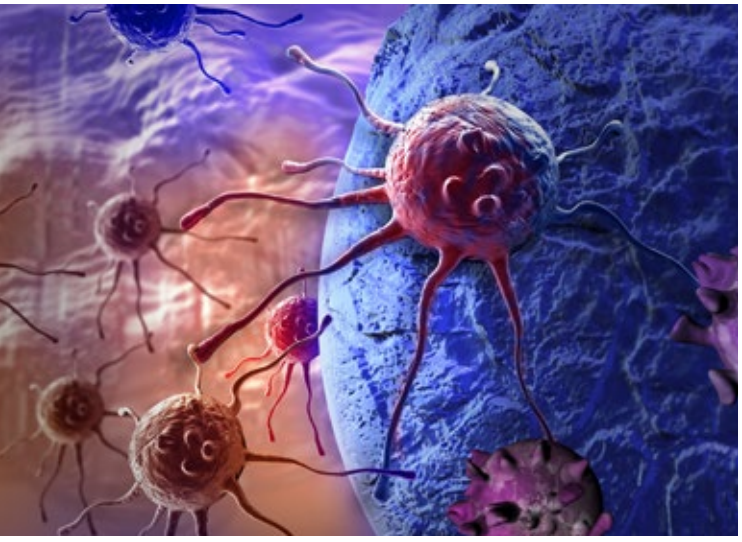
Evaluation of the system in the evaluation of 1300 samples of human urine showed an overall variation in spectral peak area response of 8%, and the mean variation in retention time reproducibility was only 1%. It has also provided good separation of amino acids and amines from human plasma with a rapid throughput and high sensitivity and precision.

Ion mobility-mass spectrometry

Ion mobility-mass spectrometry (IM-MS) has become an important analytical tool for 'omics' studies, since it has a much greater peak capacity than mass spectrometry alone, so a greater number of compounds can be identified.

Ion-mobility spectrometry separates gas phase ions on a millisecond timescale then the separated components are identified using mass spectrometry on a microsecond timescale.

It has proved especially valuable for detailed structural analysis of large and heterogeneous protein complexes. Although proteins are renowned for their precise structures, around 40% of human proteins lack a regular structure. Furthermore, many intrinsically disordered proteins play important roles in disease, such as cancer and Parkinson's disease.



It is thought that their lack of ordered structure is vital for them to function correctly. There has been extensive research into developing a mass spectrometry-based approach capable of determining the exact structures of intrinsically disordered proteins.

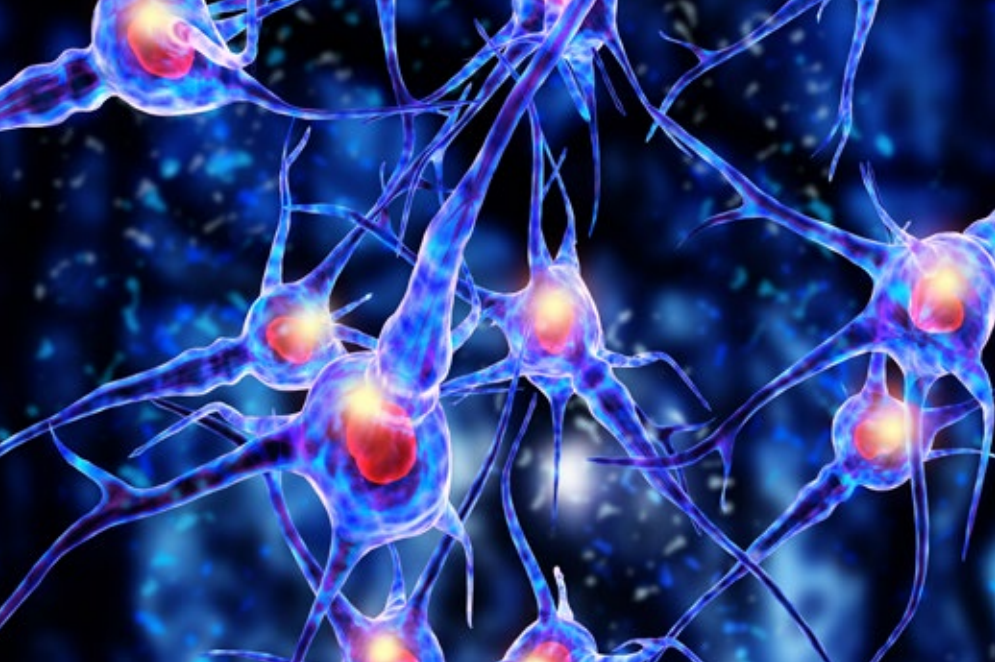
In his presentation at Pittcon 2018 entitled "Development of an Ion Mobility-Orbitrap Mass Spectrometer with a Variable Temperature Nano-ESI Source", David Russell, Professor of Applied Biosystems at Texas A&M University, will discussed the development of IM-MS instrumentation with high mobility resolution that is capable of analyzing larger proteins and protein complexes.

Liquid chromatography-mass spectrometry

Liquid chromatography mass spectrometry (LC-MS) is another key tool in metabolomics as it offers the broadest coverage of metabolites. This is largely because it is possible to use different column chemistries, as in reversed phase liquid chromatography (RPLC).

Furthermore, metabolites separated by liquid chromatography do not generally need derivatization and do not need to be volatile. Consequently, LC-MS has wide applicability in both untargeted and targeted metabolomic analyses.

The Thermo Fisher Scientific™ Exactive™ Plus EMR mass spectrometer LC-MS System provides a new level of accuracy in the study of native tertiary and quaternary protein structures. This high-resolution technology is also ideally suited to screening complex mixtures, providing accurate-mass full-scan mass spectra.



Ion mobili Secondary-ion mass spectrometry

Secondary-ion mass spectrometry (SIMS) allows analysis of the composition of solid surfaces and thin films. A focused primary ion beam is directed at the surface and the mass/charge ratios of the secondary ions reflected back are analyzed by mass spectrometry. This provides data on the elemental, isotopic, or molecular composition of the surface.

Pittcon 2018 exhibitor CAMECA presented their NanoSIMS 50L, which simultaneously delivers a range of key performance metrics that previously required had to be executed individually using a range of technologies. It has been used to quantitatively measure a subcellular slow protein turnover inside the cochlea of bullfrogs.

Mass spectrometry analysis of glycosylation

Glycosylation is one of the most common covalent modifications of proteins. It is frequently the means by which external stimuli cause physiological responses within a cell. It is thus essential for cell survival, and glycosylation errors are the cause of many human diseases, including cancer and infectious diseases.

Study of glycoproteins can therefore provide important information about cellular development and disease states.

However, due to their heterogeneity, it is exceptionally difficult to thoroughly analyze glycoproteins in complex biological samples. Recently, a range of chemical and enzymatic methods have been developed to facilitate the study of protein glycosylation using mass spectrometry. Such advances in mass spectrometry -based proteomics have provided a unique opportunity to systematically study protein glycosylation.

At Pittcon 2018, in a presentation entitled "Novel Mass Spectrometry-Based Methods to Globally and Site-Specifically Analyze Glycoproteins", Ronghu Wu of the Georgia Institute of Technology described the latest technique for studying surface glycoproteins.

Since glycoproteins are key players in many important biological processes, including disease, they are prime targets for identifying biomarkers.

Biomarkers are a valuable tool for facilitating early detection of disease and more accurate prognoses, and individually tailored treatment plans.

Investigation of site-specific protein glycosylation, enabled by advances in mass spectrometry technologies, is essential for the functional analyses of complex glycoproteins.

In his talk at Pittcon 2018, "Ultrahigh Resolution FTICR-MS for Clinical Glycomics Applications", Yuri van der Burgt of Leiden University Medical Center, described clinical glycomics studies indicating the potential prognostic value of total serum N-glycome analysis.

The high resolution of Thermo Fisher Scientific's Orbitrap Elite™ Hybrid Ion Trap-Orbitrap Mass Spectrometer facilitates analysis of low-abundance glycoproteins in complex samples. In addition, their UltiMate™ Well Plate Autosamplers delivers injections of even the smallest sample volumes with high reproducibility, reducing delay times and improving accuracy and peak resolution.

Fourier transform ion-cyclotron resonance mass spectrometry

Fourier transform ion-cyclotron resonance (FT-ICR) mass spectrometry is a type of mass spectrometry particularly suited to identifying heavy molecules, providing the highest resolving power and mass accuracy among all types of mass spectrometers. It differs from other mass spectrometry techniques in that the ions are identified according to the frequency with which they pass a detector when moving in a circular motion in a strong magnetic field.

Since this is determined by their mass, it can be used to determine their mass-to-charge ratio. The technique can be used on intact proteins without damaging them, making it an important tool proteomics and metabolomics.

References

- *Nicholson J, et al. Metabonomics: a platform for studying drug toxicity and gene function. Nat Rev Drug Discov 2002;1:153–161*
- *Waters Corporation. Stability of the Acquity UPLC system with Xevo G2-XS QTof Mass Spectrometer for metabolic phenotyping of human urine in large cohort studies. Technology Brief 2016. Available at http://www.waters.com/webassets/cms/library/docs/720005798en.pdf*
- *Wilson ID, Plumb RS. An Validated Bioanalytical Method for the Quantification of Biogenic Amines using UPLC-MS/MS. Application Note 2016. Available at http://www.waters.com/webassets/cms/library/docs/720005882en.pdf*
- *Chen SH, Russell DHJ. Am. Soc. Mass Spectrom. 2015;26:1433*
- *Corey DP. Movement and Growth of Hair-Cell Stereocilia. Presentation at "Molecular Cell Dynamics" conference, June 1, 2007, University of Münster, Germany. Available at http://www.cameca.com/markets/life-science/cell-biology-protein-turnover-nanosims*

1.9 EXTENDING THE BOUNDARIES OF "OMICS" RESEARCH

The preceding sections in this chapter have focused on the development of technologies and techniques to facilitate our understanding of cellular processes through proteomic and metabolomic research. However, they also have important applications that are directly relevant to everyday life. The instrumentation and methodologies used in research have become valuable tools for a range of real-world assessments.

These include aiding decisions regarding medical treatment strategies, determining toxicity, providing quality control, and detecting unscrupulous activity.

Metabolomics in oncology

Cancer affects normal cellular metabolism, and so tumor cells can be readily distinguished from healthy cells by their unique metabolic phenotype. Since metabolomics provides a global assessment of a cellular state, it is ideally suited to detecting and characterising cancer cells. Metabolomics also provides valuable data for informing treatment decisions and the development of novel therapeutics.

With the increasing sensitivity and specificities of nuclear magnetic resonance and mass spectrometry technologies, they are becoming important tools in the clinical management of cancer. The benefits of metabolomic analysis have already been proven with the use of metabolite imaging in the screening and diagnosis of breast and prostate cancers. Biomarkers also hold great promise for enabling earlier cancer diagnosis and predicting which treatments will be most effective for a given patient.
Elevated tissue choline levels are a reliable indicator of breast cancer. Similarly, metabolomic determination of lipid metabolic profiles by NMR identifies the presence of tumour cells with 83% accuracy. This could reduce the need for tissue biopsy. Metabolic profiling is also key to sub-typing breast cancer, providing valuable prognostic and predictive information regarding long-term outcomes for a given patient.

It is also becoming clear that metabolomic analysis can predict how likely a surgically excised tumour is to recur. If it is known that a tumour is likely to regrow quickly surgery, and its associated complications, could be avoided in patients for whom it is not likely to be curative. In cases where it is decided that clinical excision is the best option, it is often necessary to pharmacologically reduce the size of the tumour before surgery. Although pharmacological treatment can elicit a strong metabolic response in all patients, a good clinical response is not achieved in many cases.
At Pittcon 2018, Facundo Ferrnandez of Georgia

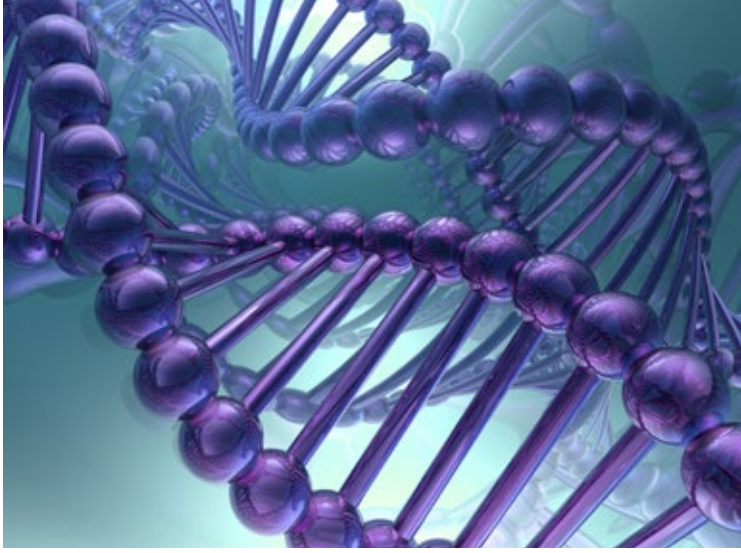
Institute of Technology gave a presentation entitled “Fused MS and NMR Metabolic Signatures of Prostate Cancer Recurrence Following Radical Prostatectomy” in which he will describe the use of nuclear magnetic resonance and liquid chromatography – mass spectrometry to make an a priori prediction of

Metabolomic NMR analysis has helped determine which metabolic profile is predictive of survival for more than 5 years.

the likelihood of prostate cancer recurrence prior to prostatectomy.

Nuclear magnetic resonance metabolomics is thus making it possible to provide clinical cancer care that is tailored on an individual patient level so that patients are provided with the treatment that is most likely to be successful against the particular cancer that they have.

Modern spectrometers readily provide an accuracy of ±5%, assuming relaxation issues are handled properly. With attention to potential sources of error, such as baseline distortions, poorly tuned instrumentation, signal to noise, accuracy can be increased to <1%.



Genomics

Proteomics research is so complex, and has such a broad scope, that a vast number of different technologies are used to achieve the required results with the necessary degree of quality. However, it is not only the instrumentation used in the analysis that is important for consistent, high-quality results. Sample preparation techniques and analytical software can both also significantly impact the data and therefore must be carefully considered in order for research to be successful.

Epigenetic profiling

A new epigenetic identification technique using bisulfite modification, polymerase chain reaction (PCR) and pyrosequencing has been developed and validated. It determines the specific pattern of DNA methylation in the sample and this can be matched with the patterns found in semen, saliva, and blood. Semen has been identified using this technique in samples containing as little as 1ng of genomic DNA.

The procedure was described at Pittcon 2018 by Bruce McCord of Florida International University, in his presentation entitled ‘Forensic Epigenetics, A Novel Method for Body Fluid Identification and Phenotyping’.

Thermo Fisher Scientific, who often exhibit at Pittcon, produced the PSQ 96 system for the analysis of DNA using pyrosequencing. It allows real-time sequencing of large numbers of short- to medium-length DNA sequences. Preparation and analysis of a sample is easily automated and results are obtained in under 2 hours.

Extragene also had a stand at Pittcon 2018 where their range of PCR equipment could be explored. Extragene PCR tubes, strips and tips are made from prime virgin polypropylenes that gives a perfect balance between transparency, softness, robustness, antistatic characteristics and gas tightness.

Short tandem repeat genotyping

Within the human genome, there are many areas of repeated DNA sequences. These repeated sequences varying in size and are classified according to the length of the repeating unit. Repeats 2-6 bases pairs long are called short tandem repeats (STRs).

STRs have several unique features that make them a valuable means of human identification. The number of repeats in STR markers varies markedly between individuals (an individual inherits an STR from each parent, which may or may not have similar repeat sizes) and have a low mutation rate. In addition, STRs are easily amplified by PCR and so have become popular DNA markers.

However, STRs can be challenging to analyze due to the propensity for errors to arise during amplification.

A novel targeted sequencing technology that simultaneously genotypes thousands of STR loci and phase proximal SNPs with significantly higher accuracy than currently available methods was described at Pittcon 2018.

In a presentation entitled 'Population Haplotype Analysis of 2,543 STRs and their Flanking SNPs Using a Massively Parallel Next-Generation Sequencing Technology' Giwon Shin from Stanford University School of Medicine presented data obtained using the new STR sequencing methodology.

Proteomics

Quantification of proteomes by mass spectrometry is commonly used in human pathology to study the effects of disease and treatments. Novel therapeutic agents are evaluated in animal models of disease before being tested in humans. A key aspect of such studies is to determine changes in protein expression.

Proteomic analysis may quantify thousands of proteins, making it a challenge to identify changes, particularly if only a small subset of proteins is affected. Furthermore, it may be difficult to distinguish which changes are in response to the treatment and which are a consequence of the disease.

It is also desirable to identify the changes in protein expression occurring at the earliest stages of the disease process since reversing these is more likely to change the course of the disease and thus have the potential to be successful drug targets.

To facilitate such studies, a new technique has been developed that enables in vivo quantitative proteomic analysis specifically of newly synthesized proteins.

The PALM (Pulse AHA Labeling in Mammals) technique allows for the first time in vivo labeling of mouse tissues to differentiate protein synthesis rates at discrete time points.

This new technique was presented at Pittcon 2018 by John Yates of the Georgia Institute of Technology in his presentation entitled "PALM (Pulse Azidohomoalanine Labeling in Mammals) Analysis for Global Analysis of Newly-Synthesized Proteins in Animal Models of Disease".

References

- *Antunes J, et al. High-resolution melt analysis of DNA methylation to discriminate semen in biological stains. Anal Biochem. 2016;494:40-45*
- *Madi T, et al. The determination of tissue-specific DNA methylation patterns in forensic biofluids using bisulfite modification and pyrosequencing. Electrophoresis. 2012;33(12):1736-1745*
- *McClatchy DB, et al. Pulsed Azidohomoalanine Labeling in Mammals (PALM) Detects Changes in Liver-Specific LKB1 Knockout Mice. J Proteome Res. 2015 Nov 6;14(11):4815-22. Available at http://pubs.acs.org/doi/ipdf/10.1021/acs.jproteome.5b00653*
- *Shin G, et al. High resolution STR analysis using next generation sequencing. Patent US 20160362751 A1. Available at https://www.google.com/patents/US20160362751*
- *Shin G, et al. CRISPR-Cas9-targeted fragmentation and selective sequencing enable massively parallel microsatellite analysis. Nat Commun. 2017;8:14291. Available at https://www.nature.com/articles/ncomms14291*
- *Silva DSBS, et al. Developmental validation studies of epigenetic DNA methylation markers for the detection of blood, semen and saliva samples. Forensic Sci Int Genet. 2016;23:55-63*

CONCLUSION

Living cells comprise a complex, ever-changing solution of small-molecule endogenous metabolites that represent the substrates and products of numerous inter-related pathways. This chemical fingerprint can therefore give an indication of which specific cellular processes are activated.

Consequently, changes in the precise combination of species present, along with changes in protein structure, reflect metabolic modifications in a cell providing insight into how a cell responds to external stimuli.

Metabolomic studies assessing metabolite composition are commonly used in conjunction with studies evaluating genetic mutations (genomics) and protein content and modification (proteomics) to obtain a holistic view of cell metabolism for full elucidation of the effects of environmental change.

Such 'omic' assessments have many applications in addition to being a valuable research tool, such as screening for and diagnosis of disease processes and forensic investigations. 'Omic' techniques are also valuable for informing treatment decisions.

They can identify gene mutations and biomarkers that predict disease course and treatment response. Such analyses allow treatment strategies to be tailored on an individual patient basis. This has proved especially important in oncology, where unpredictable efficacy and response rates had previously meant that finding an effective treatment strategy involved an element of trial and error. For example, NMR metabolic profiling can identify those patients most likely to respond to pre-surgical chemotherapy, and combined mass spectrometry and NMR metabolic signatures have been used to predict post-surgical prostate cancer recurrence.

> High-frequency NMR and mass spectrometry are the techniques most suited to metabolomic and proteomic analyses, as they can simultaneously measure numerous endogenous metabolites.

Although the analysis of highly complex mixtures is now commonplace, novel innovative uses and adaptations of these technologies continue to increase their capabilities. Ultra-high-resolution mass spectrometers, such as Orbitrap Fusion™ and Impact II™, enable accurate analysis of multiple molecules in a single process, facilitating biomarker identification.

Novel mass spectrometry based methods have also been developed to address current research needs, such as FTICR-MS for site-specific analysis of glycoproteins and non-damaging analysis of intact proteins, IM-MS for the analysis of intrinsically disordered proteins, and PALM for differentiation of protein synthesis rates at discrete time in vivo.

Mass spectrometry techniques are also used in combination with chromatographic separation, e.g.gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry, to increase resolution and facilitate peak identification.