Coherent anti-Stokes Raman scattering microscopy with dynamic speckle illumination

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Abstract. We demonstrate that dynamic speckle patterns can be utilized to improve the optical sectioning power of wide-field coherent anti-Stokes Raman scattering (CARS) microscopy. The time-dependent speckle patterns are generated by randomly moving a multimode fiber delivering one of the excitation laser pulses. The standard deviation of various CARS images with changing speckle illumination yields an enhanced axial resolution as compared with a simply averaged CARS image. The procedure makes use of the intrinsically high speckle contrast even in scattering materials.

Structured illumination techniques have been shown to enhance the resolution in wide-field light microscopy (for an overview see [1]). An improvement in the lateral resolution by a factor of two [2] can be obtained with linear methods, and a further increase is possible [3] by combining structured illumination with nonlinearities, as in stimulated emission depletion (STED) microscopy [4].

Recently, Ventalon and Mertz [5] have shown that laser speckle used as a structured illumination pattern can improve the optical sectioning power of wide-field fluorescence microscopy. An advantage of dynamic speckles illumination (DSI) is that speckles maintain a high contrast even in strongly scattering materials [6, 7], as opposed to incoherent grid patterns that are projected onto the sample [8]. The reason is that speckle patterns are not ‘projected’, but are formed within the sample volume by interference of scattered light. Thus a high contrast is maintained as long as the coherence length of the incident light exceeds the scattering length distribution [6].
Taking a sequence of different images of a sample with randomly varying speckle illumination and calculating its standard deviation (STD) suppresses out-of-focus contributions, since only the part of the sample that is sharply in focus displays a high-contrast intensity pattern that will exhibit statistical changes when modulating the speckle pattern. In other words, recording the variations, i.e. the STD, rather than the average of several images, intensifies the in-focus part of the signal, resulting in an increased optical sectioning power. It has been shown [5] that the sectioning capability with DSI is proportional to $1/|z|$ with $z$ denoting the axial depth and thus lies between a confocal ($\propto 1/|z|^2$) and an ordinary wide-field microscope (constant).

Here, the DSI method is applied to improve the axial sectioning capability of wide-field coherent anti-Stokes Raman scattering (CARS) microscopy. CARS employs a third-order nonlinear process to resonantly excite Raman-active vibrations of molecules in the sample for ‘chemical’ contrast. Two laser beams, namely a pump and a Stokes pulse with frequencies $\omega_p$ and $\omega_S$, respectively, are spatially and temporally overlapped within a sample and generate an anti-Stokes signal at $\omega_{AS} = 2\omega_p - \omega_S$. This signal is resonantly enhanced, if the frequency difference $\omega_p - \omega_S$ matches a vibrational transition of molecules inside the medium. The laser beams have to satisfy a phase matching condition given by $\Delta \vec{k} = 2\vec{k}_p - \vec{k}_S - \vec{k}_{AS} = 0$, where the $\vec{k}$-vectors correspond to the wavevectors of the three beams within the sample medium, and which has to be fulfilled simultaneously with photon energy conservation. CARS microscopy [9]–[10] is mostly realized as a scanning imaging method, where the pump and the Stokes pulses are focused on a nearly diffraction-limited spot. The method is more sensitive than spontaneous Raman scattering owing to resonant enhancement, and it is a labeling-free imaging method, avoiding problems related to photobleaching or phototoxicity of exogenous fluorescent dyes.

In non-scanning variants of CARS microscopy, i.e. wide-field CARS set-ups, the CARS signal is generated from the whole sample area simultaneously [12]–[14]. This requires no scanning devices, but has a lower spatial resolution than the quasi-confocal scanning methods. In the following paragraphs, it will be investigated how the DSI method can be implemented in such a wide-field CARS set-up in order to increase the axial sectioning capability.

The combination of DSI with wide-field CARS microscopy is not trivial. To date the DSI method has been demonstrated only in fluorescence microscopy. There, the success of the method is based on the fact that fluorescence excitation can be performed with coherent light, creating the desired speckle pattern, whereas the emitted fluorescence light is incoherent. The incoherence of the detected light is essential, since it prevents a ‘secondary’ speckle generation of signal light at the camera chip. These secondary speckles would be highly contrasted, even if they were generated by waves emitted from out-of-focus regions of the sample volume, and this would counteract the intention of DSI, i.e. the discrimination of in-focus and out-of-focus sample planes due to their different speckle contrast.

Applied to our situation of wide-field CARS microscopy, the DSI method works because one of the CARS excitation beams (the Stokes beam) is coherent and creates a speckle pattern in the sample volume, whereas the second excitation beam (the pump beam) is sufficiently incoherent to avoid speckle formation. Since the CARS signal is generated by the coherent interaction of both pump and Stokes beams, it acquires the incoherence of the pump beam. Thus, the CARS image displays the speckle pattern within the sample volume as an incoherent sharp

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1 A recent overview of CARS imaging and extensive references can be found in [11].
Figure 1. Sketch of the set-up: the pump beam (green) is guided through a fiber, passes through a dark-field condenser and enters the sample plane under a flat angle at the surface of a cone. The Stokes beam (red) is guided through another fiber and illuminates the sample from below through the objective. A CARS signal (blue) is generated in the region where the laser pulses overlap, and travels back through the microscope objective, a dichroic mirror (DM) and to an intensified CCD camera (ICCD). The inset shows the excitation geometry in more detail: whereas the Stokes pulses have a sufficiently large coherence length to generate a high-contrast speckle pattern in the whole sample volume, the low coherence of the pump pulses leads to a homogeneous illumination. The back-traveling CARS signal adopts the low coherence of the pump beam.

Figure 1 shows the set-up. A diode-pumped Nd:YAG laser (Coherent Infinity™) emits 3 ns laser pulses at a wavelength of 1064 nm with a power of 400 mJ, at a repetition rate adjustable between 1 and 100 Hz. Due to the laser design, the pulses are almost bandwidth-limited and have a coherence length of 50 cm. A 10 mJ fraction of the pulse energy is coupled out by a beamsplitter and acts as the Stokes beam in the CARS experiments. The other part of the pulse is frequency tripled (355 nm) by passing two beta barium borate (BBO) crystals, and used to pump an optical parametric oscillator (OPO; GWU Lasertechnik). The OPO performs a downconversion of the 355 nm pump photons into two other photons, namely a signal and an idler beam with a wavelength that is adjustable under computer control by tilting the OPO crystal. The total energy of the two emitted OPO pulses is approximately 20 mJ at a pulse duration of 3 ns. For the experiment, one of the OPO pulses is tuned to a wavelength of around 810 nm and used as the pump beam, in order to excite C–H stretching vibrations with characteristic wavenumbers around 3000 cm$^{-1}$. The downconversion process in the midband type-two OPO increases the bandwidth of the emitted pulses to 5 cm$^{-1}$, and correspondingly reduces their coherence length to 2 mm.

The pump and the Stokes pulses are guided to the CARS microscope by two separate 3 m long multimode step-index fibers (AMS Technology, 940 µm core diameter and numerical image, which is not superposed by a secondary speckle pattern at the camera. Altogether, the coherence properties of excitation and signal light are similar to the situation in DSI fluorescence imaging [5].
aperture (NA) = 0.22). There, the Stokes beam travels through a microscope objective (Zeiss ACHROPLAN 40 × water immersion, \( f = 3.6 \text{ mm} \), numerical aperture, NA = 0.8) and illuminates the sample from below. In the sample plane, its pulse energy is on the order of 1 mJ, distributed over a circular area of approximately 80 \( \mu \text{m} \) diameter. The same objective is used for imaging, collecting the CARS signal in a central area of 40 \( \mu \text{m} \) diameter and recording it with an air-cooled intensified CCD camera (Apogee KX1E with a gated intensifier Lambert II 18GD).

The pump beam passes through a dark-field condenser and illuminates the sample from above. The numerical aperture of the dark-field condenser covers a range between 1.2 and 1.4, such that the light travels along a cone-shaped shell. In the focal plane below the dark-field condenser, the light pulse has an energy of 0.2 mJ and is homogeneously spread across a circular disk slice with a diameter of 80 \( \mu \text{m} \) and a thickness of approximately 5–10 \( \mu \text{m} \). Above and below this slice, the light field has a ring shape with a dark center, which does not illuminate the imaged part of the sample volume. Within the disk volume, the pump beam overlaps with the Stokes beam, satisfying the wave matching condition for a range of CARS resonances within 0 and 5000 cm\(^{-1}\) [12] in such a way that the generated CARS signal counterpropagates with respect to the Stokes beam.

CARS imaging with this set-up is usually done by setting the laser frequency of the pump beam (emitted by the OPO) under computer control to a vibrational resonance, and integrating the CARS image at the CCD camera over a selected number of laser shots. Typical image acquisition times at a 10 Hz laser repetition rate are on the order of 1–10 s (single shot images are possible at the cost of a low image intensity and a correspondingly higher noise level [15]). The resulting CARS images, however, show granularity, due to laser speckle, in the excitation volume. Speckled images are usually not desired, since—at a first glance—speckles seem to reduce the resolution.

Speckles arise from the interference of light that has a random spatial phase modulation distributed over its wavefront. In the multimode fibers used for guiding the laser pulses, such a randomly modulated wavefront is formed at the fiber output faces due to the superposition of a large number \( M \) of spatial fiber modes, given by:

\[
M \approx \frac{2\pi a}{\lambda_0} \text{NA}_F. \tag{1}
\]

Here \( a \) denotes the radius of the fiber core, \( \lambda_0 \) the vacuum wavelength of light, and \( \text{NA}_F \) the numerical aperture of the fiber. Using near-infrared laser pulses and step-index silica/silica multimode fibers with \( \text{NA}_F = 0.22 \) and a core diameter of 1 mm, \( M \) becomes greater than \( 10^3 \). When traveling through the fiber, the different laser modes acquire different phase shifts and generate a speckle pattern at the end face of the waveguide. The contrast of this speckle pattern depends on the coherence length of the transmitted beam. It will vanish if the optical path length difference \( \Delta L \) between the fastest and slowest modes exceeds the longitudinal coherence length of the incoming laser beam. Thus, \( \Delta L \) corresponds to a reduction of the effective longitudinal coherence length, given by [6]:

\[
\Delta L = \frac{L}{n_2(n_1 - n_2)} \approx \frac{L}{2n_1} \text{NA}_F^2. \tag{2}
\]

Here, \( L \) is the length of the fiber and \( n_1 \) and \( n_2 \) are the refractive indices of the fiber core and cladding, respectively. For the 3 m long pump and Stokes fibers with a numerical aperture of 0.22 and \( n_1 = 1.46 \), \( \Delta L \) becomes approximately 5 cm.
Since the Nd:YAG pulses at 1064 nm acting as the Stokes beam originally have a coherence length of 50 cm, which is much larger than $\Delta L = 5 \text{ cm}$, they can pass through the fiber without losing their coherence. On the other hand, the OPO pulse (acting as the pump beam) originally has a coherence length of only 2 mm, which is smaller than $\Delta L = 5 \text{ cm}$, and thus its coherence is destroyed by the passage through the fiber. Therefore the pump beam just produces a homogeneous illumination in the sample volume, whereas the Stokes beam generates a highly contrasted speckle pattern. The lateral size of the speckle grains is determined by the light wavelength and by the numerical aperture of the objective ($d \propto \lambda / \text{NA}$), and is in our case on the order of 1 $\mu\text{m}$. The CARS signal is generated in the region where the excitation laser pulses overlap, and depends linearly on the Stokes and quadratically on the pump beam intensities. Thus, in the present set-up, the wide-field CARS image exhibits the same speckle pattern as the Stokes beam.

In earlier experiments, it turned out that the undesired CARS image granularity due to speckle can be suppressed by attaching the pulse-delivering optical fibers to a vibrating loudspeaker membrane (amplitude 1 mm and frequency 20 Hz) and integrating the images over a sufficiently high number (100 or more) of laser shots [13]. The vibrating fibers produce a dynamic change in the speckle pattern from shot to shot, which averages out during the integration. However, in the following paragraphs, we show that the usually disturbing speckle can actually be beneficial for improving the axial resolution of wide-field CARS microscopy.

In the present experiment, numerous images of the sample volume were recorded with variable speckle illumination. For this purpose, the 3 m long fiber that guided the Stokes beam was attached to the arm of a two-dimensional graphics plotter. The plotter arm could be moved under computer control by a few millimetres to different randomly chosen positions, leading to different phase delays of the various modes inside the fiber. Thus, every movement of the plotter arm changed the speckle pattern within the sample.

As a first demonstration of the principle, a sequence of 16 images of a test sample, consisting of a 15 $\mu\text{m}$ diameter polystyrene bead in water, was recorded. For imaging, the wavelength of the pump beam was adjusted to 803.2 nm, in order to match to the 3050 cm$^{-1}$ aromatic CH$_2$ stretching vibration of polystyrene. Each individual image was acquired at a fixed position of the plotter arm within 5 s by using 50 laser shots. During that time, the speckle pattern was stationary. Then, for the next image, the plotter arm was moved to another position, changing the speckle pattern, and the imaging procedure was repeated.

Then, following [5] (see also refined versions using random speckle patterns [16], or wavelet prefiltering of the images [7]), the STD of the different images was computed according to

$$I_{\text{STD}} = \frac{1}{N} \sqrt{\frac{1}{N} \sum_{n=1}^{N} I_n^2 - \left( \frac{1}{N} \sum_{n=1}^{N} I_n \right)^2}.$$  \hspace{1cm} (3)

Here, $N$ is the total number of recorded images and $I_n$ denotes the intensity pattern of the $n^{\text{th}}$ ‘raw’ image.

Figure 2 shows the results. Altogether, four image sets, each consisting of 16 CARS images, were recorded. The first set was recorded with stationary speckle illumination, i.e. without moving the plotter arm during the whole experiment, and with the sample sharply in focus. Figure 2(A) shows the average of the 16 images. Due to the fact that during the whole imaging sequence the speckle had been stationary, it shows significant ‘granularity’, which can
Figure 2. Comparison of dynamic speckle- and normal wide-field CARS images of a 15 µm diameter polystyrene bead, immersed in water. The intensity scale is the same for all the displayed images, and all images are calculated as the average (A, C, E and G) or as the STD of four sets of 16 individually recorded CARS images, respectively. The images in the upper row were taken with the sample in focus, and in the lower row with the sample displaced by 10 µm below the focal plane. The first ‘block’ of images (A, B, C and D) was recorded with stationary speckle illumination, whereas the second block (E, F, G and H) was recorded with DSI. The STD images (F) and (H), recorded with DSI, show a contrast of 2.5 : 1 between in-focus (F) and out-of-focus (H) imaging, whereas the corresponding contrast for the just averaged CARS images (E) and (G) is 1 : 1.

be quantified by the speckle contrast of the image, defined as the STD of the spatial intensity distribution (computed over the bead area) divided by its mean value. The thus defined speckle contrast of figure 2(A) is 38%.

The next image set was recorded again with stationary speckle illumination, but now with the sample bead being defocused by 10 µm. Figure 2(C) shows the mean value of the 16 images. As expected, the result is smoother than in figure 2(A) (with a speckle contrast of 10%), due to the fact that in figure 2(C) the speckle pattern is not sharply imaged at the camera. Just for comparison, figures 2(B) and (D) show the corresponding STD images of figures 2(A) and (C), respectively, calculated according to equation (3). Since all the recorded images were stationary, the STD images are almost vanishing. The residual STD-image intensity is due to electronic camera readout noise, and laser intensity fluctuations.

The third image set was recorded with DSI, changing the plotter arm position from image to image, and with the sample in focus. Figure 2(E) shows the average of the 16 images. Due to the variable speckle pattern, the averaging over 16 records leads to a smoother image appearance (speckle contrast 11%), as compared to the corresponding experiment (A) (speckle contrast 38%).

Finally, the sample bead position was again defocused by 10 µm, and a fourth set of 16 images was recorded with DSI. The average of the 16 images is displayed in figure 2(G). The average intensity of this defocused image is comparable to that of the corresponding image figure 2(C) recorded with stationary speckle illumination, whereas its speckle contrast is slightly lower (7%), as a result of the averaging. Note that the numerical averaging over the 16 different
speckle images in figures 2(E) and (G) is equivalent to the above mentioned speckle reduction method, where a dynamically changing speckle pattern is produced by attaching a fiber to a vibrating loudspeaker membrane, and the CARS image is integrated over a selected number of laser shots.

Finally, figures 2(F) and (H) demonstrate the application of the DSI method to the in-focus (F) and out-of-focus (H) CARS images, respectively. There, the STDs of the third (in-focus) and the fourth (out-of-focus) data sets were calculated according to equation (3). In contrast to the almost vanishing STD of the corresponding stationary speckle images (B and D), the focused DSI image (F) has a non-vanishing intensity that disappears in the out-of-focus case (H). As compared to the ‘just’ averaged DSI images (E) and (G), which have an equal intensity (intensity ratio 1 : 1), the STD intensity ratio between in-focus (F) and out-of-focus (H) images is 2.5 : 1, corresponding to an increased axial sectioning capability. This is due to the fact that out-of-focus speckles in the sample volume are not sharply imaged but blurred, as demonstrated in figure 2(C), i.e. their change from image to image is reduced, corresponding to a reduced STD.

Thus, the basic mechanism that provides the axial sectioning power is the depth of sharpness of the employed microscope objective, which scales proportional to NA\(^{-2}\), where NA is the numerical aperture. On the other hand, a drawback of the method becomes obvious: the increase in axial resolution is accompanied by an increase of the STD image granularity, which is, in the case of figure 2(F), on the order of 30%. Reduction of the STD image granularity requires processing of a larger number \(N\) of individual CARS images and thus a longer imaging time, where the residual granularity contrast scales as \(\propto 1/\sqrt{N}\) [7].

To further test the sectioning performance, we used a sample consisting of an oil-in-water emulsion with micron-sized oil droplets. A 15 \(\mu\)m large multilamellar vesicle with a central water inclusion was selected as a probe. The left-hand side of figure 3 shows the appearance of the structure under dark-field illumination. The outer water–oil boundary is sharply visible, whereas the inner wall of the water inclusion has a low contrast. Tuning the laser frequencies to the symmetric CH\(_2\) stretching vibration at 2850 cm\(^{-1}\) and averaging over 40 recorded CARS images with changing speckle patterns (middle image), the internal structure is revealed. The intensity of the inclusion is approximately 60% lower than that of the shell. Comparing this value to the CARS signal intensity outside the droplet, which is less than 10%, the possibility that this is attributed to the non-resonant CARS signal from water can be ruled out. Instead, the main part of the signal obtained from the water inclusion is due to the oil shell that is situated below and above the focal plane, and thus it is a consequence of the limited axial sectioning.

Applying the DSI technique, one can obtain an improvement in the axial resolution. The STD image (on the right) obtained from the 40 DSI images shows a sharper picture of the wall, which allows one to estimate its thickness to be about 3 \(\mu\)m. Figure 3 shows a 2.5-fold contrast enhancement with respect to the averaged image. The increase of the contrast is due to the fact that the oil shell of the vesicle above and below the water inclusion is not imaged anymore, i.e. the thickness of the imaged sample slice is now smaller than the radius of the water inclusion of approximately 2.5 \(\mu\)m, suggesting an improvement of the axial sectioning capability to structure sizes below 2.5 \(\mu\)m.

In summary, the presented experiments are intended as a proof-of-principle for the applicability of the DSI method to wide-field CARS imaging. It could be demonstrated that the axial discrimination of ‘normal’ wide-field CARS in the present set-up could be improved by DSI from about 5 \(\mu\)m to less than 2.5 \(\mu\)m. This is already in the range of the larger sub-cellular...
structures and thus it extends the range of applications of wide-field CARS in biophysical imaging.

To date the method has the disadvantage that a long imaging time is required in order to record enough individual DSI images to get a smooth processed STD image. The reduction of the image granularity scales as $\propto 1/\sqrt{N}$, where $N$ is the number of acquired speckle images [7]; for example, it requires 100 individual speckle images to reduce the spatial image intensity fluctuations of a homogeneous sample to 10%. In the present case, the image acquisition time was limited by the image transfer rate of the employed CCD camera to about 1 image per second, which required a long imaging time of some minutes during which the sample had to be stationary. On the other hand, the method can gain more practical interest in combination with faster camera systems, which may work at video rate.

As explained in the text, the possibility of applying the DSI method in wide-field CARS is not trivial, since—in order to be analogous to fluorescence DSI imaging—it requires that one of the excitation beams is coherent enough to generate speckle, and the other not. We assume that the DSI method will not work if both of the excitation beams are coherent, although to date we cannot demonstrate this due to experimental limitations.

However, we plan to investigate improvements of the axial resolution that might be achieved by producing the speckle pattern with the pump beam instead of the Stokes beam. Since the dependence of the CARS signal on the pump beam intensity is quadratic, this would lead to a higher speckle contrast within the CARS images, and thus to a possibly improved sectioning power of the setup. The experiments also open up the way for applying more advanced structured illumination schemes, like illumination with a morphologically constant but moving speckle pattern [16], that reduce the number of DSI images that have to be recorded.
and may further increase the sectioning power of wide-field CARS imaging to near the level of the scanning CARS methods.

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