Surface-Enhanced Raman Scattering Holography

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ABSTRACT: Nanometric probes based on surface-enhanced Raman-scattering (SERS) are promising candidates for all-optical environmental, biological and technological sensing applications with intrinsic quantitative molecular specificity. However, the delicate trade-off between particle-size, stability and brightness of effective SERS probes has so far hampered their wide application in SERS-imaging methodologies. In this article, we introduce holographic Raman microscopy, which allows single-shot 3D single particle localization. We validate our approach by simultaneously performing Fourier transform Raman spectroscopy of individual SERS nanoparticles, and Raman holography, using shearing interferometry to extract both the phase and the amplitude of wide-field Raman images, and ultimately localise and track single SERS nanoparticles inside living cells in three dimensions. Our results represent the first step towards multiplexed single-shot 3D concentration mapping in many different scenarios, including live cell and tissue interrogation and complex anti-counterfeiting applications.

During the last 40 years SERS spectroscopy[1–4] has emerged as a fast and reliable ultrasensitive technique for the confident and precise identification of molecular systems in a variety of complex samples[4–8]. Notwithstanding, to date, the major drawback is the low SERS efficiency and the resulting long acquisition times. State-of-the-art Raman spectrometers acquire spectra of complex plasmonic nanostructures within 100 ms. While this acquisition times may be more than enough for applications requiring single spectra acquisitions, such as the detection and/or quantification of biomolecules[9], toxic pollutants[10], explosives[11], or even microorganism[12] and cells[13], it completely hinders its applicability to other purposes, such as chemical imaging, where its prominent spatial resolution can excel. Unlike wide-field fluorescence microscopy, SERS imaging is still predominantly performed using scanning confocal microscopes. Thus, contrary to fluorescence where a snapshot image can be acquired in milliseconds to seconds, SERS imaging is registered serially point-by-point, increasing the acquisition times to hours[14,15]. In fact, this procedure not only restricts the area to be mapped, preventing 3D tomographic images, but also dramatically increases the damage of the mapped surface due to an excessive exposure to the excitation light. These lack of minimally phototoxic and rapid imaging modalities has prevented both potential applicability of SERS to the 3D study of living cells at the sub-cellular level, and technological applications, such as anti-counterfeiting.

Shortening of acquisition times requires the engineering of ultrabright SERS particles, together with alternative imaging spectroscopies for more effective data acquisition. In this article, we combine novel concepts in both directions. On the SERS particle side, plasmonic nanoparticle superclusters, obtained from small nanoparticle building blocks[16], are an ideal particle-choice to generate very strong electric field in a restricted cluster size. The supercluster approach allows miniaturising the probe, thus increasing the spatial resolution, while simultaneously reducing negative impacts on cell viability[17]. On the imaging side, digital holography is an ideal candidate not only for volumetric imaging, but especially for the tracking of hundreds of individual particles in large 3D volumes which can be obtained from a single image[18–20]. However, holographic techniques crucially rely on coherent light as they require interference between a so-called signal and a reference field to recover the important phase information[21,22]. Thus, for spontaneous Raman scattering, an incoherent signal, holography seems impossible at first glance. Indeed, it is highly nontrivial to generate a suitable reference, and holographic imaging of incoherent radiation has, therefore, remained a curiosity. One way to nevertheless obtain the desired phase information is to rely on self-interference phenomena, which are accessible via shearing interferometry, and which have recently successfully been applied in 3D imaging of fluorescent emitters down to the single molecule limit[23].

Here, we show that holographic imaging of spontaneous Raman signals is possible. We simultaneously record the phase and the amplitude of wide-field images of multiple SERS nanoparticles, alongside their respective Raman spectra by coupling a Michelson interferometer with a shearing interferometer-based holographic microscope. Moreover, our work enables digital image propagation for 3D localization of multiple SERS nanoparticles, positioned at different z-positions, from a single image. This approach validates our proof-of-concept demonstrations and enables spectral multiplexing and Raman-band specific image decomposition.

For the preparation of suitable plasmonic material in terms of size SERS cross-section and intensity reproducibility, we synthetized highly homogeneous gold nanoparticles of 16 nm (Figure S1a), encoded with a variety of different SERS active molecules (Figure S2) and assembled them into superclusters of around 100 nm (Figures 1b and S1a, b). These superclusters are characterized by a localized surface plasmon resonance (LSPR) with a maximum at 792 nm (Figure S1d) and enhancement factor of 2 x 10^6 as compared with the initial particles (Supplementary Information S2). These bright and robust superclusters of nanoparticles with different SERS codes are an ideal system for fast multiplexed imaging, featuring reproducibility, easy preparation, colloidal stability and straightforward surface functionalization.

A microscope extended by a Raman shearing interferometer images the samples and allows accessing the complex electric field of widefield images of SERS emitters as well as their Raman spectra (Figure 1c). In brief, we focus a 785 nm laser into the microscope's back-focal-plane thus widefield illuminating the sample consisting of the previously mentioned nanoparticles doped with mercaptobenzoic acid. The red-shifted Raman signal is collected by the objective lens and separated from residual Rayleigh scattering with an 808 nm longpass filter before being imaged onto a 2D sCMOS-camera. A Michelson interferometer equipped with a linear-actuator , placed between the microscope objective and the imaging-lens, serves as a Fourier-transform spectrometer that directly accesses the frequency content of the radiation emitted from the particles (Supplementary Information S3). To measure the phase and the amplitude of the widefield images we, furthermore, employ a 2D 0-π transmission phase-grating (Supplementary Information S1) that is relay imaged onto the camera[24,25]. Slightly offsetting the grating with respect to the intermediate image plane allows directly determining the images' phase-gradients as we will discuss in detail later.

Fourier-transform SERS imaging

Figure 2a shows a representative SERS particle-image where the individual point-spread-functions (PSFs) are reminiscent of sub-diffraction-limited emitters with the characteristic checkerboard pattern due to the 0-π phase-grating induced self-interference[23]. The pronounced amplitude-heterogeneity between individual particles is most likely caused by the strong SERS-signal dependence on the precise positioning of individual molecules in the plasmonic hotspots of the metallic nanoparticles[26,27]. To verify that the observed signal is indeed Raman scattering and not due to particle luminescence we record image-stacks while varying the length of one arm in the Michelson interferometer (Figure 2a). The delay-dependent integrated signal-intensity of most emission-sites exhibits the expected interferometric fringes with pronounced beating patterns reminiscent of spectral signatures with multiple frequency components (Figure 2b). Overall, the emission-intensity of most of the particles is extremely stable, and often constant over tens of minutes to hours, without signs of blinking or photobleaching. However, some particles show dramatic emission instabilities, reminiscent of single-molecule behaviour, which indicates that even the medium intensity SERS-emission might originate from individual molecules. To obtain single particle spectra we perform discrete Fourier transformations of the respective time-delay traces (Figure 2c). These spectra show good quantitative agreement with an ensemble Raman-spectrum measured with a, conventional, Czerny–Turner spectrometer. We, furthermore, note that essentially the full emission-intensity is contained within the Raman bands with negligible presence of broadband components, such as fluorescence, that might be present for poorly-coupled, and hence unquenched, molecules.

After verifying the SERS-nature of the signal, we will now attempt spectral decomposition of the single-color image into individual spectral bands. Figure 3a shows the spectral content of the entire image-stack, obtained analogous to Figure 2c but by summing the Fourier transformation of all camera-pixels. As expected, the particles' Raman spectrum is visible on top of a residual background caused by experimental noise such as stochastic emission-fluctuations and shot-noise. Based on the spectrum, we select three spectral bands at 520, 1075 and 2674 cm^{-1}, respectively. The former two bands, which we formally assign to being due to CH-bending and ring-breathing modes of the adsorbed molecules, serve as references for weak and strong Raman signals and the latter as an estimate for background-induced signal. At each image pixel we Fourier filter the spectrum with a 17.7 cm^{-1} hard-aperture bandpass centred at the wavenumbers of interest, then extract the amplitude of the inverse Fourier transformation to yield the multiplexed images (Figure 3b). As all SERS-particles are doped with the same molecules, the decomposed images qualitatively agree with the spectrally integrated version (Figure 2a), with some intensity differences between spectral bands. Surprisingly, the 2674 cm^{-1} image shows the presence of all particles, albeit at a drastically reduced intensity, even though no Raman-signal is present in the averaged spectrum (Figure 3a,b).

To rationalise this observation, we examine the phase term of the inverse Fourier transformation (Figure 3c). Here, only the spectrally observable bands at 520 and 1075 cm^{-1} show a distinct Raman-phase signal, with minor deviations from zero phase for all particles, which is due to a minor misalignment of the Michelson interferometer, resulting in a pathlength differences of ~100 nm across the image. The absence of a well-defined phase signal in the 2647 cm^{-1} image suggest that the amplitude observed is merely due to the increased total photon number detected at the locations of individual emitters. Here, shot-noise results in a spectrally uniform noise-increase that reflects itself in an apparent signal in the amplitude-term of the complex signal, which is, by definition, positive:

$A = \sqrt{x^2 + y^2}$. The Raman-phase-term, however, fluctuates freely and no signal is observed which serves as a robust guide for discriminating Raman emission in a specific wavenumber range from shot-noise induced signals, due to Raman signals being present in a different part of the spectrum.

Following the spectral analysis of the SERS images, we now turn our attention towards the shearing-interferometer that enables phase-measurements of the image. Importantly, we would like to stress that the Raman-phase discussed until now is distinctly different from the phase of the image which we are going to discuss in the following paragraphs. The former is solely determined by the time-delay in the Michelson interferometer; the latter contains information about the propagating electric field of the sample or, in other words, the image formation process. Deliberately different image-colour schemes are therefore used for the representation of the respective data.

Hologram processing and phase extraction

As mentioned previously, the slightly offset 2D 0-π phase grating (Figure 1c) allows indirect determination of the phase of an image in a single shot[25]. As the wavefront passes the grating, four identical image copies are generated as the first diffraction orders (+1/+1, +1/-1, -1/+1, -1/-1). These copies exhibit distinctly different k-vectors. A relay imaging-system recombines the diffraction orders on a camera where self-interference takes place[24,28]. As the grating is offset with respect to the relay-imaged conjugate image-plane, the spatial overlap of the images is a function of the grating position (Figure 4a). If we consider a slightly out-of-focus emission site with spherical phase, it becomes apparent that the grating induced image-displacement directly translates into a phase-gradient measurement (Figure 4b). If the image-copies are not displaced, identical parts of the image interfere, and all phase information is lost. However, as soon as a shift is introduced, different parts start interfering. The resulting phase-difference resembles a linear function with non-zero slope, which is equivalent to the derivative of the spherical phase $\varphi(x, y) = (x^2 + y^2)$ we assumed previously. As the grating-generates several diffraction orders displaced in both x- and y-dimensions, simultaneous gradient-measurements in both dimensions are performed.

To isolate the gradients, we rely on the k-vector induced information-shift of the interference terms, which we isolate in k-space via simple Fourier filtering[29]. The isolation-workflow is schematically outlined in Figure 4c. We Fourier transform the raw image, which yields a k-space image consisting of the DC term alongside interference terms at different spatial frequencies. We isolate the terms containing either x- or y-direction gradients, $\Delta\varphi_x$ and $\Delta\varphi_y$, by multiplication with a circular hard-aperture, followed by shifting to DC. Inverse Fourier transformation yields the phase-gradient as well as the amplitude in the respective dimensions (Figure 4c).

The images' phase is retrieved by means of a modified spiral phase integration algorithm which combines $\Delta\varphi_x$ and $\Delta\varphi_y$ (Figure 4d, see Supplementary Information S4 for the integration-code)[30,31]. As the phase-gradients are strongly dependent on the distance between grating and conjugate image plane (Figure 4e), scaling and removal of potentially present residual background-phases is necessary before the final phase-image is obtained (Figure 4d, Supplementary Information S5).

Spectrally multiplexed 3D localisation

Having analysed the spectral information as well as the image phase and amplitude we conclude with a "proof-of-concept" experiment, employing two kinds of SERS particles which are doped with different dye-molecules and then immobilized in a 3D-PVA matrix obtained by drop-casting and drying of an 8% aqueous PVA solution. Figure 5a shows a representative image on a logarithmic scale to account for the strongly varying emission intensities. As previously, we determine the spectra of individual particles which exhibit distinctly different Raman activity around 1006 cm^{-1} (Figure 5b), an observation that is also reproduced in spectrally decomposed images (Figure 5c), a multiplexing advantage that makes our approach an attractive contender for SERS imaging in general (Supplementary Information S6).

After Fourier filtering and gradient-based phase-integration we obtain the respective amplitude and phase images. A direct comparison between the images reveals particles with almost identical PSFs but very different phase values, reminiscent of concave and convex areas (Figure 5d). To understand these observations, we examine the phase-profile along the path shown in Figure 5d. Here, two adjacent emitters exhibit essentially inverted phase-terms (Figure 5e). This situation can be qualitatively understood by considering the wavefront of a focusing beam. Depending on the observer's position with respect to the focus the wavefront will appear as either being convex or concave. Translated to the image formation this observation is indicative of one particle being positioned below and another above the focal plane of the image.

Beyond qualitative estimates of the relative z-position of individual emitters with respect to the focal-plane of the image it is furthermore possible to re-focus the entire image, at different z-positions, by relying on a suitable propagation kernel. Here, we propagate the complex NxN image to a new z-position by convolving with the following kernel:

$$K(x,y,z) = e^{-\left(iz\sqrt{k_m^2+k_x^2+k_y^2}\right)},$$

with $\left(k_x,k_y\right) = \frac{2\pi}{n\Delta px(x,y)}$ for $\left(\frac{-N}{2} \leq x,y < \frac{N}{2}\right)$ and $k_m = \frac{2n\pi}{\lambda}$ with Δpx being the magnified image-size corresponding to one camera pixel, n the refractive index and λ the signal wavelength. Based on this kernel we focus the images 1110 nm above and 1800 nm below the original image plane (Figure 5f) which shows that particles are indeed localized at different 3D locations within the PVA matrix. We, furthermore, verified that the particles are indeed present by manually re-focusing the images at the respective positions by moving the z-focus of the microscope by the distances mentioned above. For all propagations we assume a signal-wavelength of 860 nm which corresponds to 1193 cm^{-1}, close to the approximate wavenumber-mean of the Raman spectra of all observed particles (Figure 5e). Clearly, the SERS signals are not single-wavelength and nanometric miss-localizations might occur for propagation distances exceeding several wavelengths with the absolute error being directly related to the discrepancy between assumed and real mean-wavelength. Given the spectral agreement between individual particles such problems can, however, be corrected for by either computing the appropriate spectral mean or by 3D tracking the SERS particles in isotropic media and ensuring that the z-component of the estimated diffusion coefficient, which is solely determined by the quality of the computational propagation, matches the x- and y-components. Once determined the same particles can be used in the system of interest.

Live cell tracking

To conclude we present preliminary 3D holographic live cell tracking experiments (Figure 6). In brief, we incubate live HeLa cells with the doped SERS superclusters for 3h at 37^{o}C and then acquire time-lapse SERS as well as brightfield images to capture both the SERS particles' as well as the cells' movements. Supplementary Video 1 shows a representative 10 min live-cell SERS-particle tracking video obtained by superimposing the aforementioned imaging channels, with minor displacement-differences between the two channels being due to the fact that brightfield and SERS images are acquired consecutively with a delay of 500 ms between acquisitions. To obtain the 3D trajectories of the individual SERS probes we perform Raman holography-based 3D single-particle localisation by relying on the propagation Kernel introduced above. Using the holographically measured amplitude and phase information of the SERS signal we generate 3D image stacks over a total z-range of $\pm 3\ \mu m$ by propagating in steps of 50 nm. Once obtained, we determine the precise 3D positions of all particles by a combination of Gaussian fitting and maximum-amplitude estimation (Supplementary Information S7). The individual particle positions are then linked[32] to generate the 3D trajectories shown in Figure 6 which show a combination of confined 3D diffusion with periods of active 3D transport within the live cell (Supplementary Video 1).

Conclusion

To summarize, we have presented the first holographic measurements of spontaneous Raman images and, simultaneously, measured the spectral content of all individual SERS supercluster emitters. These experiments have been as proof-of-principle demonstrations and enabled the initial SERS-supercluster based live-cell tracking experiments. We have demonstrated that the observed emission is indeed due to Raman scattering and not a result of background luminescence or poor filter choices. State-of-the-art Raman sensors for intracellular sensing mainly rely on the ratio of two distinct Raman bands and the simultaneous measurement of said bands can be implemented by spectrally manipulating some of the four diffraction orders generated by the 2D grating. Depending on its experimental implementation such strategies could allow simultaneously imaging up to six Raman bands for more elaborate multiplexing arrangements[33] which would allow complex chemical quantification of living systems in real time. Importantly, even though multiplexed experiments allow directly accessing highly complex phenomena it is important to rationally design the system-dependent SERS probes to avoid that shot-noise contributions from strong, but experimentally irrelevant, Raman transition mask the marker bands of interest.

A spectrally multiplexed implantation of single-shot Raman holography for chemical sensing inside living cells as well as of complex codes for anticounterfeiting applications, are currently being implemented and will ultimately enable precise 3D mapping of cellular activity with chemical sensitivity. We expect such mapping capabilities to facilitate the generation of detailed chemical libraries of the metabolic processes in cells, providing us with direct access to intracellular concentration equilibria and their dependence on cellular health.. These observations will allow both detecting early stage infections and facilitate the discovery of novel cell-based therapies to fight recurrent malignancies such as cancer. In addition, the versatility and speed offered by the combination of superclusters and SERS holography enables the generation (and fast acquisition) of complex patterns with great technological applicability in fields such as the anticounterfeiting of money documents and commercial labels as it enables encoding spectral information in complex 3D patterns.

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Data Availability: The materials and data that support the findings of this study are available from the corresponding authors on request.

Code Availability: The software used for data analysis is available from the corresponding authors on request.

Additional Information: Supplementary information is available in the online version of the paper. Reprints and permission information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to M.L.

Figure 1: Bright SERS superclusters for spontaneous Raman holography. a) Localized hotspots and strong interactions between molecules and the SERS substrate give rise to enhanced Raman signals. b) TEM overview image (left) alongside a detailed view (right) of one of the SERS encoded gold supercluster particles used in this study. c) Schematic of the spectrally resolved holographic widefield microscope composed of a Michelson and a shearing interferometer enabling simultaneously spectrally resolved imaging and image-phase measurements. The sample is widefield illuminated at 785 nm, using a fluence of 2.3 kW/cm^2, and the red-shifted SERS signal separated from residual laser light with a dichroic beamsplitter and a longpass filter. After propagating through a Michelson interferometer a conjugate image is formed that is relay-imaged onto an sCMOS camera. A 2D 0-π phase grating is placed slightly offset with respect to the conjugate image plane and generates multiple diffraction orders which propagate through the relay imaging system. A hard aperture placed into the Fourier plane isolates the four first diffraction orders which ultimately self-interfere on the camera. The insets highlight the grating-induced change in PSF (left vs right) alongside a schematic of the Fourier filter (Supplementary Information S1 for more detail).

Figure 2: Wide-field time-domain SERS spectroscopy. a) Stack of SERS images recorded by varying the time-delay in one arm of the Michelson-interferometer. Several emission sites alongside a background-region (BG) are highlighted. The real-space image is plotted on an amplitude scale (square-root of the recorded intensity) for representation purposes only. b) Time-delay dependent integrated emission intensity of the respective regions highlighted in (a). The images are recorded by changing the time-delay in one arm of the Michelson interferometer, the overall setup is otherwise as outlined in Figure 1c c) Raman spectra obtained by discrete Fourier-transformation of the data shown in (b). An ensemble reference spectrum recorded on a conventional Raman spectrometer (black) is shown for comparison.

Figure 3, Spectral image multiplexing. a) Fourier transformation of a recorded SERS image interferogram alongside a magnification of the spectral Raman window. The reduced noise at high wavenumbers is most likely due to the step-size correction-algorithm of the Michelson interferometer which relies on interpolation of adjacent time-points thus artificial apodizing values close to Nyquist. b) Fourier-filtered radius-images obtained by plotting the intensity for three different spectral bands. The black numbers indicate the relative intensity scaling factors. c) Corresponding phase of the spectral Raman bands. Note that the background shows no phase response.

Figure 4, Extracting spatial phase information from Raman-images. a) PSF displacements as recorded on the camera depending on the offset of the 2D grating with respect to the conjugate image plane in the shearing interferometer. The further the grating is separated from the conjugate image plane the larger the separation between the individual diffraction orders on the camera. b) Phase-gradient scaling due to PSF displacements explained by considering a defocused emitter with resulting spherical phase. Blue: field amplitude, purple: phase, pink phase-derivative. c) Schematic of the steps necessary for phase-gradient extraction from an image recorded with the shearing interferometer: The raw image is Fourier transformed which reveals the momentum-shifted self-interference terms of interest. To isolate the image phase gradients, we separately hard-aperture filter either the $\Delta\varphi x$ and $\Delta\varphi y$ terms, shift them to zero momentum and inverse Fourier transform. d) Raw phase image obtained from a) by 2D integration. The final phase is obtained by scaling, to account for the 2D-grating position, and removal of residual aberrations. e) Phase gradients $\Delta\varphi_x$ for multiple 2D-grating to conjugate image plane distances (lines) measured across a slightly defocused SERS emitting particle (light blue). The grating is moved by 200 µm between individual measurements.

Figure 5, Multiplexed Raman phase-images. a) Logarithmic representation of a SERS image with differently doped particles (NP_A or NP_B) embedded in a 3D PVA matrix. b) Raman spectra of the indicated particles. c) Wavenumber-dependent spectral decomposition of the image shown in a) obtained by only showing intensity contained in the wavenumber regions indicated. The spectral window exclusive to the NP_A particles clearly identifies the respective particle-fraction. d) Amplitude and phase images obtained from a) by means of Fourier-decomposition and 2D integration. e) Phase (blue) and amplitude (pink) of the cross-section indicated in d). The phases of the two particles show opposite curvature, as one is located below and the other above the focal plane as can be rationalized in analogy to an inverting spherical wavefront in the vicinity of a focusing laser beam as shown in the inset. f) Computational re-focusing of d) allows 3D particle localisation.

Figure 6, Live cell SERS particle tracking. The Raman signal (pink), recorded at a fluence of 1.8 kW/cm^2 and an integration time of 250 ms, is superimposed onto a brightfield image, recorded using Koehler illumination with a 470 nm fibre-coupled LED, of the cells. The trajectories of individual SERS particles are colour-coded to show the respective z-positions which are obtained by 3D localising the particles from image stacks generated via numerical propagation (Supplementary Information S7). 40 time-points are recorded per minute.