This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

Multiphoton Microscopy and Ultrafast Spectroscopy: Imaging meets Quantum (MUSIQ)

Roadmap

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Published on 30.09.2023

Abstract

In April 2019 the EU Marie Skłodowska-Curie Actions (MSCA) Innovative Training Networks (ITN) MUSIQ officially started. The network brought together a unique team of world-leading academics and industrial partners at the forefront of optical micro-spectroscopy and ultrafast laser technology developments merged with fundamental studies of coherent light-matter interaction phenomena, development of quantitative image analysis tools beyond state-of-the-art, and biomedical/pharmaceutical real-world applications. The unique vision of MUSIQ has been to develop and apply the next-generation optical microscopy technologies exploiting quantum coherent nonlinear phenomena. This Roadmap has been written collectively by the MUSIQ early-stage researchers and their supervisors. It provides a summary of the achievements within MUSIQ to date, with an outlook towards future directions.

Table of Contents

Introduction
Paola Borri
[1] Chemical sensing at the nanoscale: Local field enhanced CARS
Martina Elisena Recchia and Paola Borri
[2] Single-molecule spectroscopy of fluorescent proteins
Vikramdeep Singh and Wolfgang Langbein
[3] Four-wave-mixing microscopy for live cell imaging at the single particle level
Nicole Slesiona and Peter Watson
[4] Ultrafast Holographic Microscopy
Martin Hörmann, Margherita Maiuri and Giulio Cerullo
[5] Ultrafast coherent spectroscopy of light-harvesting systems
Vasilis Petropoulos, Margherita Maiuri and Giulio Cerullo
[6] Polarization-sensitive multiphoton microscopy
Eleanor Munger and Sophie Brasselet
[7] High-Resolution Wide-Field Coherent Anti-Stokes Raman Scattering Microscopy
Eric Michele Fantuzzi and Hervé Rigneault
[8] Plasmonic antenna arrays for high-throughput single-molecule fluorescence detection
Ediz Herkert and Maria F. Garcia-Parajo
[9] Ultrasmall and ultrafast: Molecules, nanocavities and fs pulses
Saurabh Borkar and Niek F. van Hulst
[10] Stimulated Raman scattering microscopy with electronic pre-resonance enhancement
Andrea Pruccoli and Andreas Zumbusch
[11] Quantitative analysis of hyperspectral stimulated Raman scattering microscopy
Rushikesh Laxmikant Burde and Andreas Zumbusch
[12] 2D mid-IR/vibrational micro-spectroscopy of organic molecules
Thomas Deckert and Daniele Brida
[13] Calculating the response functions for multidimensional electronic spectroscopy
Frank E. Quintela Rodriguez, Filippo Troiani and Elisa Molinari
[14] Combine optical and mass spectrometry imaging techniques to characterise biological components at sub-cellular resolution
Jan Majer, Steve Hood and Peter Watson
[15] Dual frequency and multi-foci coherent Raman scattering microscopy
Dominykas Gudavičius and Wolfgang Langbein
Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

Introduction

MUSIQ started in April 2019, designed as an innovative and pioneering training network, with the ambitious vision of developing the next-generation optical microscopy exploiting quantum coherent nonlinear phenomena. It brought together a unique team of world-leading academic groups and high-tech companies at the forefront of nonlinear optical microscopy and ultrafast coherent light-matter interaction phenomena. 15 early-stage researchers (ESRs) were recruited to work towards 3 mains scientific objectives: O1. Investigate nonlinear optical phenomena originating from the intrinsic response of natural biomolecules, to achieve label-free imaging and overcome artefacts from sample staining/fluorescence methods; O2: Combine nonlinear (i.e. multiphoton) imaging with ultrafast two-dimensional spectroscopy to increase specificity and unravel quantum coherences in biomolecules; O3: Achieve single molecule detection and super-resolution in coherent nonlinear imaging via a combination of approaches, including the enhancement of the light field in the vicinity of metallic (plasmonic) nanostructures.

To tackle these objectives a multidisciplinary team of physicists, optical engineers, biologists, and chemists was formed. Innovative advances within MUSIQ include the design, development and implementation of novel microscopes, both in terms of image contrast methods as well as excitation/detection schemes. Nonlinear phenomena including coherent Raman scattering (CRS, see Sections 1,7,10,14,15), second harmonic generation (SHG, Section 6), and electronically-resonant four-wave mixing (FWM, Section 3) were exploited. A range of innovative instrument configurations were implemented, including heterodyne interferometry (Section 1), wide-field imaging (Sections 4, 7), multi-color/multi-foci (Sections 7,15), mid-IR excitation/detection (Section 12), polarisation sensitive schemes (Section 6), and the combination of CRS with mass spectrometry imaging (Section 14). An important pioneering push within MUSIQ has been to increase molecular detection sensitivity (Sections 1,8,10), eventually reaching the single molecule limit in microscopy and spectroscopy (Sections 2,9). Key to single molecule detection has been the exploitation of the local field enhancement occurring in the vicinity of a plasmonic nanostructure, and the design of optimised metallic nanoantennas for this purpose (Sections 1,8,9). In addition to instrumentation/hardware development, significant effort was devoted towards quantitative image analysis as well as simulations, computational and theoretical approaches (Sections 1,11,13).

Real world applications addressed i) the trafficking of nanoparticles via endocytic pathways inside living cells, of importance for fundamental biology and drug delivery (Section 3), ii) genetically encoded fluorescent proteins, as model systems to understand electronic-vibrational coupling and as potentially promising new probes for vibrational microscopy (Section 2), iii) light-harvesting complexes to unravel the physical processes regulating their efficiency, of importance for the development of sustainable energy solutions (Section 5), and iv) animal tissue models for drug discovery and development pipelines (Section 14).

Expanding from the first Roadmap[1] authored by the MUSIQ consortium in 2021, this second Roadmap has been written collectively by the ESRs and their supervisors. It provides a summary of the main scientific achievements reached by the end of the MUSIQ programme, with an outlook towards future directions.

References

[1] Herkert E. *et al* (2021), Roadmap on bio-nano-photonics. *Journal of Optics* **23**, 073001. DOI: https://doi.org/10.1088/2040-8986/abff94.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

1. Chemical sensing at the nanoscale: Local field enhanced CARS

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Coherent anti-Stokes Raman scattering (CARS) micro-spectroscopy is a promising technology for label-free chemical sensing and imaging at high-speed. It has been extensively developed over the last two decades,[1] allowing for mapping of endogenous biomolecules in living systems, from single cells to large area tissues, without the drawback of staining artefacts and photobleaching associated with fluorescence microscopy, at speeds up to video rate.[2,3,4] Despite these advantages, it is still not possible to achieve single-molecule detection with a CARS based technique. The strong enhancement of the light field occurring at the nanoscale region near the surface of a metallic nanostructure has helped to overcome this limitation.[5,6,7] In plasmon-enhanced CARS, the generated anti-Stokes signal is the result of the locally enhanced pump and Stokes laser fields with an additional amplification of the generated anti-Stokes field by the plasmonic nanostructure.

In our recent work,[8,9,10,11] we have demonstrated local-field enhanced (LFE) CARS, exploiting a novel scheme where CARS is detected interferometrically in epi-geometry (epi heterodyne CARS - eH-CARS), with the local field enhancement of the CARS field provided by a plasmonic nanoantenna. Two types of nanoantennas were explored, namely silica-coated colloidal gold nanorods (SiAuNRs) and nano-fabricated gold nanobowties (AuNBs). Silica coating was introduced to prevent reshaping of the gold nanorods upon laser excitation, a known problem with these nanoparticles.[12] Since colloidal synthesis typically generates a variety of sizes and shapes, we investigated the optical proprieties of the SiAuNRs via single particle optical extinction microscopy measurements.[13,14,15] Notably, the geometrical characteristics are key to determine the nanostructure optical properties, therefore we developed an "optical sizing" tool able to quantify the rod shape and size by comparing the measured extinction cross-sections with simulations. The latter were obtained via an elaborate model developed with the commercial software COMSOL Multiphysics, reproducing the experimental measurements.[16]

Experimentally, we quantified the CARS enhancement for different SiAuNR sizes, using a bulk medium of known Raman spectrum (namely silicon oil) as material surrounding the antenna for proof-of principle. Additionally, we characterized the dependence of such enhancement on the power of the incident beams and on the wavenumber detuning between the CARS driving fields and the vibrational resonance under study. Via COMSOL modelling, we then compared simulated and experimental LFE-eHCARS. Notably, the gold antenna itself contributes to the measured signal via a non-resonant CARS emission (due to electronic four-wave mixing) and we were able to infer the value of the third order susceptibility of gold reproducing our experiment. In fact, in the literature, the reported values of the third-order susceptibility of gold span over several order of magnitudes. This is due to the various techniques utilized to measure the gold third order nonlinearity, which employed different laser pulses durations, thus probing different contributions to the gold nonlinear response over various time scales.[17] Exploiting COMSOL simulations, we also investigated the nonresonant CARS contribution to our measurements arising from the silica shell. Importantly, incoherent processes such

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as photoluminescence from the metal nanoparticles[18] are suppressed by the interferometric heterodyne detection employed.

Overall, this work demonstrated that LFE-eHCARS can be achieved with AuNRs, and simulations showed local enhancement factors in the order of 1000 at the nanorod tips. Experimentally however, the study highlighted the shape instability of the AuNRs under laser excitation (hence the need to coat them with a silica shell), and the non-resonant contribution from gold.

After the proof-of-principle demonstration of LFE-eHCARS using SiAuNRs surrounded by a static bulk medium, we performed dynamic sensing experiments, with polystyrene (PS) beads moving in and out of the antenna LFE volume. In this case, we used AuNBs as antennas and performed correlative fluorescence and LFE-eHCARS sensing measurements by exploiting fluorescently-labelled PS beads. This study was followed by a sensing experiment with living cells expressing a membrane receptor (P2X7), thought to partition in lipid nanodomains/rafts, rich in cholesterol and saturated lipids.[19] Overall, we found evidence of LFE-eHCARS correlatively with fluorescence when measuring with the PS beads, while the study in cells proved more difficult to analyse and interpret.

A considerable limitation of the available setup is that eH-CARS spectra can be acquired only sequentially, with consequent reduction in acquisition speed and chemical specificity. As an outlook, going forward it would be very useful to implement a spectrally-resolved broadband eH-CARS, in order to interrogate and detect multiple vibrational modes in one spectral acquisition simultaneously. An important aspect in implementing this configuration is related to the need of tailoring, both temporally and spectrally, the pump, Stokes and reference fields such that multiple vibrations can be excited simultaneously and distinguished spectrally. This could be implemented using an appropriate choice of spectral bandwidth (hence femtosecond pulses) and chirp combinations. A broadband eH-CARS implementation would bring also the significant advantage of distinguishing between the enhanced CARS signal from the biological molecules and the signal coming from the electronic FWM process in the antenna, as the latter would not exhibit a vibrational resonance and could thus be separated as a spectrally constant contribution. An additional improvement going forward, would be the design and fabrication of optimal plasmonic nanoantennas, in terms of shape stability, enhancement factors, and ability to enhance multiple vibrational resonances.

References

[1] Cheng J. X., and Xie X. S. (2004), Coherent anti-Stokes Raman scattering microscopy: instrumentation, theory, and applications. *The Journal of Physical Chemistry B* **108**, 827-840.

[2] Evans C. L., Potma E. O., Puoris'haag M., Côté D., Lin C. P., and Xie X. S. (2005), Chemical imaging of tissue in vivo with video-rate coherent anti-Stokes Raman scattering microscopy. *Proceedings of the National Academy of Sciences* **102**, 16807-16812.

[3] Liao C. S., and Cheng J. X. (2016), In situ and in vivo molecular analysis by coherent Raman scattering microscopy. *Annual Review of Analytical Chemistry* **9**, 69-93.

[4] Saar B. G., Freudiger C. W., Reichman J., Stanley C. M., Holtom G. R., and Xie X. S. (2010), Video-rate molecular imaging in vivo with stimulated Raman scattering. *Science* **330**, 1368-1370.

[5] Koo T. W., Chan S., and Berlin, A. A. (2005), Single-molecule detection of biomolecules by surface-enhanced coherent anti-Stokes Raman scattering. *Optics Letters* **30**, 1024-1026.

[6] Zhang Y., Zhen, Y. R. Neumann, O. Day, J. K. Nordlander P., and Halas N. J. (2014), Coherent anti-Stokes Raman scattering with single-molecule sensitivity using a plasmonic Fano resonance. *Nature Communications* **5**, 4424.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

[7] Zong C., Premasiri R., Lin H., Huang Y., Zhang C., Yang C., ... and Cheng, J. X. (2019), Plasmon-enhanced stimulated Raman scattering microscopy with single-molecule detection sensitivity. *Nature Communications* **10**, 5318.

[8] Recchia M. E., Harlow D.S, Langbein W.W, and Borri P. (2022), Coherent Raman detection at the nanoscale via the local field enhancement at a single plasmonic Nanorod' *Focus on Microscopy (FOM) 2022*, Online conference. Poster presentation.

[9] Recchia M. E., Harlow D.S, Langbein W.W, and Borri P. (2022), Nanoscale coherent Raman detection via the local field enhancement at a single plasmonic nanorod. *International Conference on Raman Spectroscopy (ICORS) 2022*, Long Beach, California, USA. Oral presentation.

[10] Recchia M. E., Harlow D.S, Langbein W.W, and Borri P. (2022), Nanoscale coherent Raman detection via the local field enhancement at a single plasmonic nanorod. *UK Conference on Photonics PHOTON 2022*, Nottingham, UK. Oral presentation.

[11] Recchia M. E., article manuscript in preparation and PhD thesis in progress.

[12] González-Rubio G., Guerrero-Martínez A., and Liz-Marzán L. M. (2016), Reshaping, fragmentation, and assembly of gold nanoparticles assisted by pulse lasers. *Accounts of Chemical Research* **49**, 678-686.

[13] Payne L. M., Albrecht W., Langbein W., and Borri P. (2020). The optical nanosizer–quantitative size and shape analysis of individual nanoparticles by high-throughput widefield extinction microscopy. *Nanoscale* **12**, 16215-16228.

[14] Payne L. M., Langbein W., and Borri, P. (2013), Polarization-resolved extinction and scattering cross-sections of individual gold nanoparticles measured by wide-field microscopy on a large ensemble. *Applied Physics Letters* **102**, 131107.

[15] Herkert E. K., Bermeo Alvaro D. R., Recchia M., Langbein W., Borri P., and Garcia-Parajo M. F. (2023), Hybrid Plasmonic Nanostructures for Enhanced Single-Molecule Detection Sensitivity. *ACS Nano* **17**, 8453-8464.

[16] Zilli A. (2018), Measuring and modelling the absolute optical cross-sections of individual nano-objects (Doctoral dissertation, Cardiff University).

[17] Boyd R. W., Shi Z., and De Leon I. (2014). The third-order nonlinear optical susceptibility of gold, *Optics Communications* **326**, 74-79.

[18] Fast A., and Potma E. O. (2019). Coherent Raman scattering with plasmonic antennas. *Nanophotonics* **8**, 991-1021.

[19] Garcia-Marcos M., Peérez-Andreés E., Tandel S., Fontanils U., Kumps A., Kabreé E., ... and Pochet S. (2006), Coupling of two pools of P2X7 receptors to distinct intracellular signaling pathways in rat submandibular gland. *Journal of Lipid Research* **47**, 705-714.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

2. Single-molecule spectroscopy of fluorescent proteins

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The role of optical microscopy and imaging is pivotal in many domains, including life sciences, nanotechnology, material sciences and engineering. From the wide variety of different optical microscopy methods available, fluorescence-labelled microscopy and imaging techniques are the pedestals for modern life science research, enabling high contrast and selective imaging of specific structures or molecules of specimens using different fluorescent dyes, and proteins. The discovery and demonstration of green fluorescent protein (GFP), as a tool for marking different proteins and their expressions, revolutionised the microscopy and imaging of biological specimens[1]. This led to the development of a huge library of different fluorescent tags in the visible and near-infrared spectrum, which can be coupled to different proteins to understand their cellular dynamics, interactions with other components and transport. Despite the extensive growth of the fluorescent proteins (FP) library and its use, less focus has been given to studies interpreting the photophysics of different FP. For an accurate assessment of different biological and biochemical processes probed by FP, an understanding of the photophysics of these markers is essential. Some of the photophysical properties can only be assessed in studies addressing single molecules, such as time trace of emission intensity giving information on changes in molecular orientation, photobleaching, transient transitions to dark states and changes in quantum yield. Single-molecule spectroscopy (SMS) is the zenith of studying the interaction of optical electric fields with matter to uncover the hidden heterogeneities on an individual molecule level, which cannot be accessed in ensemble measurements. Exactly one single molecule is addressed at a time, to collect the actual distribution of parameters rather than a collective averaging from a set of molecules.

Almost three and half decades ago, single-molecule optical spectroscopy in condensed matter at low temperatures was realised[2]. Over time, this field has grown from studies of molecular impurities in solids in the early stages to the observation of single-molecule absorption and emission at low- and room-temperature in different media, solutions, polymers, and cells. The very first studies utilised the idea that at low temperatures, well-localized molecular impurities have very sharp absorption resonances due to spectral fine structure arising from local fluctuations in the host matrix. By frequency modulation of the excitation laser, different sets of individual molecules could be addressed which were in resonance with excitation. Single-molecule studies of GFP showed novel observation of hidden heterogeneities- blinking, spectral diffusion, and emission recovery by exciting at a different wavelength[3].

Single-molecule spectroscopy studies are not limited to linear spectroscopy techniques only where a static picture of the dynamic world is achieved. The evolution of various non-linear optical spectroscopy techniques to understand the transient dynamics on femtosecond timescales gave a real picture of complex temporal evolution[4]. Various non-linear optical spectroscopy tools have been employed for SMS, including fluorescence-detected pump-probe spectroscopy on DBT molecules[5], coherent anti-stokes Raman spectroscopy with single-molecule sensitivity using plasmonic enhancements[6], and surface-enhanced Raman spectroscopy[7] of GFP.

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In our research project, we investigated two genetically encoded FPs, mCherry and mRhubarb720, with their optical emission in the red and near-infrared spectrum. Genetically encoded FPs are proteins with tailored fluorescence properties compared to natural FPs, and are attached to proteins of interest for labelling. We conducted a thorough study of mRhubarb720. Room-temperature single-molecule imaging was done to understand the blinking and photobleaching dynamics of plasmonically enhanced FP embedded in the polymer matrix. Low-temperature high-resolution fluorescence emission spectroscopy measurements revealed sharp Raman lines due to the strong enhancement of the individual fluorophores by plasmonic gold nanorods[8]. A temperature-dependence measurement series was conducted to evaluate the linewidth of Raman lines at different temperatures. A polarization-resolved detection scheme was employed using a polarising beam displacer to detect two orthogonal polarisations simultaneously. The molecular orientation of the fluorophores was probed by investigating switching and contrast between two polarisations for different emission lines. The phenomenon of fluorescence intermittency is observed at low temperatures where the molecule switches between dark and bright states. These jumps are random, and molecules can stay in a dark state for seconds. This timescale is vastly longer than the dark state lifetime, as predicted by Bohr's on-off exponential distribution of states' lifetimes. In addition to single-molecule measurements, ultrafast pump-probe and two-dimensional electronic spectroscopy measurements were conducted on the ensemble of mCherry and mRhubarb to understand the transient dynamics at room temperature.

This project has addressed the challenge of achieving single-molecule enhanced fluorescence and Raman spectroscopy. The fluorescence intermittency in single-molecule fluorescence emission at low temperatures is still not well explained with current models in the case of FPs. The advancement of SMS is poised to enhance our understanding of fundamental science such as vibrational coherence and excitation-emission pathways, and will also push boundaries in molecular imaging, molecular electronics, nanotechnologies, and many other fields.

References

[1] M. Chalfie, Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher (1994), Green Fluorescent Protein as a Marker for Gene Expression. *Science* **263**, 802-805.

[2] W. E. Moerner and L. Kador (1989), Optical detection and spectroscopy of single molecules in a solid. P*hysical Review Letters* **62**, 2535-2538.

[3] R. M. Dickson, A. B. Cubitt, R. Y. Tsien and W. E. Moerner (1997), On/off blinking and switching behaviour of single molecules of green fluorescent protein. *Nature* **388**, 355-358.

[4] T. S. Rose, M. J. Rosker and A. H. Zewail (1988), Femtosecond real-time observation of wave packet oscillations (resonance) in dissociation reactions. *The Journal of Chemical Physics* **88**, 6672-6673.

[5] M. Liebel, C. Toninelli and N. F. van Hulst (2017), Room-temperature ultrafast nonlinear spectroscopy of a single molecule. *Nature Photonics* **12**, 45-49.

[6] Y. Zhang, Y.-R. Zhen, O. Neumann, J. K. Day, P. Nordlander and N. J. Halas (2014), Coherent anti-Stokes Raman scattering with single-molecule sensitivity using a plasmonic Fano resonance. *Nature Communications* **5**, 1-7.

[7] S. Habuchi, M. Cotlet, R. Gronheid, G. Dirix, J. Michiels, J. Vanderleyden, F. C. De Schryver and J. Hofkens (2003), Single-Molecule Surface Enhanced Resonance Raman Spectroscopy of the Enhanced Green Fluorescent Protein. *Journal of the American Chemical Society* **125**, 8446-8447.

[8] V. Singh, O. Evans, D. Jones, O. Aksakal, P. Borri, and W. Langbein (2023), Low temperature resonant Raman scattering of single mRhubarb720 reveals long vibrational coherence and vibrational spectral wandering. *In preparation*.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

3. Four-wave-mixing microscopy for live cell imaging at the single particle level

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Single gold nanoparticle (GNP) tracking in live cells has the potential to guide us into a new era of imaging, offering unprecedented resolution and clarity in visualizing cellular and molecular interactions in real-time. Unlike traditional fluorescence-based imaging techniques which can suffer from drawbacks like photobleaching and autofluorescent background, GNP-based single particle tracking (SPT) provides sustained long-term imaging capabilities. The nonlinear optical properties of GNPs allow for superior detection of biological processes. Prior to this project, the only practical imaging applications capable of non-fluorescence-based single-nanoparticle tracking in living cells were photothermal imaging[1] and parallax-DIC microscopy[2]. In the last four years, both interferometric single particle tracking (iSPT) which is based on interferometric scattering microscopy (iSCAT)[3], and now with our current developments, Four-Wave-Mixing imaging (FWMi) have emerged as live-cell single particle tracking modalities.

In its current configuration, FWMi is capable of detecting single GNPs down to 10 nm diameter with high signal-to-noise ratio in live cellular environments[4–6]. FWMi exploits the third-order optical nonlinearity of gold for specificity, and is detected free from linear scattering backgrounds using a heterodyne scheme. Interferometric detection also eliminates autofluorescent background. With an imaging speed of 0.1–1 ms per pixel, FWMi allows us to follow internalisation events at the single particle level. A main limiting speed factor is its point sample-scanning configuration, where the exciting laser beam is focussed on a specific location of the sample and the latter is moved in 3D using a piezoelectric stage. Current developments within our consortium might lead to faster imaging speeds of this modality, as multiple data points could be acquired at the same time, in either a multi-foci (see Section 15) or wide-field imaging geometry (see Section 4).

FWM already shows great potential for unravelling GNP trafficking pathways label-free due to its specificity and compatibility with live-cell imaging[4]. Previous works have shown that the labelling of proteins with fluorophores alters their behaviour, toxicity and trafficking pathway within living cells[7,8] and furthermore it has been shown that there is little to no colocalization between fluorophore and particle signals[4,9]. Further application and development of this new imaging modality will increase our understanding of how GNPs traffic throughout the cell, give more detail about accumulation of GNP in cells and tissues, and reveal how fluorophore labelling influences the targeting capabilities of GNPs. Next to validating fluorescence-based imaging modalities, FWM is also capable of assisting with the validation of data acquired with synchrotron-based x-ray methods, electron microscopy such as transmission electron and scanning electron microscopy[10,11].

Apart from looking at GNPs as pure labelling agents, they also hold great promise as fundamental tools for understanding cell biology and as drug delivery vectors in the clinic, however as we have seen within our project[12], controlling, characterising and understanding fabrication parameters of functionalised particles will be key to interpreting data obtained from any source, imaging or otherwise. Surface functionalisation along with the size and shape of the nanoparticle itself need to

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be correlated. Addition of spikes to nanoparticles has been shown to increase internalisation by regulating myosin IIA recruitment at the cell membrane during cellular uptake[13], highlighting that just small changes to nanoparticle geometry can impact interactions between nanoparticles and the cell. This is further complicated by the large variation possible in proteins binding to the surface of nanoparticles as they encounter biological material. Efforts are being made to understand how the nanoparticle surface changes within a biological environment, specifically with the formation of a protein corona[14] that can impact and affect the way in which nanoparticles bind to, and traffic within cells. One further advantage of using gold nanoparticles is the possibility of using surface enhanced Raman scattering to be ability to identify chemical signatures from the local environment. This has been used to explore pathway entry of GNPs into mammalian cells[15], with specifical chemical signatures being identified, and modified through the use of pathway specific inhibitors across a number of cell lines. Again, one complication here is the protein corona, which under different biological conditions (i.e. concentration and type of media) can impact the route of NP uptake. A recent review on NPs from a group of cell biologists however gives an alternative perspective on this problem, in that "the identification of a specific pathway might not always need to be a priority and the focus should instead be on establishing that internalization occurs, its relative efficiency, and showing where in the endocytic pathway internalized NP are located at specific times after endocytosis"[16]; Due to the non-invasive, high signal to noise and long timeframes for acquisition available, FWM is ideally placed to be able to answer exactly these questions within cells.

References

[1] Lasne D., Blab G. A., Berciaud S., Heine M., Groc L. Choquet D., Cognet L., Lounis B (2006), Single Nanoparticle Photothermal Tracking (SNaPT) of 5-Nm Gold Beads in Live Cells. *Biophys. J.* **91**, 4598–4604. https://doi.org/10.1529/biophysj.106.089771.

[2] Chen K., Gu Y., Sun W., Bin Dong, Wang G., Fan X., Xia T., and Fang N (2017), Characteristic Rotational Behaviors of Rod-Shaped Cargo Revealed by Automated Five-Dimensional Single Particle Tracking. *Nat. Commun*. **8**, 887. https://doi.org/10.1038/s41467-017-01001-9.

[3] Taylor R. W., Mahmoodabadi R. G., Rauschenberger V.. Giessl A.. Schambony A. and Sandoghdar, V (2019), Interferometric Scattering Microscopy Reveals Microsecond Nanoscopic Protein Motion on a Live Cell Membrane. *Nat. Photonics* **13**, 480–487. https://doi.org/10.1038/s41566-019-0414-6.

[4] Giannakopoulou N., Williams J. B., Moody P. R., Sayers E. J., Magnusson J. P., Pope I., Payne L., Alexander C., Jones A. T., Langbein W., Watson P., and Borri P (2020), Four-Wave-Mixing Microscopy Reveals Non-Colocalisation between Gold Nanoparticles and Fluorophore Conjugates inside Cells. *Nanoscale* **12**, 4622–4635. https://doi.org/10.1039/C9NR08512B.

[5] Pope I., Ferreira N. G. C., Kille P., Langbein W., Borri P (2023), Background-Free Four-Wave Mixing Microscopy of Small Gold Nanoparticles inside a Multi-Cellular Organ. *Appl. Phys. Lett.* **122**, 153701. https://doi.org/10.1063/5.0140651.

[6] Zoriniants G., Masia F., Giannakopoulou N., Langbein W., and Borri P (2017), Background-Free 3D Nanometric Localization and Sub-Nm Asymmetry Detection of Single Plasmonic Nanoparticles by Four-Wave Mixing Interferometry with Optical Vortices. *Phys. Rev. X* **7** 041022. https://doi.org/10.1103/PhysRevX.7.041022.

[7] Birch D., Christensen M. V., Staerk D., Franzyk H., Nielsen H. M (2017), Fluorophore Labeling of a Cell-Penetrating Peptide Induces Differential Effects on Its Cellular Distribution and Affects Cell Viability. *Biochim. Biophys. Acta Biomembr*. **1859**, 2483–2494. https://doi.org/10.1016/j.bbamem.2017.09.015.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

[8] Zsila F (2022), Far-UV Circular Dichroism Signatures Indicate Fluorophore Labeling Induced Conformational Changes of Penetratin. *Amino Acids* **54**, 1109–1113. https://doi.org/10.1007/s00726-022-03149-1.

[9] Miles B. T., Greenwood A. B., Benito-Alifonso D., Tanner H., Galan M. C., Verkade P., and Gersen H (2017), Direct Evidence of Lack of Colocalisation of Fluorescently Labelled Gold Labels Used in Correlative Light Electron Microscopy. *Sci. Rep*. **7**, 44666. https://doi.org/10.1038/srep44666.

[10] Pan S., *et al* (2023), Gold Nanoparticle-Enhanced X-Ray Microtomography of the Rodent Reveals Region-Specific Cerebrospinal Fluid Circulation in the Brain. *Nat. Commun*. **14**, 453. https://doi.org/10.1038/s41467-023-36083-1.

[11] Ahn S., Jung S. Y., Lee S. J (2013), Gold Nanoparticle Contrast Agents in Advanced X-Ray Imaging Technologies. *Molecules* **18**, 5858–5890. https://doi.org/10.3390/molecules18055858.

[12] Slesiona N., Payne L., Pope I., Borri P., Langbein W., and Watson P (2023). Correlative Extinction and Single Fluorophore Bleaching Microscopy for Ligand Quantification on Gold Nanoparticles. *Preprint arXiv*. https://doi.org/10.48550/arXiv.2210.00942.

[13] Huang L., *et al* (2023), Nanoparticle Spikes Enhance Cellular Uptake via Regulating Myosin IIA Recruitment. *ACS Nano* **17**, 9155–9166. https://doi.org/10.1021/acsnano.2c12660.

[14] Ngo W., *et al* (2022). Identifying Cell Receptors for the Nanoparticle Protein Corona Using Genome Screens. *Nat. Chem. Biol*. **18**, 1023–1031. https://doi.org/10.1038/s41589-022-01093-5.

[15] Yılmaz D., Culha, M (2021), Investigation of the Pathway Dependent Endocytosis of Gold Nanoparticles by Surface-Enhanced Raman Scattering. *Talanta* **225**, 122071. https://doi.org/10.1016/j.talanta.2020.122071.

[16] Griffiths G., Gruenberg J., Marsh M., Wohlmann J., Jones A. T., Parton R. G (2022), Nanoparticle Entry into Cells; the Cell Biology Weak Link. *Adv. Drug Deliv. Rev*. **188**, 114403. https://doi.org/10.1016/j.addr.2022.114403.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

4 - Ultrafast Holographic Microscopy

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Within our MUSIQ project, we combined femtosecond transient absorption (TA) spectroscopy with widefield off-axis holographic microscopy. The general motivation has been to acquire femtosecond TA data with spatial information to unveil the ultrafast coherent spatiotemporal evolution of complex systems. In a recently published work, we directly imaged the ultrafast diffusion of carriers in a hybrid organic-inorganic perovskite[1]. To that end, we developed a technique whereby we simultaneously photoexcited hundreds of diffraction-limited spots covering a large field-of-view. Thanks to this, we can either probe effects of sample heterogeneity or average the response of all spots to vastly increase the sensitivity[1].

Another part of our work consisted in implementing a three-dimensional imaging technique, which we did employing optical diffraction tomography (ODT) with femtosecond pulses. The core problem was to enable the holographic imaging necessary for ODT with broadband pulses and their intrinsically short coherence times. We showed experimentally how to solve this problem by performing spectrally resolved ODT with a bandwidth of 100nm in the visible[2].

In the future these results will be combined, aiming at performing femtosecond three-dimensional imaging experiments by adding an excitation pulse to the tomographic microscope. Also, these systems are a convenient platform to study heterogeneity and dynamics of hot and cold charge carriers in new (organic) semiconductors such as perovskites or transition metal dichalcogenides. Finally, we aim to extend the holographic transient microscope to perform time-resolved Faraday rotation imaging, aiming to resolve spin dynamics.

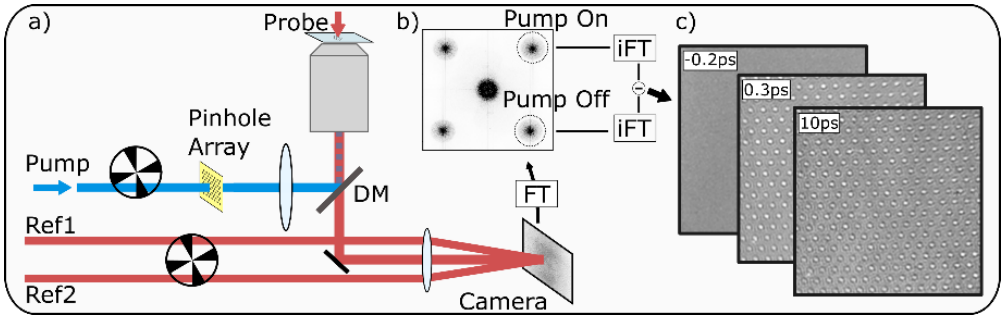

Figure 1: (a) Sketch of the experimental setup. DM: dichroic mirror. (b) Processing of the on and off signatures in k-space. (c) Transient images of a polycrystalline thin film of methylammonium lead bromide perovskite for negative and positive delays.

References

[1] Hörmann M., *et al* (2023), High-Sensitivity Visualization of Ultrafast Carrier Diffusion by Wide-Field Holographic Microscopy." *Ultrafast Science* **3**, 0032

[2] Hörmann M., *et al*. (2022), From Ultrafast Holography to Ultrafast Tomography. *Digital Holography and 3-D Imaging* 2022, Technical Digest Series (Optica Publishing Group, 2022) paper **Th1A.3**.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

5. Ultrafast coherent spectroscopy of light-harvesting systems

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Sustainable energy systems thrive on the abundant potential of solar energy. However, the crucial challenge lies in efficiently harnessing and distributing this energy. Bridging this gap requires technological advancements which heavily depend on answering crucial questions regarding the efficiency of natural light harvesting complexes.

Ultrafast coherent spectroscopy enables the study of intact natural systems, facilitating the exploration of their complex dynamics and providing insights that can be transferred from model systems to real-world applications. Advanced techniques like coherent multidimensional and transient absorption spectroscopy using sub-10 femtosecond pulses, are able to map the primary excited state pathways of natural complexes. By employing these techniques, we can additionally observe the fate of impulsively generated wavepackets, unveiling the nuclear and electronic motions that drive ultrafast events.[1]

During our project within the MUSIQ consortium, our main breakthrough was unravelling the 60-fs internal conversion (IC) within the low-lying Q-states in a functionalized porphyrin monomer while monitoring the vibrational motions promoting that process (Figure 1a). The IC process is accompanied by a re-arrangement of vibrations, where the initially high amplitude C-H vibrations in the central core of the porphyrin ring (1270 cm^{-1}, shown in green) transition towards in-plane C-C stretching motions (1514 cm^{-1}, shown in red) distributed in the periphery of the porphyrin ring (Figure 1b).[2] Further exploration of molecule functionalization as a viable pathway to achieve IC modulation at the single molecule level shows promise. The introduction of substituents rich in high-frequency vibrations can effectively speed up the ultrafast internal conversion process, simultaneously lowering the energy barrier between the Q-states.

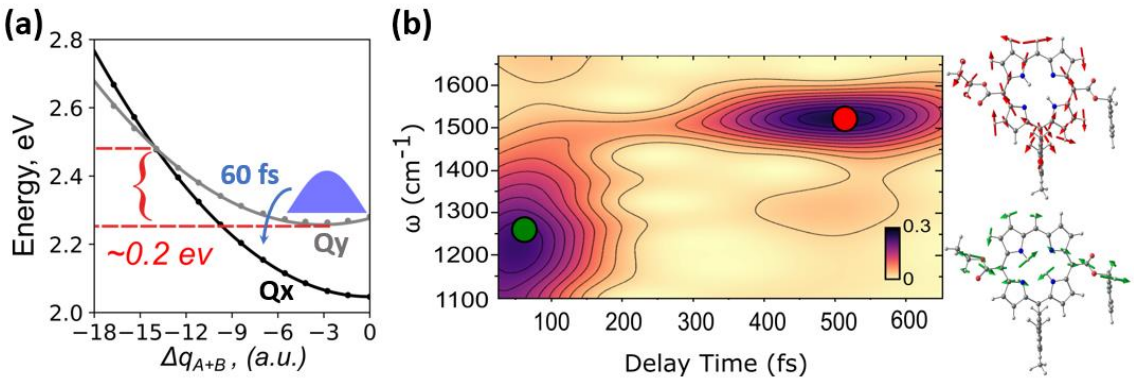

Figure 1: (a) Potential energy surfaces of Qx and Qy electronic states of a functionalized porphyrin, highlighting the crossing region. A photoinduced wavepacket on the Qy surface is funneled to the Qx within 60 fs. (b) SWFT map scanning a Gaussian window of 250 fs width along the excited-state beating signal. The corresponding motions of the dominant modes at 1270 cm-1 and 1514 cm-1 are visualized.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

Based on our findings and the current progress of ultrafast coherent multidimensional spectroscopy, a promising future perspective emerges: utilizing the knowledge acquired from ultrafast spectroscopy to design and optimize artificial systems that exhibit enhanced performance and sustainability.

Obtaining a more comprehensive understanding of energy flow within light-harvesting systems will require further groundbreaking advancements in the field. The integration of multidimensional coherent spectroscopic tools with complementary techniques, such as single-molecule spectroscopy, time-resolved X-ray scattering and spatially resolved techniques, holds great potential in providing a holistic insight into the intricacies of light-harvesting systems.[3]

References

[1] F. D. Fuller *et al.* (2014), Vibronic coherence in oxygenic photosynthesis. *Nat Chem* **6**, 706-711, doi: 10.1038/nchem.2005.

[2] V. Petropoulos *et al.* (2022), Unveiling Vibronic Coupling within the Q-bands of a Free-base Porphyrin, in *The International Conference on Ultrafast Phenomena (UP) 2022*, Washington, D.C.: Optica Publishing Group, 2022, p. **M1A.3**. doi: 10.1364/UP.2022.M1A.3.

[3] M. Maiuri, M. Garavelli, and G. Cerullo, (2020), Ultrafast Spectroscopy: State of the Art and Open Challenges. *J Am Chem Soc* **142**, 3–15, doi: 10.1021/jacs.9b10533.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

6 - Polarization-sensitive multiphoton microscopy

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Polarization-sensitive microscopy techniques using fluorescence and second harmonic generation are useful approaches to provide structural information on biological samples at the molecular level. Measuring the orientation of fluorescent labels directly reports on the underlying biological structure if they are rigidly linked to the biomolecules of interest. This is for instance the case for fluorescent labels attached to proteins via small linkers, but also for lipid probes which are sensitive to the surrounding lipids orientations in membranes. An ensemble of fluorescent molecules, when measured at the focus of a microscope by one- or two-photon fluorescence (TPF), is sensitive to the incident light polarization and also provokes an anisotropic polarized emission based on dipole orientations. Similarly in second harmonic generation (SHG), aligned repetitive subunits in collagen fibers create a strong sensitivity of the SHG excitation and emission to the orientation of these subunits. The advantage of TPF and SHG for imaging is the nonlinear nature of their excitation, improving axial resolution in 3D imaging.

The goal of our MUSIQ project was to overcome one of the important limits of all current polarization-sensitive TPF/SHG methods, namely that they probe only a 2D projection in the sample plane of the 3D molecular orientation information, and introduce biases to the resulting orientation readouts. This project applied a concept first developed to measure the 3D orientation of single molecules, based on the manipulation of light at the Fourier plane of a microscope, to ensemble measurements in scanning TPF/SHG microscopy[1]. First, we demonstrated the possibility to access 3D molecular orientation information in the focal volume of a nonlinear microscope. The scheme is based on the splitting of their emission into four polarization directions (4polar), two of which use an amplitude mask at the Fourier plane of the detection path. We showed that both mean 3D orientation of the molecules as well as their order (angular extent) can be determined by the 4polar method. This study included the important aspect of the effect of the excitation polarization on the measured signals[2], as well as influence of noise on the precision and accuracy of the retrieved parameters. Next, we addressed a modular two-photon microscope that we constructed, and considered the challenges of calibrating polarization-sensitive components in a scanning microscope. We performed 3D molecular orientation imaging of fluorescent orientational probes in lipid membranes, as well as for fluorescent molecules bound to F-actin in cells in 2D and 3D cell cultures[1]. Moreover, we showed preliminary results on a label-free measurement of the 3D orientation of molecular subunits of collagen using SHG 4polar imaging[1]. The prospective applications of this novel 4polar technique coupled with multi-photon ensemble measurements are to gain molecular information in 3D biological samples such as collagen structure in tissue and roles of actin for cells interacting with a 3D environment.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

References

[1] E. Munger (2023), Nonlinear imaging of molecular organization in 3D in biological media. **PhD thesis**, Aix Marseille University France, defended **April 13th 2023**.

[2] E. Munger, M. Sison and S. Brasselet (2023), Influence of the excitation polarization on single molecule 3D orientation imaging. *Opt. Communications* **541**, 129480 DOI: 10.1016/j.optcom.2023.129480

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

7- High-Resolution Wide-Field Coherent Anti-Stokes Raman Scattering Microscopy

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Within MUSIQ, we have been working on different nonlinear wide-field microscopy techniques[1]. The first technique is based on the combination of coherent anti-Stokes Raman scattering (CARS) microscopy[2] and random illumination (speckle) microscopy[3] (RIM). On the one hand, CARS provides the chemical sensitivity required to distinguish the various chemical elements in the sample under examination. On the other hand, the use of RIM makes it possible to increase spatial resolution and implement axial resolution at the same time. By using pump and Stokes wide field illumination with speckle fields we have implemented the first RIM-CARS microscope[4]. It has been tested on several samples, among which tissue biopsies and mixtures of plastics are the most relevant applications in biomedical and environmental fields. Thanks to the rapid data acquisition of this microscope (20Hz imaging) and the relatively fast data processing, RIM-CARS is a possible precursor to further wide-field microscopes for medical diagnostic and scientific applications. RIM-CARS video rate is particularly relevant for biopsy investigation as it allows the structure of the sample to be studied in real time and its sanity determined. This avoids the classical staining procedure typical of histopathology.

Being the first demonstrator of this kind, several improvements can be made on RIM-CARS, especially in terms of field of view (FOV) and signal-to-noise ratio (SNR). i.e. increasing the laser power, together with a large active surface spatial light modulator (SLM) would bring benefit to both the FOV and SNR.

The second investigated wide-field nonlinear technique takes the name of Fourier ptychography second harmonic generation microscopy (FP-SHG) and it is based on the work of Heuke et al. in Ref.[5]. Also in this case, two different techniques are combined. The first is Fourier ptychography[6] (FP), which is a linear computational technique that allows the spatial resolution of a sample to be increased from several large field of view, low-resolution images of the sample taken with different illumination angles. The second is second harmonic generation[7] (SHG), a nonlinear technique widely used to study collagen and other structures in which an inversion symmetry is absent. Computational studies on this FP-SHG technique yielded positive results and we then moved on to the experimental implementation and on to the study of a biopsy section (containing collagen). Despite the estimation of the experimental parameters, the computational reconstruction of the high-resolved image failed. This might be related to an incorrect assessment of the experimental angles of incidence. Further studies are thus necessary.

The natural extension of FP-SHG is in[5] FP-CARS, in which two colors are involved and the CARS signal is probed. Following this, it is possible to think about using three different wavelengths and experimentally explore the world of CARS Fourier tomography already theoretically presented by Heuke *et al*. in Ref.[8].

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

References

[1] E. Fantuzzi (2022), High-Resolution Wide-Field Coherent Anti-Stokes Raman Scattering Microscopy. **PhD thesis**, Aix Marseille University France, defended **Dec 8th 2022**;

[2] Andreas Zumbusch, Gary R. Holtom, and X. Sunney Xie (1999), Three-dimensional vibrational imaging by coherent anti-Stokes Raman scattering. *Physical Review Letters* **82**, 4142.

[3] Thomas Mangeat *et al*. (2021), Super-resolved live-cell imaging using random illumination microscopy. *Cell Reports Methods* **1** , 100009.

[4] Hervé Rigneault, Eric Fantuzzi, Sandro Heuke, Randy A. Bartels, Anne Sentenac, Dominykas Gudavičius (2023), Random illumination wide field coherent anti-Stokes Raman scattering microscopy (RIM-CARS), Proceedings Volume PC12392, Advanced Chemical Microscopy for Life Science and Translational Medicine 2023; https://doi.org/10.1117/12.2648081, Event: SPIE BiOS, 2023, San Francisco, California, United States (Conference Presentation).

[5] Sandro Heuke *et al*. (2019), Coherent anti-Stokes Raman Fourier ptychography. *Optics Express* **27**, 23497.

[6] Guoan Zheng, Roarke Horstmeyer et Changhuei Yang (2013), Wide-field, high resolution Fourier ptychographic microscopy. *Nature Photonics* **7**, 739-745.

[7] Riccardo Cicchi et al. (2012) From molecular structure to tissue architecture: collagen organization probed by SHG microscopy. *Journal of Biophotonics* **6**, 129-142.

[8] Sandro Heuke, Hervé Rigneault, and Anne Sentenac (2021) 3D-Coherent anti-Stokes Raman scattering Fourier ptychography tomography (CARS-FPT). *Optics Express* **29**, 4230-4239.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

8- Plasmonic antenna arrays for high-throughput single-molecule fluorescence detection

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A great variety of nanophotonic biosensor architectures based on plasmonic nanoantennas have already demonstrated their suitability for highly sensitive single-molecule fluorescence detection due to their capability to enhance and confine light in subwavelength volumes. While the different sensor architectures can vary strongly in their design, they essentially establish different trade-offs between signal enhancement, background reduction, throughput, and scalability in terms of fabrication cost and time [1–4]. In this project, we have demonstrated that the so-called antenna-in-box (AiB) architecture, consisting of a nanoantenna located within a nanoaperture, stands out due to its unique ability to simultaneously provide very high fluorescence enhancement together with background reduction [1]. Their relevance for biosensing applications was further extended by overcoming many standing limitations of conventional AiBs [5]. In particular, we established new fabrication processes, material combinations, and probing schemes that enable parallelized multicolor single-molecule detection for instance through aluminum-based hexagonally close-packed AiB (HCP-AiB) arrays.

HCP-AiB arrays hold the potential to provide insights into previously inaccessible and scarce biological dynamics as they allow to probe single-molecule events at physiologically relevant concentrations with a readout of > 1000 antennas simultaneously enabling an unpreceded high-throughput and thus the acquisition of robust statistics. Nevertheless, the HCP-AiB architecture comes with two yet untackled challenges, being the simplification of the complex fabrication process and their application to a wider range of relevant biosensing applications. These two challenges are not completely unrelated to each other as a simple and cost-effective fabrication process would allow the scientific community to easily adapt the novel architecture for their biosensing experiments so that solving the former challenge could also help overcoming the latter.

To simplify the current HCP-AiB fabrication process, three promising directions are worth exploring. The most straightforward would be the identification of a suitable high-resolution negative-tone resist suitable for lift-off processes. However, due to the axial exposure profile of negative-tone resists they are usually unsuitable for high-resolution lift-off processes. Inorganic negative-tone resists such as hydrogen silsesquioxane (HSQ) can provide the required resolution but rely on corrosive process chemicals that prohibit the use of many metals, including aluminium [6]. If a suitable negative-tone resist with a resolution of below 30 nm in lift-off processes could be identified, the number of required electron-beam lithography (EBL) steps could be reduced from three to one. A second promising approach to fabricate HCP-AiB arrays in a single step is an EBL process based on a positive-tone resist as a mask for reactive ion etching (RIE) [7]. This approach relies on the availability of chlorine-based

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etching gases during the RIE processes and a sufficiently etch-resistant mask. Finally, a direct pattern transfer through focused ion-beam (FIB) etching is another viable option. While conventional FIB does not provide sufficient throughput to fabricate large HCP-AiB arrays, emerging technologies like ion-beam lithography (IBL) could overcome this limitation [8].

HCP-AiBs arrays provide the most advantages in scenarios in which signals are weak, backgrounds are strong, and dynamics take place in the millisecond-to-second regime. The observation of binding kinetics such as receptor-ligand interactions could therefore be a biologically highly relevant class of interactions to explore using HCP-AiBs. In this regard, it is important to understand that the current temporal limitation is not intrinsic to HCP-AiBs but originates from the relatively slow camera-based readout of the HCP-AiB arrays [9]. Using HCP-AiBs optimized for subarray readout of recent sCMOS cameras could very well allow exploring interactions lasting only tens of microseconds. The fluorescence enhancement and high Purcell factors provided by the HCP-AiBs enable higher excitation and emission rates so that sufficient signal can be acquired on these short time scales.

The use of the current aluminum-based HCP-AiB design for real-world biosensing will reveal if further refinement of the design is required. Such modifications could include the use of ultrathin passivation layers such as alumina to minimize unspecific interactions between the nanostructures and biological specimens as well as planarization strategies, for instance through template stripping [6,10]. Passivation and planarization strategies have been explored as part of this project and could be included in the HCP-AiB architecture if required.

References

[1] Punj D., Mivelle M., Moparthi S. B., Van Zanten T. S., Rigneault H., Van Hulst N. F., García-Parajó, M. F., Wenger, J. (2013), A Plasmonic “antenna-in-Box” Platform for Enhanced Single-Molecule Analysis at Micromolar Concentrations. *Nat. Nanotechnol.* **8**, 512–516. https://doi.org/10.1038/nnano.2013.98.

[2] Flauraud V., Van Zanten T. S., Mivelle M., Manzo C., Garcia Parajo M. F., Brugger J (2015), Large-Scale Arrays of Bowtie Nanoaperture Antennas for Nanoscale Dynamics in Living Cell Membranes. *Nano Lett.* **15**, 4176–4182. https://doi.org/10.1021/acs.nanolett.5b01335.

[3] Levene H. J., Korlach J., Turner S. W., Foquet M., Craighead H. G., Webb W. W (2003), Zero-Mode Waveguides for Single-Molecule Analysis at High Concentrations. *Science* **299**, 682–686. https://doi.org/10.1126/science.1079700.

[4] Kinkhabwala A., Yu Z., Fan S., Avlasevich Y., Müllen K., Moerner W. E (2009), Large Single-Molecule Fluorescence Enhancements Produced by a Bowtie Nanoantenna. *Nat. Photonics* **3,** 654–657. https://doi.org/10.1038/nphoton.2009.187.

[5] Herkert E. K., Bermeo Alvaro D. R., Recchia M., Langbein W., Borri P.; Garcia-Parajo M. F. (2023), Hybrid Plasmonic Nanostructures for Enhanced Single-Molecule Detection Sensitivity. *ACS Nano* **17***,* 8453–8464. https://doi.org/10.1021/acsnano.3c00576.

[6] Flauraud V., Regmi R., Winkler P. M., Alexander D. T. L., Rigneault H., Van Hulst N. F., García-Parajo M. F., Wenger J., Brugger J (2017), In-Plane Plasmonic Antenna Arrays with Surface Nanogaps for Giant Fluorescence Enhancement. *Nano Lett.* **17**, 1703–1710. https://doi.org/10.1021/acs.nanolett.6b04978.

[7] Ansaryan S., Liu Y. C., Li X., Economou A. M., Eberhardt C. S., Jandus C., Altug H. (2023), High-Throughput Spatiotemporal Monitoring of Single-Cell Secretions via Plasmonic Microwell Arrays. *Nat. Biomed. Eng.* **7**, 943–958. https://doi.org/10.1038/s41551-023-01017-1.

[8] Hentschel M., Karst J., Giessen H (2020), Tailored Optical Functionality by Combining Electron-Beam and Focused Gold-Ion Beam Lithography for Solid and Inverse Coupled Plasmonic Nanostructures. *Adv. Opt. Mater.* **8,** 2000879. https://doi.org/10.1002/adom.202000879.

This project has received funding from the European Union’s Horizon 2020 research and innovation programme under grant agreement No 812992

[9] Winkler P. M., van Zanten T. S., Mayor S., Garcia-Parajo M. F. (2022), Multiplexed Plasmonic Nanoantennas for High Throughput Single Molecule Nanoscale Dynamics in Living Cells. *Preprint* https://doi.org/10.48550/arXiv.2201.12068.

[10] Kelly C. V., Wakefield D. L., Holowka D. A., Craighead H. G., Baird B. A (2014), Near-Field Fluorescence Cross-Correlation Spectroscopy on Planar Membranes. *ACS Nano* **8**, 7392–7404. https://doi.org/10.1021/nn502593k.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

9. Ultrasmall and ultrafast: Molecules, nanocavities and fs pulses

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Strong molecule-light coupling at room temperature is now a reality due to plasmonic field confinement. Especially the NanoParticle on Mirror (NPoM) platform is gaining recognition as a stable, easy-to-fabricate, and reliable alternative to the intricate nanofabrication processes traditionally explored for strong light-matter coupling in plasmonic cavities[1]. However, a major drawback of the NPoM platform is that the yield of strong-coupling is limited to less than 2%.

Within the scope of the MUSIQ project, numerous groundbreaking proof-of-principle experiments have been conducted. A most notable achievement is the first optical (fs pulses) control of strong coupling. In this experiment, we utilized fs pulses to effectively change the signature of strong coupling in plasmonic cavities. This work represents a significant advancement in the field. We observed the jump in coupling strength resulting from the bleaching of molecules with fs pulses.

These observations were possible due to a unique platform build: combining single particle scattering spectroscopy with SERS microscopy on the same spot. Thus, we were able to detect remarkably high Raman intensity from typically faint bands and track the molecular Raman response upon fs control of the SERS (enhanced or decreased) and the final bleach of the molecule[2]. This observation opens the door to conduct transient Raman spectroscopy on plasmonic NPoM cavities[3,4]. The successful utilization of fs pulses in enhancing very weak Raman bands not only advances our understanding but also provides a promising avenue for further investigations in this field by enabling transient Raman experiments on NPoM cavities.

In our proof-of-concept demonstration, we fine-tuned the resonances of plasmonic cavities by employing various solvents (from air to oil), thereby influencing the refractive index. Through the manipulation of the refractive index, we demonstrated the ability to tune the resonance of plasmonic cavities, subsequently influencing the strong coupling regime. We still intend to conduct experiments utilizing continuous solvent tuning, allowing us to observe the corresponding changes in coupling strength. This investigation will provide valuable insights into the dynamic behavior of plasmonic cavities and their coupling characteristics such as Rabi splitting.

In tuning the molecular and plasmonic resonances, the spectral phase of the nanoparticles plays an important role in the nature of the coupling. To date, there have been no reports on the phase of strongly coupled systems, indicating the complexity of this task. Indeed, observation of the absolute spectral phase of nano-objects is not trivial. Here, we have solved this by performing in-line holography of strongly coupled plasmonic structures, observing the double phase delay due to the two interacting resonances.

A prominent question within the nanoscience community revolves around the coherent interaction, the Rabi oscillations, and temporal decay dynamics of strongly coupled states[5]. And ultimately, which

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perishes first: the molecule or the nanocavity? To answer this question, we are setting up ultrafast experiments on a strongly coupled system (fabricated at ICFO) on a high peak power (repetition rate ~1kHz) interferometric microscope (with our collaborators at Poli Milano). These experiments employ the basics of interferometry, a technique previously employed in the study of phase, to unravel the questions surrounding the temporal decay dynamics.

References

[1] R. Chikkaraddy, B. de Nijs, F. Benz, S. J. Barrow, O. A. Scherman, E. Rosta, A. Demetriadou, P. Fox, O. Hess and J. J. Baumberg (2016), *Nature* **535**, 127–130.

[2] Saurabh Borkar, Jana Ockova, Matz Liebel and Niek F. van Hulst (2023), in preparation.

[3] F. Benz, M. K. Schmidt, A. Dreismann, R. Chikkaraddy, Y. Zhang, A. Demetriadou, C. Carnegie, H. Ohadi, B. de Nijs, R. Esteban, J. Aizpurua and J. J. Baumberg (2016), *Science* **354**, 726–729.

[4] R. Chikkaraddy, V. A. Turek, Q. Lin, J. Griffiths, B. Nijs and J. J. Baumberg (2021), *J. Raman Spectrosc.* **52**, 348–354.

[5] S. Yampolsky, D. A. Fishman, S. Dey, M. Banik, E. O. Potma and V. A. Apkarian (2014), *Nat. Photonics* **8**, 650–656.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

10 – Stimulated Raman scattering microscopy with electronic pre-resonance enhancement

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Our work in the MUSIQ project has shown that stimulated Raman scattering (SRS) microscopy with electronic pre-resonance (epr) enhancement is an attractive approach.[1-3] It allows the recording of microscopy images with high spatial resolution and contrast generated based on vibrational spectra of the sample molecules. The sensitivities that can be achieved are similar to those obtainable from fluorescence imaging albeit with much more specific spectral information.

The main obstacle preventing a broader application of epr-SRS microscopy is the lack of suitable laser excitation sources in the visible part of the optical spectrum. The realization of such sources therefore is a first major goal for the further development of epr-SRS microscopy. Suitable schemes for such systems can be based on the components already established in this project. To that end, we set up epr-SRS microscopy with visible excitation by using a commercial laser system with two optical parametric oscillators (OPO) providing two outputs with wavelengths tunable from 680 – 900 nm and 900 – 1350 nm, respectively (see also Section 15). By frequency doubling the second output to 640 nm, we obtained a pump beam in the visible spectral region that together with the other output serving as Stokes beam could be used for epr-SRS microscopy of molecules with absorptions ranging from 500 – 580 nm. The tunability of the other laser output allows the recording of vibrational spectra over the whole relevant energy scale. Especially the possibility to monitor both the fingerprint region, where most of the resonance enhanced bands are found will be of great interest.[4]

To make investigations of a broader range of sample molecules possible, it is desirable to also frequency double the first OPO output. In this manner, one could then investigate a broad range of chromophores. This opens the possibility to study the spectroscopic properties of many fluorescent compounds that would be impossible with classical spontaneous Raman due to the overwhelming fluorescence background that does not play a role in SRS microscopy.

One should note here that one of the major selling points of using non-linear vibrational microscopy techniques such as SRS microscopy has always been the possibility to examine unlabelled samples. This advantage over fluorescence microscopy had to be sacrificed when using epr-SRS microscopy with laser excitation available today. Yet, the extension to visible laser excitation outlined above will make it possible to also investigate molecules with electronic absorptions in between 350 and 500 nm, which are abundant in unlabelled biological and material scientific samples.

The second major goal in the further development of epr-SRS microscopy therefore is the demonstration of its usefulness in real world applications. Since the resonance enhancement only requires electronic absorption, but not fluorescence of the sample molecules,[5] a broad range of applications can be envisioned ranging from biomedical applications such e.g. as the monitoring of

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

melanin distribution to investigations of material scientific samples as e.g. the optical microscopy of organic photovoltaic devices.

Finally a third interesting goal for the further development of epr-SRS microscopy is the achievement of even higher detections sensitivities. To date, the detection limit of epr-SRS microscopy of dissolved sample molecules is on the order of 1µM. Taking the size of the excitation volume into account, this corresponds to roughly 1000 detected molecules. Also in this respect, the proposed blue shifting of the excitation promises to lead to significant improvements due to the scaling of the Raman cross sections with the fourth power of the excitation frequency.

References

[1] Wei L., and Min W. (2018), Electronic Preresonance Stimulated Raman Scattering Microscopy, *J. Phys. Chem. Lett*. **9**, 4294-4301.

[2] Bi Y. L., Yang C., Chen Y. G., Yan S., Yang G., Wu Y. Z., Zhang G. P. and Wang, P. (2018), Near-resonance enhanced label-free stimulated Raman scattering microscopy with spatial resolution near 130 nm. *Light Sci. Appl.* **7**, 81.

[3] Fimpel P., Choorakuttil A. J. X., Pruccoli A., Ebner L., Tanaka S., Ozeki Y., Winterhalder M. J. and Zumbusch, A. (2020). Double modulation SRS and SREF microscopy: signal contributions under pre-resonance conditions, *Phys Chem Chem Phys* **22**, 21421-21427.

[4] Choorakuttil A.J.X., Pruccoli, A., Winterhalder M.J., Zirak,P., Gudavičius D., Martynaitis G., Petrulionis D., Samsonas D, Kontenis L, Zumbusch A. (2023), Electronically preresonant stimulated Raman scattering microscopy in the visible; submitted for publication.

[5] Pruccoli A., Kocademir M., Winterhalder M.J., Zumbusch A. (2023), Electronically preresonant stimulated Raman scattering microscopy of weakly fluorescing chromophores. *J. Phys. Chem. B* **127**, 6029–6037.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

11- Quantitative analysis of hyperspectral stimulated Raman scattering microscopy

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Within the MUSIQ project, we performed quantitative comparisons between different multivariate spectral unmixing methods for the analysis of hyperspectral stimulated Raman scattering (SRS) microscopy datasets of various model systems. We showed that for the selection of essential components as the first important step in the application of these methods, using first order autocorrelation yields good results.[1] Of the methods taken into account, principal component analysis turned out to be of limited use for the construction of abundance maps since the derived spectra do not allow a chemical interpretation. This is not the case for vertex component analysis (VCA), N-FINDR, and multivariate curve resolution (MCR) which were also tested. The main difference which we found for these methods is the susceptibility of N-FINDR to noise in the hyperspectral imaging data. This leads to a slightly inferior performance of N-FINDR compared to the other two methods. Both VCA and MCR performed well. In comparison, however, MCR stands out as it can factorize the input data into abundance maps which are suitable for finding relative concentrations of different chemical compounds. In experiments on test samples, we find that already for limited spectral range data recorded from simple model systems as in our case, multivariate analysis delivers results of superior quality, revealing more details in the images.

The hyperspectral data sets used for the most of our studies were limited to the CH stretch vibration region. In general for hyperspectral SRS imaging data, the analysis of the fingerprint region is not yet as common as for spontaneous Raman data. Since the fingerprint region contains much more specific spectral information, it will be interesting to extend the type of analysis described above also to the spectral region from 800 to 1750 cm^{-1}. While our first data from simple model systems imaged in this spectral region yielded results comparable to those obtained from the CH stretch region with VCA and MCR clearly performing best, it can be expected that the full potential of multivariate approaches will only be realized in more complex samples in which many different chemical species with overlapping vibrational spectra are present. In this respect, the possibility to record hyperspectral imaging date including the full fingerprint region from complex biological and material scientific samples by using state-of-the-art SRS microscopy systems is a great promise for the future.

Going forward, we worked on developing SRS microscopy under electronically pre-resonant (epr) excitation conditions. We could show that this leads to a significantly enhanced detection sensitivity down to 1 µM concentrations.[2-3] With respect to the spectral analysis of SRS data, epr-SRS is of great interest since the enhancement will not affect all vibrational bands but only those that couple strongly to the electronic transitions. This will lead to an important simplification of the vibrational spectra especially of complex samples such as biological cells and tissues. One can anticipate that this will also allow facilitate the spectral unmixing of such samples using the approaches explored in this project.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

References

[1] Ragupathy I.C., Schweikhard V., Zumbusch A. (2021), Multivariate analysis of hyperspectral stimulated Raman scattering microscopy images. *J. Raman. Spec*. **52**, 1630-1642.

[2] Fimpel P., Choorakuttil A. J. X., Pruccoli A., Ebner L., Tanaka S., Ozeki Y., Winterhalder M. J. and Zumbusch A. (2020), Double modulation SRS and SREF microscopy: signal contributions under pre-resonance conditions. *Phys Chem Chem Phys* **22**, 21421-21427.

[3] Pruccoli A., Kocademir M., Winterhalder M.J., Zumbusch A. (2023), Electronically preresonant stimulated Raman scattering microscopy of weakly fluorescing chromophores. *J. Phys. Chem*. *B* **127**, 6029–6037.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

12 – 2D mid-IR/vibrational micro-spectroscopy of organic molecules

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Ultrafast multidimensional mid-infrared (mid-IR) spectroscopy is an advanced spectroscopy technique that allows probing of characteristic vibrational modes in the 'fingerprint' region (3-20 µm) of biomolecules with unprecedented spectral and temporal resolution[1]. Contrary to conventional pump-probe spectroscopy, the precise energy resolution of the excitation process is preserved to probe energetic couplings and coherences, which provides a major part of experimental evidence of energy migration pathways in complex biological and chemical systems. In a complementary approach, the exploitation of plasmonics in the mid-IR based on heavily doped semiconductors such as germanium (Ge) is a crucial addition to study biomolecules through the field enhancement provided by plasmonic nanostructures[2,3].

The combination of ultrafast two-dimensional (2D) mid-IR (2DIR) spectroscopy techniques and plasmonics in the mid-IR held the enticing promise to probe dynamics in very diluted amounts of the biological systems of interest towards the single molecule limit and, e.g., track chemical reactions, protein folding mechanisms, or interactions between molecules. In parallel, alternative promising plasmonic materials are being introduced to provide large field enhancement. In this context, a large free electron nonlinearity is theoretically predicted in heavily doped indium phosphite[4], which remains to be confirmed experimentally with antenna geometries at various doping levels. Furthermore, a complementary extension to 2DIR is the so-called 2D electronic-vibrational spectroscopy, which tracks the dynamics of couplings between electronic and vibrational states through visible excitation and mid-IR detection. This is particularly important in light-harvesting complexes, where energy transfer processes can be tracked upon absorption of visible radiation and their influence on vibrational modes and structural changes can be quantified. Another promising new research direction is the coherent coupling of single biomolecules to plasmonic nanostructures or to excitons and other quasiparticles in low dimensional condensed matter systems. Depending on the nature of coupling (weak or strong), hybridization effects can play a crucial role for the ultrafast response[5]. These dynamics can be fully tracked experimentally with high spectral and temporal resolution through 2D electronic spectroscopy (2DES). Such hybrid systems show high potential to be utilized as optical photoswitches through selective optical excitation or preparation of the molecule. If molecules show circular dichroism, the handedness of light polarization can alter the molecule optical response and thus the nature of coupling transiently. Molecule isomerization and its reverse reaction, for example triggered through UV or visible radiation in spiropyran, promises similar effects[6]. This would allow to selectively switch between different (polariton) coupled states and suppress or enhance them. Similarly, peculiar valley polarization states in transition metal dichalcogenides could potentially be enhanced or minimized through the coupling with dichroic molecules. With advancements in all these fields, exciting results in real-time tracking of energy migration pathways, hybrid (polariton) states, protein conformation, chemical reactions, and similar dynamics are to be expected. The outcome will have a strong impact on fundamental research that targets several scientific fields including biology, chemistry, physics, and medicine.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

References

[1] Deckert Thomas, Allerbeck Jonas, Kurihara Takayuki and Brida Daniele (2022), Ultrafast Multidimensional Spectroscopy with Field Resolution and Noncollinear Geometry at Mid-Infrared Frequencies, *New Journal of Physics* **24**, 023005.

[2] J. Frigerio, A. Ballabio, G. Isella, E. Sakat, G. Pellegrini, P. Biagioni, M. Bollani, E. Napolitani, C. Manganelli, M. Virgilio, A. Grupp, M. P. Fischer, D. Brida, K. Gallacher, D. J. Paul, L. Baldassarre, P. Calvani, V. Giliberti, A. Nucara, and M. Ortolani (2016), Tunability of the dielectric function of heavily doped germanium thin films for mid-infrared plasmonics. *Phys. Rev. B* **94**, 85202.

[3] M. P. Fischer, C. Schmidt, E. Sakat, J. Stock, A. Samarelli, J. Frigerio, M. Ortolani, D. J. Paul, G. Isella, A. Leitenstorfer, P. Biagioni, and D. Brida (2016), Optical Activation of Germanium Plasmonic Antennas in the Mid-Infrared. *Phys. Rev. Lett*. **117**, 47401.

[4] F. de Luca, M. Ortolani, and C. Ciracì (2021), Free electron nonlinearities in heavily doped semiconductors plasmonics. *Phys. Rev. B* **103**, 115305.

[5] J. Krumland and C. Cocchi (2021), Conditions for electronic hybridization between transition-metal dichalcogenide monolayers and physisorbed carbon-conjugated molecules," *Electron. Struct.* **3**, 44003.

[6] J. Kuttruff, M. Romanelli, E. Pedrueza-Villalmanzo, J. Allerbeck, J. Fregoni, V. Saavedra-Becerril, J. Andréasson, D. Brida, A. Dmitriev, S. Corni, and N. Maccaferri (2023), Sub-picosecond collapse of molecular polaritons to pure molecular transition in plasmonic photoswitch-nanoantennas. *Nat Commun* **14**, 3875.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

13 – Calculating the response functions for multidimensional electronic spectroscopy

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Coherent multidimensional spectroscopy represents a powerful tool for investigating the interplay between electronic and vibrational degrees of freedom in ultrafast photophysical and photochemical processes[1,2]. In spite of the complexity of the investigated systems, the observed spectra are typically interpreted in terms of model Hamiltonians, possibly derived from microscopic approaches.

A large part of our MUSIQ research project has focused on the derivation of analytical expressions for the response function of widely used models, accounting for the electronic-state dependent displacement[3], frequency shift and mixing (Duschinsky rotations) of the vibrational modes[4]. The derivations are based on a quantum optical formalism, and on the fundamental observation that the vibrational states are – within such models and for each pathway – always given by coherent or squeezed coherent states. These can be described by a limited number of complex numbers, whose dependence on the Hamiltonian parameters and on the waiting times is explicitly given in our analytical solutions.

This approach is susceptible of further extensions and generalizations to other models, for example to systems where the Franck-Condon principle does not apply (Herzberg-Teller coupling), or where anharmonicity plays a significant role. Besides, the explicit derivation of the vibrational states opens the possibility of relating their quantum properties to quantities that can be experimentally accessed in multidimensional spectroscopy. In particular, the linear superposition of different coherent states corresponding to different pathways can evolve into highly nonclassical states, with properties resembling those of the bosonic Schrödinger cats. More in general, the knowledge of the vibrational states allows to address questions of general interest, such as the role played by nonclassical states in different photophysical or photochemical processes. Finally, the use of a quantum optical formalism can be used to establish a connection with theoretical and experimental tools that have been developed in that context, relating, for example, specific response functions that are relevant in coherent multidimensional spectroscopy to phase space descriptions of the bosonic modes, such as those obtained through coherent state tomography.

References

[1] P. Hamm and M. T. Zanni (2011), Concepts and Methods of 2D Infrared Spectroscopy, Cambridge University Press.
[2] M. Maiuri, M. Garavelli, and G. Cerullo (2020), *J. Am. Chem. Soc*. **142**, 3.
[3] F. E. Quintela Rodriguez and F. Troiani (2022), *J. Chem. Phys*. **157**, 034107.
[4] F. E. Quintela Rodriguez and F. Troiani (2023), *preprint*, arXiv:2306.08886.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

14 – Combine optical and mass spectrometry imaging techniques to characterise biological components at sub-cellular resolution

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This MUSIQ project addressed the challenging ambition to combine chemically specific stimulated Raman scattering (SRS) microscopy with mass spectrometry approaches, specifically matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI MSI). The view was to merge the advantages in spatial resolution brought by SRS with the exquisite chemical/molecular specificity of mass spectrometry.

The methodology for data acquisition of a biological sample using MALDI MSI and SRS microscopy was established. It was found that the conductive elements necessary for successful ionization in MALDI were a major hindrance to SRS microscopy. To circumvent this issue, non-conductive glass slides were used for SRS imaging, which were then modified with conductive DIUTHAME membranes[1], replacing the role of conductive slides and matrix.

It is worth mentioning that DIUTHAME dictated that SRS had to be conducted prior to MALDI MSI. Desorption electrospray ionization[2] (DESI) MSI should be considered in studies where MSI data acquisition is required as the initial step because it does not warrant the use of conductive substrates.

Apart from animal tissues, DIUTHAME was successfully applied in surface-assisted laser desorption ionization (SALDI) MSI of cells and complex *in vitro* models. The combination of MSI and SRS was performed using SRS to generate artificial H&E histological images, which were spatially overlapped with hyperspectral MSI data. The next step in the SRS-to-MSI studies should be an establishment of data analytical pipeline, which would exploit the information from two hyperspectral datasets, that were proven to improve the machine learning-based techniques for tissue segmentation, as shown in other Raman-to-MALDI work.[3]

Bridging of the two technologies in tissue analyses has the potential to significantly reduce the time between tissue collection and data interpretation by eliminating the necessity of using multiple tissue sections to carry out individual analyses. Moreover, with the development of MALDI-2 instruments, which bring significant improvements to system sensitivity and spatial resolution,[4] the use of a single sample has become necessary to reduce spatial mismatches between individual tissue sections.

Finally, although highly specific fluorescence experiments after SALDI MSI were not performed, these experiments are expected to be feasible using epifluorescence confocal systems, which would avoid interference with the conductive DIUTHAME membrane. This 3-in-1 sample analysis has great potential in studies of liver diseases, which are often associated with lipid dysregulation.[5] The distribution and concentration of lipids and drugs can be quantified using[6] SRS, whereas SALDI MSI can shed light on lipid species composition with high specificity.[7] Moreover, fluorescence microscopy can be used to detect the proteins and nucleic acid sequences of interest with high epitope

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specificity.[8,9] Such a direct combinatory approach would have a positive impact on correlative studies, which could further speed up the decision-making process during drug development.

Irregularities in the hyperspectral SRS data were found during laser tuning, which affected the machine learning segmentation of cells in a 2D culture using a Leica SP8 SRS microscope with a wavelength-tuning-based picosecond laser.[10] The tuning effect was partially reduced by using water as an internal standard in the CH Raman region. However, improved internal standards should be explored in future analyses to improve correction efficiency. Such standards should be bio-orthogonal, yet chemically stable, and Raman-active. Alternatively, spectral focusing-based SRS systems[11] should be considered for cell classification analyses between samples.

References

[1] Naito Y., Kotani M. and Ohmura T (2018), A novel laser desorption/ionization method using through hole porous alumina membranes. *Rapid Communications in Mass Spectrometry* **32**, 1851-1858. https://doi.org:https://doi.org/10.1002/rcm.8252

[2] Towers M. W., Karancsi T., Jones E. A., Pringle S. D. and Claude E (2018), Optimised Desorption Electrospray Ionisation Mass Spectrometry Imaging (DESI-MSI) for the Analysis of Proteins/Peptides Directly from Tissue Sections on a Travelling Wave Ion Mobility Q-ToF. *J Am Soc Mass Spectrom* **29**, 2456-2466. https://doi.org:10.1007/s13361-018-2049-0

[3] Ryabchykov O., Popp J. and Bocklitz T (2018). Fusion of MALDI Spectrometric Imaging and Raman Spectroscopic Data for the Analysis of Biological Samples. *Frontiers in Chemistry* **6**. https://doi.org:10.3389/fchem.2018.00257

[4] Barré F. P. Y. *et al* (2019), Enhanced Sensitivity Using MALDI Imaging Coupled with Laser Postionization (MALDI-2) for Pharmaceutical Research. *Analytical Chemistry* **91**, 10840-10848. https://doi.org:10.1021/acs.analchem.9b02495

[5] Deprince A., Haas J. T. and Staels B (2020), Dysregulated lipid metabolism links NAFLD to cardiovascular disease. *Molecular Metabolism* **42**, 101092. https://doi.org:https://doi.org/10.1016/j.molmet.2020.101092

[6] Cao C., Zhou D., Chen T., Streets A. M. and Huang Y (2016), Label-Free Digital Quantification of Lipid Droplets in Single Cells by Stimulated Raman Microscopy on a Microfluidic Platform. *Analytical Chemistry* **88**, 4931-4939. https://doi.org:10.1021/acs.analchem.6b00862

[7] Müller W. H. *et al.* (2021), Dual-polarity SALDI FT-ICR MS imaging and Kendrick mass defect data filtering for lipid analysis. *Analytical and Bioanalytical Chemistry* **413**, 2821-2830. https://doi.org:10.1007/s00216-020-03020-w

[8] Zhu H. and Hamachi I (2020), Fluorescence imaging of drug target proteins using chemical probes. *Journal of Pharmaceutical Analysis* **10**, 426-433. https://doi.org:https://doi.org/10.1016/j.jpha.2020.05.013

[9] Sato H., Das S., Singer R. H. and Vera M (2020), Imaging of DNA and RNA in Living Eukaryotic Cells to Reveal Spatiotemporal Dynamics of Gene Expression. *Annual Review of Biochemistry* **89**, 159-187. https://doi.org:10.1146/annurev-biochem-011520-104955

[10] Wei L. *et al.* (2014), Live-cell imaging of alkyne-tagged small biomolecules by stimulated Raman scattering. *Nature Methods* **11**, 410-412. https://doi.org:10.1038/nmeth.2878

[11] Gagnon J. R. *et al.* (2023), Spectral focusing-based stimulated Raman scattering microscopy using compact glass blocks for adjustable dispersion. *Biomedical Optics Express* **14**, 2510. https://doi.org:10.1364/boe.486753

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

15 – Dual frequency and multi-foci coherent Raman scattering microscopy

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Since the first practical application of coherent anti-Stokes Raman scattering (CARS) microscopy[1] in the 1980's, most research and instrumentation was using a point scanning approach where a single pump-Stokes beam pair is overlapped and scans the sample in a raster-scan type pattern to create an image, similar to other laser scanning microscopes. For such an approach, a typical laser source set-up consists of a Ti:Sapphire or Yb oscillator pumping a tuneable wavelength optical parametric oscillator (OPO) with pulse energies in the nJ range and repetition rates in the 100 MHz range. Such devices generate a wavelength-tuneable signal and idler pair and one of these beams from the OPO is used for a typical CARS instrument. With the recent development of dual-OPO device (Light Conversion CRONUS 2P) it is possible to generate two independently tuneable wavelengths, and together with the fundamental oscillator wavelength, address three separate vibrational regions at the same time. To demonstrate the idea, in this MUSIQ project experiments were performed by imaging cells, separating deuterated lipids in the cell silent region and non-deuterated lipids in the C-H stretch region simultaneously, without any motion artefacts[2].

CARS microscopy has an intrinsic non-resonant background from electronic transitions and higher frequency vibrational resonances. To avoid this background, one can use stimulated Raman scattering (SRS) which started to attract attention in the late[3,4] 2000's. It measures the homodyne interference of the coherent Raman scattering (CRS) and one of the exciting pulses, being sensitive to the one of the two quadratures of the complex CRS. This quadrature is proportional to the imaginary part of the third-order susceptibility, which is only present in a resonant response. CARS instead measures the absolute square of the CRS field, including its real part which contains the non-resonant background. The presence of the exitation beam in the detected intensity requires isolation of the CRS via a modulation scheme, and a shot-noise limited laser system is needed to obtain a similar sensitivity as in CARS. During a MUSIQ secondment within this project, it was determined that the CRONUS 2P is shot-noise limited[5] at 2.5 MHz, and imaging with this system of a quality comparable with the more complex Ti:Sapphire and OPO based system presently used was shown.

To increase the speed in single-beam raster-scanning a short pixel dwell-time is used, and to get sufficient signal the pulse energy need to be increased which can lead to sample damage since all the energy is concentrated into a single focal volume at a given time. Furthermore, the CRS process is saturating at intensities around 10^{13} W/cm^2. To avoid these issue, a wide-field approach can be used where energy is distributed across a certain area on the sample, but this method lacks sectioning capability and due to the coherent character of CRS imaging is affected by unwanted phase contrast. To retain the sectioning and avoid the phase contrast, a multifocus technique can be used, as in spinning disk confocal microscopy. Instead of a single focal point, a lenslet array is used to generate

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multiple focal points. To use a large number of foci (100-1000), the available pulse energy from an OPO is insufficient to give each focus sufficient pulse energy for efficient CRS generation. For more pulse energy, an amplified oscillator can be used for a fixed wavelength and an optical parametric amplifier (OPA) for tuneable wavelength. Within the project, we have developed such an imaging platform [6], showing its reduced damage at a given imaging speed and the potential for faster imaging. With the growing variety and accessibility to OPAs we expect that the future will bring more interest to multi-foci and other wide-field approaches for non-linear microscopy.

References

[1] M. D. Duncan, J. Reintjes, and T. J. Manuccia (1982), Scanning coherent anti-Stokes Raman microscope. *Opt. Lett*. **7**, 350, doi: 10.1364/ol.7.000350.

[3] D. Gudavicius *et al* (2023), Dual CARS microscopy across the vibrational spectrum enabled by single pump dual optical parametric oscillators and spectral focusing. *Submitted*

[2] E. Ploetz, S. Laimgruber, S. Berner, W. Zinth, and P. Gilch (2007), Femtosecond stimulated Raman microscopy. *Appl. Phys. B Lasers Opt*. **87**, 389–393, doi: 10.1007/s00340-007-2630-x.

[4] Freudiger C. W., Min W., Saar B. G., Lu S., Holtom G. R., He C., Tsai J. C., Kang J. X., Xie X. S. (2008), *Science* **322**, 1857-1861. DOI:10.1126/science.1165758.

[5] D. Gudavicius et al (2023), Spectral focusing coherent Raman scattering microscopy - comparison of two laser sources. *In preparation*

[6] D. Gudavicius et al (2023), Thousand foci coherent anti-Stokes Raman microscope. *In preparation*

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 812992

Acknowledgements

This Roadmap shocases the research vision and ambition of the European Training Network MUSIQ, funded by the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 812992.