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Wide-field coherent anti-Stokes Raman scattering microscopy using random illuminations (CARS-RIM)

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Abstract: Coherent Raman microscopy is the method of choice for label-free, real-time characterization of the chemical composition in biomedical samples. The common implementation relies on scanning two tightly focused laser beams across the sample, which frequently leads to sample damage and proves slow over large fields of view. The few existing wide-field techniques for their part, feature a reduced lateral resolution and do not provide axial sectioning. To solve these practical limitations, we developed a robust wide-field nonlinear microscope that combines Random Illumination Microscopy (RIM) with coherent anti-Stokes Raman scattering (CARS) and sum frequency generation (SFG) contrasts. Based on a comprehensive theoretical study, CARS-RIM provides super-resolved reconstructions and optical sectioning of the sample from the second-order statistics of multiple images obtained under different speckled illuminations. We experimentally show that multimodal CARS-RIM and SFG-RIM achieve wide-field nonlinear imaging with a 3 micron axial sectioning capability and a 300 nm transverse resolution, while effectively reducing the peak intensity at the sample compared to conventional point scanning CARS. We exemplify the label free, highly contrasted, chemical imaging potential of CARS-RIM and SFG-RIM wide-field microscopy in 2D, as well as 3D, for a variety of samples such as beads, unstained human breast tissue, and a mixture of chemical compounds.

1. Introduction

Tissue and cell imaging heavily relies on the use of chemical markers, fluorescent probes or dyes, to reveal different chemical species like DNA, proteins, or lipids. These staining techniques are time consuming, may cause unwanted perturbations of the samples and are not adapted to situations where markers cannot be used such as in some medical applications. Conversely, label free microscopy techniques, such as coherent anti-Stokes Raman scattering (CARS), offer chemical sensitivity, intrinsic 3D sampling, and real-time imaging within a limited field of view. CARS is a spectroscopic technique based on a nonlinear four-wave mixing process that is sensitive to the vibrational levels of chemical bonds [1]. In point scanning CARS microscopy [2], the sample is illuminated by two high power laser beams (the pump and the Stokes) that are scanned across the sample to produce an image from the CARS scattered power. Molecular-specific image structure is obtained when the pump and Stokes frequency difference matches the frequency of vibration of a given molecule. At this condition, the nonlinear light-matter interaction becomes

47 resonant within the targeted chemical bond and results in a surge of coherent radiation at a
48 third frequency (the anti-Stokes light) [3]. Because CARS detects molecular signatures without
49 requiring the introduction of labels, it has been extensively used for imaging of biochemical
50 constituents in a variety of samples ranging from material science to biology and medicine [4].
51 CARS microscopy implemented with focused NIR beams provides good penetration in water,
52 high lateral resolution (~ 300 nm) [2], and optical sectioning of \sim few microns. This explains
53 its growing success for chemical imaging of fixed or living biological samples [5–7] and more
54 recently for histological applications [8].

55 In practice, CARS imaging is performed by scanning the sample with the superposition of
56 tightly focused pump and Stokes beams and the anti-Stokes light is collected with a light sensitive
57 detector, such as a photomultiplier tube. Signal is collected from each focus point in the sample
58 for long enough dwell time to reach a useful image quality. Speeding up the scanning process, to
59 cover large regions or for *in vivo* studies, requires the diminution of the detector integration time
60 per focus point and therefore leads to the inevitable increase of the laser powers to compensate
61 for the loss of anti-Stokes signal. Working at few images per second over few hundred microns
62 field of view requires tens to hundreds of milliwatts focused onto diffraction limited spots [5].
63 These power levels are often above the photo-induced sample damage threshold [9], which limits
64 the illumination power and drastically restricts the scope of applications of rapid point scanning
65 CARS imaging.

66 To reduce the photo-toxicity, the excitation power can be dispersed over a large area of the
67 sample and then image the anti-Stokes light onto a camera. In this wide-field scheme, the
68 necessary long integration time is compensated by the simultaneous acquisition of the signal
69 on the multiple camera pixels. Several wide-field CARS microscopes were developed in which
70 the sample was illuminated by collimated pump and Stokes laser beams, either in a collinear or
71 folded-box geometry [10–13]. These schemes, however, introduced new difficulties. First, the
72 collimated illuminations at the sample plane lead to focused beams at the objective back-focal
73 plane, a situation that is likely to damage the optics when using high-peak laser power - see Fig. 1a.
74 Second, wide-field CARS does not provide the optical sectioning and transverse resolution of
75 point scanning CARS.

76 In this context, speckled illuminations, obtained by scattering coherent light with a diffuser,
77 seem ideally suited for replacing the collimated beams in a wide-field imaging configuration.
78 Speckles can be viewed as a multitude of randomly placed focused spots that probe the sample
79 in parallel, and that are suitable for achieving high resolution due to their wide spread in
80 spatial-frequency space. As the beam intensity is simultaneously spread across a large area in
81 both the spatial and spatial frequency domains, the power of the laser beams can be increased
82 without damaging the sample or the optics – a necessity for scaling the field of view. In this
83 work, we developed a wide-field CARS microscope that combines speckled illuminations with
84 the principles of random illumination microscopy (RIM) for precise computation of the sample
85 density. We show that random illumination CARS microscopy (CARS-RIM) achieves molecular
86 specificity with axial and transverse resolutions comparable to that of scanning-CARS, but
87 obtained in a wide-field configuration with reduced illumination peak intensity.

88 2. Theory of CARS-RIM

89 RIM was initially developed as a super-resolved wide-field fluorescence imaging technique in
90 which multiple images of a fluorescent sample are recorded under different random speckled
91 illuminations. A super-resolved image of the sample is recovered numerically from the variance
92 of the speckled images using a variance matching procedure based on the knowledge of the
93 speckle auto-correlation. It was shown that the RIM image processing doubles the transverse
94 resolution and provides an axial resolution comparable to that of an ideal confocal fluorescence
95 microscope [14–16]. The RIM concept was developed for incoherent imaging systems in which

96 the intensity recorded by the camera can be modeled as the sum of the intensities emitted by each
 97 point-source of the sample. In particular, when the speckled illuminations are changed, the bright
 98 spots corresponding to in-focus object planes show larger signal variations than the larger blurred
 99 spots coming from out-of-focus object planes, Fig. 1c. However, CARS is a coherent scattering
 100 process. The recorded image is a complex interference pattern of the fields radiated by all the
 101 nonlinear induced CARS dipoles and there is no distinction between the out and in-focus sources,
 102 Fig. 1b. Transferring the principles of RIM to coherent nonlinear microscopy is, therefore, not
 103 straightforward.

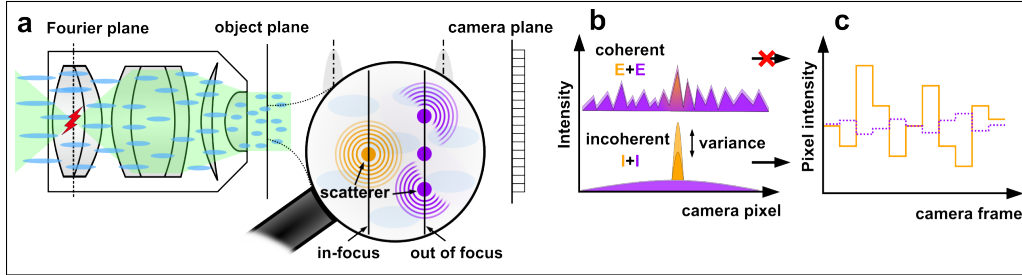


Fig. 1. Challenges in nonlinear wide-field microscopy: a) a high power plane-wave illumination (in green) easily damages the objective lens while a wide-field speckled illumination (in blue) avoids focusing at any plane. b) In coherent imaging, interference between the coherent radiation arising from scatterers placed at different object planes (orange and purple) leads to a pronounced signal variation in the image camera plane. In incoherent imaging, the intensities stemming from the different sources are summed. The light coming from out of focus sources forms blurred spots at the camera plane and contributes less to the overall image variance. c) Optical sectioning in incoherent images: when illuminated with varying speckle patterns, the image from in-focus scatterers (orange) leads to a larger frame to frame intensity variation (large standard deviation) than that of out of focus scatterers (purple).

104 In the following, we show how it is possible to take advantage of the independence between
 105 the random speckled pump and Stokes beams involved in the CARS process to generate a
 106 "quasi-incoherent" CARS emission. The idea is to vary rapidly the pump speckle while the Stokes
 107 speckle is kept static such that each CARS active speckle grain, generated by the superposition
 108 of pump and Stokes speckle grains, acquire a randomly and rapidly varying phase (due to the
 109 rapidly changing pump speckle). If the resulting CARS speckle grain phase variation is fast
 110 compared to the camera integration time, one expects the recorded CARS radiation to have some
 111 similarity with the light stemming from incoherent sources. More precisely, the phase of two
 112 CARS scatterers is fully random as soon as they are displaced from each other by more than
 113 the square of the speckle pump grain. This combination was proposed more than ten years
 114 ago by Heinrich *et al* [17] but in absence of theoretical grounds and of satisfactory results,
 115 the approach was abandoned. In this work, we developed the theory of CARS imaging under
 116 speckled illuminations. In brief, we modeled the CARS image obtained under fixed pump and
 117 Stokes illuminations and derived its expression when the pump speckles are varied during the
 118 camera integration time. Then, we studied the conditions under which this expression resembles
 119 the fluorescence image model.

120 We consider a transmission microscope similar to the one presented schematically in Figs. 2a
 121 and 3. The sample is defined by its nonlinear susceptibility $\chi_{aS}^{(3)}$ and is illuminated by two
 122 co-propagating, fully developed, pump and Stokes speckled fields, E_p and E_S passing through
 123 condensers with numerical apertures NA_S and NA_p . For simplification, the susceptibility
 124 tensor and the excitation fields are assumed to be scalar. The pump and Stokes fields are

125 modeled as random complex variables of zero mean and known correlation functions which
 126 depend on the numerical aperture of the illumination condenser objectives [18], $C_{p,S}(\mathbf{r}_1 - \mathbf{r}_2) =$
 127 $\langle E_{p,S}(\mathbf{r}_1)E_{p,S}^*(\mathbf{r}_2) \rangle$, where $\langle \cdot \rangle$ stands for the averaging over different realizations of the speckles.
 128 The CARS emitted field, E_{aS} , is collected in the forward direction, through an objective of
 129 numerical aperture NA_{aS} and a tube lens, onto a camera. The CARS intensity, I_{aS} , at the pixel \mathbf{R}
 130 of the image plane reads [19],

$$I_{aS}(\mathbf{R}) = |E_{aS}|^2(\mathbf{R}) = \left| \iiint G_{aS}(\mathbf{R}, \mathbf{r}) \chi_{aS}^{(3)}(\mathbf{r}) E_p^2(\mathbf{r}) E_S^*(\mathbf{r}) d\mathbf{r} \right|^2, \quad (1)$$

131 where $G_{aS}(\mathbf{R}, \mathbf{r})$ is the anti-Stokes Green function that describes propagation of the anti-Stokes
 132 field, with wavelength λ_{aS} , that is radiated from a point source located at \mathbf{r} in the sample and
 133 propagates to a point \mathbf{R} in the image plane. Note that the Fourier supports of G_{aS} , E_p and E_S
 134 are all caps of sphere defined by their radius $1/\lambda_{aS,S,p}$, with their projection onto the transverse
 135 (x, y) plane disks of radius $\text{NA}_{aS,S,p}/\lambda_{aS,S,p}$. Also, the Fourier supports of C_p and C_S are the
 136 same as that of E_p and E_S (see the supplemental information 1 part 1).

137 As the pump and Stokes speckled beams are formed independently, by choosing a suitably
 138 long camera integration time, it is possible to record the average of I_{aS} over many realizations of
 139 the pump speckled field, while the Stokes field is kept constant. The intensity averaged over the
 140 pump speckles (denoted as $\langle \cdot \rangle_p$) reads,

$$\langle I_{aS} \rangle_p(\mathbf{R}) = \iiint \iiint G_{aS}(\mathbf{R}, \mathbf{r}_1) \chi_{aS}^{(3)}(\mathbf{r}_1) E_S^*(\mathbf{r}_1) G_{aS}^*(\mathbf{R}, \mathbf{r}_2) \chi_{aS}^{(3)*}(\mathbf{r}_2) \langle E_p^2(\mathbf{r}_1) E_p^{2*}(\mathbf{r}_2) \rangle E_S(\mathbf{r}_2) d\mathbf{r}_1 d\mathbf{r}_2, \quad (2)$$

141 where a^* stands for the conjugate of a and $\langle E_p^2(\mathbf{r}_1) E_p^{2*}(\mathbf{r}_2) \rangle$, the characteristic function of the
 142 pump speckle grain, is equal to $2C_p^2(\mathbf{r}_1 - \mathbf{r}_2)$ [18]. Hereafter, the pump averaged image $\langle I_{aS} \rangle_p$
 143 will be called a speckle-CARS image. It corresponds to the image that is recorded on the camera
 144 when the Stokes speckled illumination is static and the pump speckles vary rapidly during the
 145 integration time.

146 If the sample is made of isolated point-like scatterers that are separated by more than the width
 147 of C_p^2 , Eq. (2) can be simplified to,

$$\langle I_{aS} \rangle_p(\mathbf{R}) = I_{aS}(\mathbf{R}) = 2I_p^2 \iiint |G_{aS}|^2(\mathbf{R}, \mathbf{r}) |\chi_{aS}^{(3)}|^2(\mathbf{r}) |E_S|^2(\mathbf{r}) d\mathbf{r}. \quad (3)$$

148 Equation (3) corresponds to the model of a fluorescence image obtained under a speckled
 149 illumination which is used in RIM [14, 16], $g_{aS} = |G_{aS}|^2$ is the standard fluorescence point
 150 spread function at emission wavelength λ_{aS} , $|E_S|^2$ is the speckle intensity for each static Stokes
 151 speckle illumination, and $|\chi_{aS}^{(3)}|^2$ is analogous to the fluorescence density. This result can be
 152 understood by noting that each CARS point-scatterer is tagged by the uncorrelated random
 153 phases of the pump field so that, when averaging over many pump speckled illuminations, the
 154 field emitted by each CARS source add in intensity on the camera in the same way as that of
 155 fluorophores. Yet, this result implicitly requires the sample to be sparse. Interestingly, and less
 156 restrictively, we show in the supplementary information 1 part 1 that the same fluorescence-like
 157 model can be obtained if $\chi_{aS}^{(3)}$ varies slowly in space over the width of C_p^2 . In this case, g_{aS}
 158 is slightly different and accounts for the coherent radiation of the CARS sources over the pump
 159 speckle excitation grains.

160 Following the RIM approach, CARS-RIM requires the recording of multiple speckle-CARS
 161 images for different Stokes speckled illuminations. A first estimate of the sample, hereafter called

162 average-CARS, can be obtained by simply averaging these speckle-CARS images. Note that the
 163 averaging can be implemented experimentally by simultaneously varying both the Stokes and
 164 pump speckles during the camera integration time. Recalling that $\langle |E_S|^2 \rangle$, is constant throughout
 165 the field of view, average-CARS $\langle \langle I_{aS} \rangle_p \rangle_S$, is similar to the image provided by a fluorescence
 166 microscope using homogeneous illumination. The average-CARS image follows the model
 167 of incoherent wide-field imaging, which is proportional to the sample density (here $|\chi_{aS}^{(3)}|^2$)
 168 convolved with g_{aS} . Because of the missing cone of the Fourier support of g_{aS} , average-CARS
 169 does not provide optical sectioning. Yet, its resolution is expected to be better than that of
 170 widefield 'coherent' microscopes using collimated laser beams (assimilated to plane waves).
 171 Indeed, in coherent imaging, the image, given by the square of Eq. (1), is related to the sample
 172 convolved with G_{aS} which has a significantly larger footprint than $g_{aS} = |G_{aS}|^2$ and explains
 173 the poor lateral resolution of the existing widefield CARS microscopes.

174 In the RIM technique, the sample is not reconstructed from the average of the speckled images
 175 but from their variance [16]. In CARS-RIM, the variance of the speckle-CARS images reads,

$$\mathcal{V}[I_{aS}] (\mathbf{R}) = \iiint \iiint g_{aS}(\mathbf{R}, \mathbf{r}_1) |\chi_{aS}^{(3)}(\mathbf{r}_1)|^2 g_{aS}(\mathbf{R}, \mathbf{r}_2) |\chi_{aS}^{(3)}(\mathbf{r}_2)|^2 g_S(\mathbf{r}_1 - \mathbf{r}_2) d\mathbf{r}_1 d\mathbf{r}_2, \quad (4)$$

176 where $g_s = |C_S|^2$ is the autocorrelation of the Stokes speckle intensity. Since \mathcal{V} depends
 177 quadratically on $|\chi_{aS}^{(3)}|^2$, recovering the sample from the speckle-CARS image variance is a
 178 non-trivial operation. CARS-RIM uses an iterative reconstruction algorithm, algoRIM, that
 179 estimates $|\chi_{aS}^{(3)}|^2$ so as to minimize the distance between the empirical variance and its model,
 180 Eq. (4). AlgoRIM requires the knowledge of g_{aS} and g_S and is based on a fast calculation of Eq.
 181 (4), which is detailed in the supplementary information 1 part 2. In theory, algoRIM is able to
 182 recover the sample spatial frequencies over the Fourier support of g_{aS}^2 , which corresponds to the
 183 resolution of an ideal confocal fluorescence microscope [15].

184 To begin RIM iterative inversion procedure, the initial estimate of the sample is set as
 185 the empirical standard deviation of the recorded speckle-CARS images. This simple data
 186 processing, named DSI-CARS, in reference to the Dynamic Speckle Illumination approach (DSI)
 187 in fluorescence microscopy [20] generally yields a better starting point than the average-CARS
 188 image. The optical sectioning arises in DSI because at the camera imaging plane the incoherent
 189 CARS speckle grains are in focus and the intensity for each camera pixel varies a lot for different
 190 speckle illuminations. On the contrary for out of focus camera imaging plane, the blurred CARS
 191 speckle grains generate poor camera pixel intensity variations for different speckle illuminations.
 192 Indeed, we observed that DSI-CARS provided optical sectioning, as illustrated in Fig. 1c and in
 193 the supplemental information 1 part 3.

194 3. Results

195 The axial and transverse resolutions of CARS-RIM reconstructions were first studied on synthetic
 196 data obtained with a CARS microscope simulator [19]. In Fig. 2b, a simulated sample is
 197 considered that is composed of an axially thin, homogeneous layer featuring a uniform transverse
 198 spatial density of $|\chi_{aS}^{(3)}|^2$. CARS-RIM and average-CARS reconstructions were obtained for
 199 different axial positions of the layer with respect to the detection focal plane, yielding a z-profile
 200 of the sample. As expected, the average-CARS profile is a constant whereas the CARS-RIM
 201 profile peaks when the thin layer is in focus. CARS-RIM axial resolution, given by the full
 202 width at half maximum (FWHM) of the latter was about $\approx 1.6\mu\text{m}$, which is close to that of a
 203 confocal fluorescence microscope $1.4\lambda_{aS}/NA^2 = 1.78\mu\text{m}$ [21]. Note that in this particular case,
 204 RIM iterative processing did not improve the z-profile given by the initial estimate, DSI-CARS,
 205 because the full axial resolution gain had already been achieved.

206 The transverse resolution of CARS-RIM is studied in Fig. 2c. We considered a thin inhomoge-

207 neous layer in which the scatterer concentration formed a star-like pattern in the transverse plane.
 208 CARS-RIM and average-CARS images were compared to the image provided by a coherent
 209 widefield CARS microscope using Stokes and pump collimated beam illumination (plane waves)
 210 propagating along the optical axis. As expected, we observe a significant improvement of the
 211 resolution from coherent CARS to average-CARS and from average-CARS to CARS-RIM.

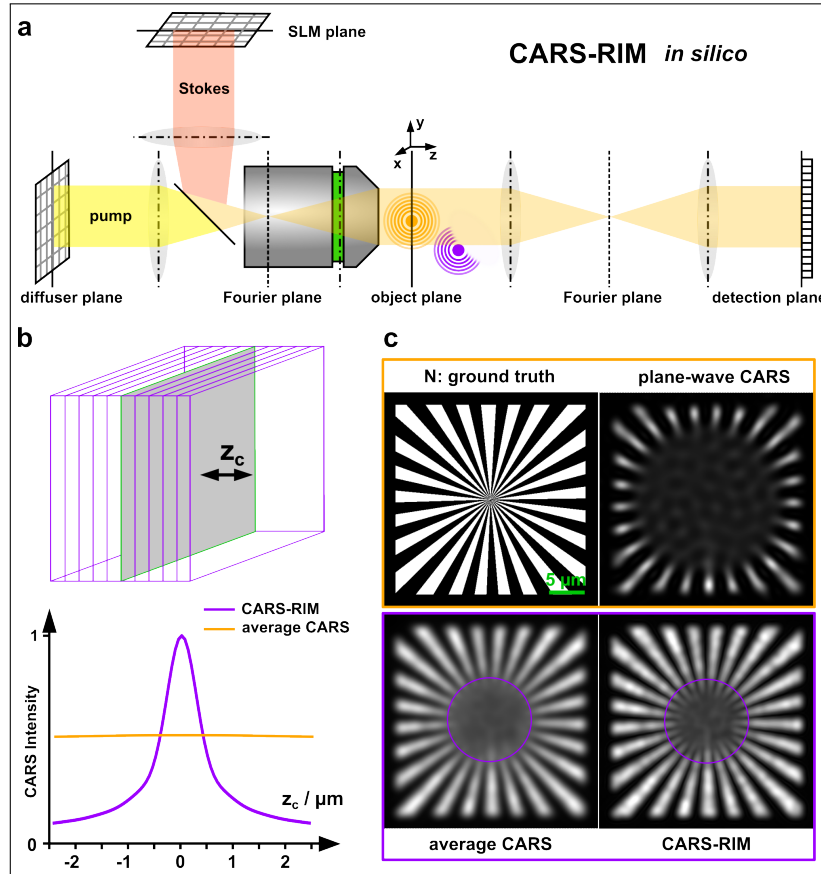


Fig. 2. CARS-RIM *in silico*: a) Schematic representation of the CARS-RIM microscope using a diffuser to generate the pump speckles and a spatial light modulator (SLM) to generate the Stokes speckles. The sample is imaged on the camera sensor in transmission. b) Study of the axial resolution of CARS-RIM with a single homogeneous thin layer that is scanned along the z optical axis. Speckled images are recorded for different positions z_c of the layer with respect to the focal plane ($z_c = 0$). Average-CARS yields a constant intensity profile as a function of z_c whereas CARS-RIM profile peaks when the layer is in focus. c) Study of the transverse resolution. Images of a thin layer featuring star-like inhomogeneous nonlinear scatterer density placed in-focus for different wide-field CARS imaging modalities (i) plane wave illumination, (ii) averaged-CARS (iii) and CARS-RIM. CARS-RIM resolution is better than that of average-CARS, which is superior to plane-wave CARS.

212 The analysis of the experimental performance of CARS-RIM was conducted on a home-made
 213 wide-field transmission microscope. A high-peak-power laser with a repetition rate of 515 kHz
 214 was used as the Stokes beam to drive the CARS four-wave-mixing process. The laser also pumped
 215 an optical parametric amplifier (OPA) to generate the color-tunable pump beam - see Fig. 3. The

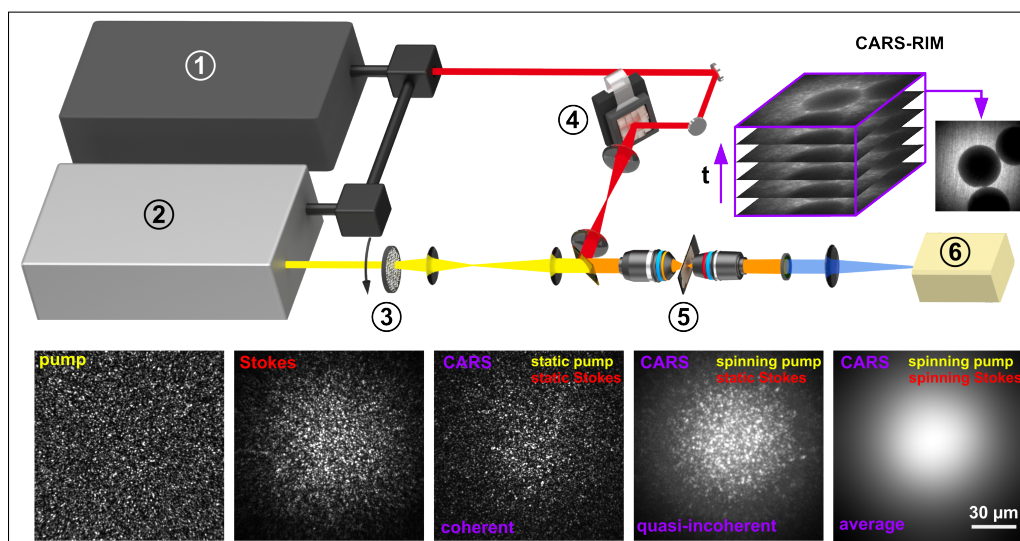


Fig. 3. Experimental setup: - 1 High peak-power laser, 2 optical parametric amplifier (OPA), 3 fast spinning diffuser, 4 spatial light modulator (SLM), 5 excitation and collections optics with the sample in between, 6 CMOS camera. Right top corner: stack of speckle-CARS images for different Stokes speckled illuminations and their CARS-RIM reconstruction. The lower part of the figure shows from left to right: a pump speckle; a Stokes speckle; CARS raw image of an olive oil thin film obtained with a static pump and static Stokes speckle illuminations at 2850 cm^{-1} CH_2 symmetric stretching vibration; speckle-CARS image obtained by averaging the CARS raw images over a large number of pump speckles (keeping the Stokes speckles static); average-CARS image obtained by averaging the CARS raw images over a large number of pump *and* Stokes speckles.

216 Stokes speckled patterns were generated by a Spatial Light Modulator (SLM), while the pump
 217 speckles were formed through a rapidly spinning diffuser. Both the pump and Stokes beams
 218 passed through the same condenser to illuminate the sample. The anti-Stokes light was detected
 219 through a high-NA objective and was recorded with a camera placed at the image plane of a 4f
 220 imaging system. The speckle-CARS images were obtained by keeping the Stokes speckled beam
 221 static while averaging over the continuously varying pump speckles. The Stokes patterns were
 222 changed with the SLM every 50 ms (20 Hz). Supplementary video 1 shows raw live CARS (and
 223 SFG) images recorded by the camera with spinning pump speckle and 20Hz updating Stokes
 224 speckle. Note that this video is intended to exemplify the data acquisition to perform CARS-RIM
 225 (that require static Stokes speckle). Depending on the sample, the average-CARS or CARS-RIM
 226 images were reconstructed using 600 to 2000 CARS speckle images.

227 The axial resolution of CARS-RIM was studied using a thin film of olive oil sandwiched
 228 between two cover slips. The 3D image was obtained by translating the sample in steps of $1\ \mu\text{m}$
 229 along the axial direction. As expected, CARS-RIM was able to localize the oil film contrary to
 230 average-CARS, see Fig. 4a. The axial resolution of the reconstructed image stack, estimated from
 231 its spatial frequency cut-off along the axial spatial frequency direction, was approximately $3\ \mu\text{m}$.
 232 CARS-RIM 3D imaging was also tested on $30\ \mu\text{m}$ diameter silica beads immersed in oil, see
 233 Fig. 4b and the supplementary video 2. The CARS-RIM images at different depths, Fig. 4b (left)
 234 are significantly crispier than that obtained with average-CARS. A set of CARS-RIM images
 235 taken by translating the sample along the optical axis was processed to recover the 3D shape of
 236 the spheres, Fig. 4b (right).

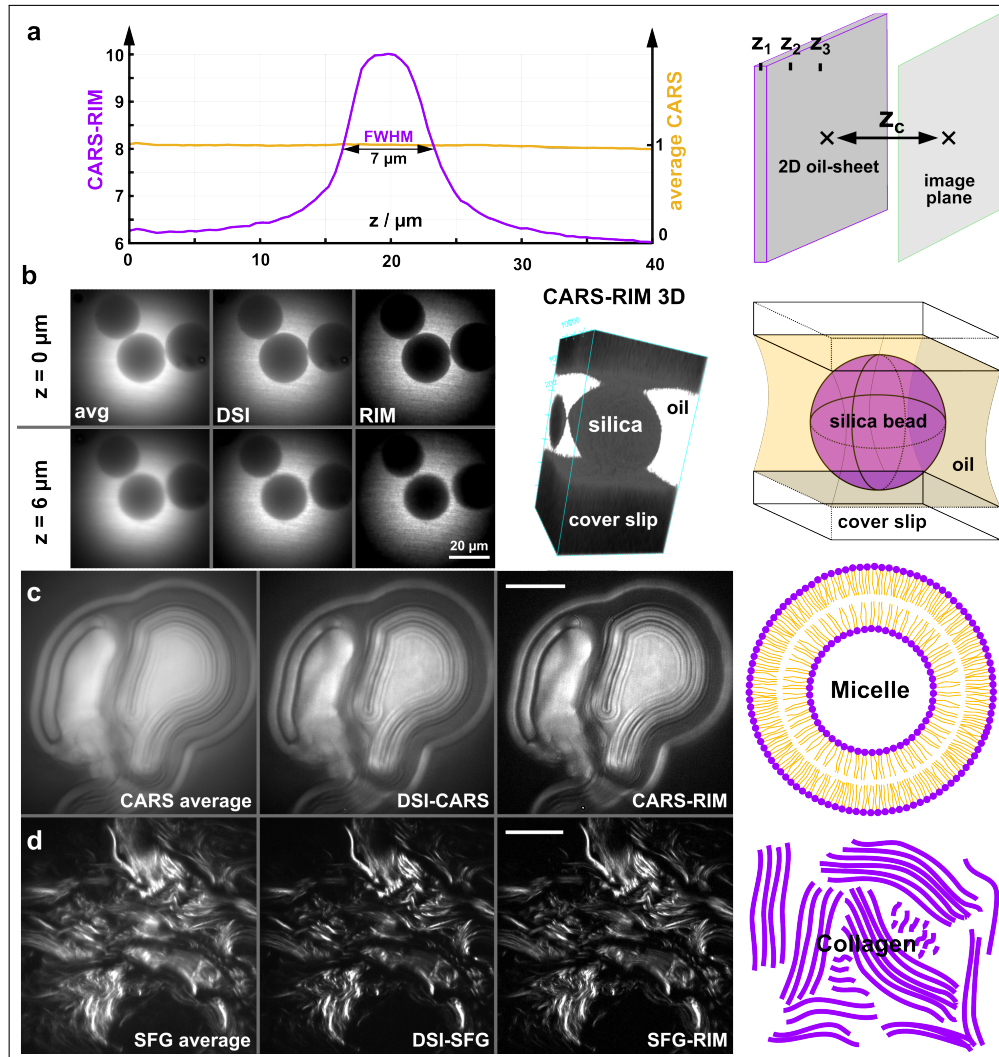


Fig. 4. Comparison between average-CARS, DSI-CARS, and CARS-RIM reconstructions of different samples represented schematically in the rightmost column. a) Average-CARS and CARS-RIM z -profiles of an approximately $5 \mu\text{m}$ thick sheet of olive oil sandwiched between 2 cover slips. The spatial frequency cut-off of the profile was found to be $\sim 1/3 \mu\text{m}^{-1}$, yielding an axial resolution of about 3 microns. b) $30 \mu\text{m}$ diameter glass beads in olive oil imaged with CARS contrast at 2850 cm^{-1} for two different depths (left) and CARS-RIM 3D reconstruction (right). The bead is clearly visible. c) Multi-lamellar vesicles imaged with CARS at 2850 cm^{-1} . d) Collagen tissue imaged with sum-frequency generation (SFG) contrast. In all cases, the CARS-RIM and SFG-RIM reconstructions are better resolved and better contrasted than average-CARS or DSI-CARS. The transverse resolution, was about 300 nm for the RIM images while an estimate of 650 nm was obtained for the average-CARS and -SFG images. In b), c) and d) are presented the average-CARS (or SFG) images, the standard deviation of the speckled images (DSI) and the RIM images. Average power in each beams $\sim 250 \text{ mW}$. The white scale bar is $20 \mu\text{m}$.

237 The resolution gain of CARS-RIM was further explored with samples showing more pronounced
238 transverse spatial structure, such as multilamellar vesicles and human connective tissues, as
239 shown in Fig.4c,d. Note that in Fig.4d, the CARS-RIM technique was applied to sum-frequency
240 generation (SFG), this SFG contrast highlights the distribution of collagen in the sample. In the
241 SFG case, the optical filter behind the collection objective lens is changed to detect the light at a
242 frequency given by the sum of the pump and Stokes beam frequencies. The transverse resolution
243 of CARS-RIM and SFG-RIM images in Fig. 4c,d were estimated with image cross sections (data
244 not shown), to be 300 nm, while the resolution of average-CARS and average-SFG was estimated
245 at about 650 nm. As expected there is a two-fold improvement of the resolution between average
246 and RIM images.

247 The capability to excite both the CARS and SFG contrasts using the same experiment allowed
248 us to acquire multi-modal images with both of these modalities. Fig. 5a shows a mosaic image
249 of a 20 μm thick human breast tissue section covering the connective tissue and the epithelial
250 layer down to the stratum corneum. For each image tile in the figure, 2000 speckle-CARS and
251 speckle-SFG images were acquired sequentially. Clearly, CARS-RIM and SFG-RIM images
252 exhibited better optical sectioning and transverse resolution than average-CARS and average-SFG.

253 Lastly, in Fig. 5b, we show that the resolution gain of CARS-RIM is compatible with
254 spectroscopic applications. We imaged a mixture of a powder of polypropylene (PP) and 60 μm
255 diameter polystyrene beads (PS) that were suspended in deuterated water (D_2O). Speckle-CARS
256 images were acquired at the Raman resonance frequencies 2400 cm^{-1} , 2850 cm^{-1} and 3050 cm^{-1}
257 matching the stretching vibrations of D_2O , the methylene groups ($-\text{CH}_2-$) within PP and the
258 aromatic rings ($=\text{C}-\text{H}$) within PS, respectively. The CARS-RIM images nicely distinguished the
259 different chemical bonds.

260 4. Discussion

261 Wide-field nonlinear microscopy is still in its infancy, but the advent of high power lasers
262 oscillators and optical parametric amplifiers makes it a promising technique for imaging large
263 samples with molecular specificity at high acquisition rates. Up to now, the few attempts to
264 develop wide-field nonlinear imaging were plagued by plane wave illumination schemes leading
265 to (i) low spatial resolution, (ii) the lack of optical sectioning, and (iii) experimental difficulties
266 due to harmful beam focusing at the fragile back-focal plane of objective lenses. CARS-RIM
267 and SFG-RIM have broken free of these limitations. Using speckled illuminations followed by
268 statistical reconstruction, they provide images with (i) optical z sectioning and (ii) enhanced spatial
269 resolution as compared to plane-wave non-linear imaging. In the supplementary information
270 section 4 we show that for the same average power, a speckle wide-field illumination (with
271 independent pump and Stokes speckles) generates ~ 2 times more CARS signal than a plane-wave
272 illumination for thin samples. This is because the CARS signal generation in bright speckle
273 grains overcompensates for dark speckle grains due to the cubic dependency of the CARS signal
274 on the excitation fields. In addition, the speckle illumination scheme is harmless to the condenser
275 and objective lenses and is extremely resilient to beam misalignment and optical or specimen
276 aberrations. This is in sharp contrast with point scanning nonlinear microscopy that requires
277 delicate beam alignment, spatial beam superposition and low-aberration numerical condenser
278 objectives to activate the CARS and SFG contrasts. Furthermore, CARS-RIM reconstruction
279 algorithm needs only the spatial frequency support of the condenser to estimate the sample,
280 rather than the full complex pupil, including aberrations, that is required for widefield harmonic
281 optical tomography or ptychography [19, 22].

282 CARS-RIM microscopy is based on a theoretical analysis showing that averaging the CARS
283 signal over the pump speckles while keeping the Stokes speckled beam static can yield a
284 speckle-CARS image similar to that obtained in Random Illumination fluorescence Microscopy
285 (RIM), with $|\chi_{aS}^3|^2$ standing for the fluorescence density and the Stokes intensity standing for

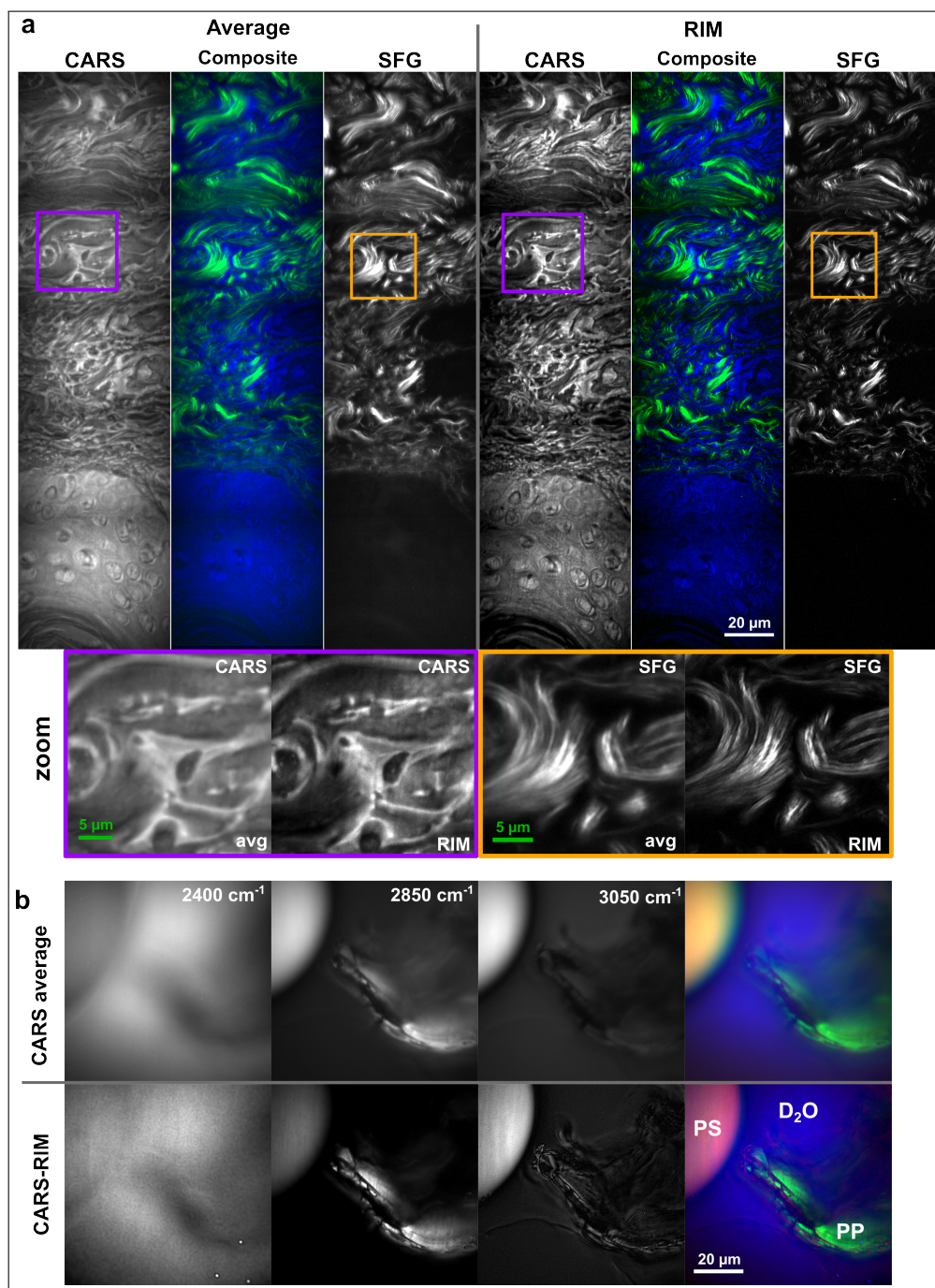


Fig. 5. a) Multimodal imaging of connective tissue of a 20 μm thick human skin sample. The CARS-RIM and SFG-RIM images (right column) are compared to average-CARS and average-SFG images. The composite image corresponds to the superposition of SFG and CARS signal. b) Spectroscopic CARS-RIM images of polystyrene beads in a suspension of polypropylene powder diluted in deuterated water. The CARS signal is detected at Raman resonances 2400cm⁻¹, 2850cm⁻¹ and 3050cm⁻¹ matching the stretching vibrations of deuterated water (D₂O), the methylene (-CH₂-) group within polypropylene (PP) and the aromatic =C-H breathing vibration of polystyrene (PS), respectively. Average power in each beams ~250 mW.

286 the speckled illumination [16]. The fluorescence-like model is valid for slowly varying samples
287 or dispersed point-scatterers. It requires the Stokes speckle grains to be much larger than the
288 pump grains (which is the case if the pump speckles are generated through an objective with a
289 numerical aperture close to unity). CARS-RIM statistical data processing consists in forming
290 the variance of multiple (600 to 2000) statistically independent speckle-CARS images obtained
291 under different random Stokes speckled patterns. It is shown that the variance is a complex
292 quadratic functional of $|\chi_{aS}^3|^2$, which depends on the Stokes speckle auto-correlation and the
293 anti-Stokes point spread function. These two functions are usually well-known and can be
294 computed or measured experimentally. The sample is then estimated iteratively by minimizing
295 the distance between the experimental variance and the variance model. It was demonstrated
296 that such data processing yields reconstructions that are linear to the sample with transverse
297 resolution and optical sectioning comparable to that of a confocal microscope [14, 15]. In practice,
298 CARS-RIM provided sample reconstructions with a resolution estimated to 3 μm axially and 0.3
299 μm laterally, Fig. (4). These results place CARS-RIM spatial resolution at the level of scanning
300 CARS microscopy [2] with the advantage of a robust set-up requiring minimal control over the
301 co-propagating pump and Stokes speckled beams.

302 In our experimental implementation, the field of view was about $80 \times 80 \mu\text{m}^2$ and the temporal
303 resolution of CARS-RIM (using several hundred to thousands of speckled images) was about a
304 few tens of seconds, this is two orders of magnitude slower than state-of-the-art scanning CARS.
305 Yet, there is clear room for improvement. Faster SLMs and cameras could reduce the acquisition
306 time per speckled image to 1ms (instead of the present 50 ms). In addition, one could reduce
307 the number of speckle images per reconstruction to 50 at the cost of some granular artifacts in
308 the reconstruction due to the residual inhomogeneity of the illumination on average [16]. Using
309 complementary speckles [23] could help reduce these artifacts by forming a uniform illumination
310 with only few speckles. We expect that combining kHz SLMs and cameras as well as fast signal
311 processing would yield CARS-RIM images at a few frames per second.

312 Furthermore, we showed in the supplementary information section 4, that with our laser
313 average power and repetition rate, widefield CARS-RIM requires theoretically ~ 4 times lower
314 peak intensity density on the sample as compared to point scanning CARS (for equal total
315 CARS signal and equal acquisition time) leading to fewer toxic multi-photon absorption events.
316 Interestingly, we show that it is possible to adjust the laser parameters (average power and
317 repetition rate) of wide-field CARS to perform faster image acquisition than in point scanning
318 (for equal peak-intensity density), or to diminish the peak-intensity density (and the heating) for
319 an equal image acquisition time than in point scanning (see supplementary information sections 4
320 and 5). This versatility is not possible in point scanning CARS where the acquisition time and the
321 peak-intensity density (and its induced phototoxicity) cannot be dissociated. Finally, whereas only
322 1/3 of the pump and Stokes speckle grains overlap with pump and Stokes independent speckles,
323 we show that this leads to ~ 2 times more CARS signal than using plane wave illumination (see
324 supplementary information section 4).

325 CARS-RIM principle works for any two-color nonlinear process. In this work, it was applied
326 to CARS and non-degenerate three-wave mixing (SFG) imaging. Still, it could also be readily
327 extended to four-wave mixing processes such as non-degenerate third harmonic generation. We
328 obtained multimodal images of human skin samples where collagen (SFG signal), nuclei and
329 borders of epithelial cells (CARS signal) were clearly distinguished, Fig. (5)a. We believe these
330 multi-modal images could be diagnostically relevant [24, 25] and useful for medical applications.
331 In another experiment, the spectroscopic ability of CARS-RIM combined with its confocal axial
332 resolution permitted to distinguish beads embedded in a powder suspension, Fig. (5b), paving
333 the way towards three-dimensional spectroscopic analysis of materials in a wide-field scheme.
334 Implemented with the spectral focusing scheme [26, 27] CARS-RIM could address a narrower
335 vibrational band and generate hyper-spectral images with reduced non-resonant background

336 contribution [28] enabling its potential for *in vivo* applications in biology and medical sciences.

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341 **Author contributions**

342 E.M.F prepared the samples and performed the experiments. S.H. conceived the idea, assisted the
343 experiments and performed the numerical calculations. S. L. developed the RIM reconstruction
344 algorithm, algoRIM. D.G. assisted the experiments and installed the laser source. A.S. conceived
345 the idea and derived the analytical description of CARS-RIM with the help of R.B. H.R. conceived
346 and supervised the project. S.H. A.S. H.R. R.B wrote the manuscript. All authors discussed the
347 results and commented on the manuscript.

348 **Competing financial interests**

349 The authors declare no conflict of interest.

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419 **Methods**

420 **4.1. Numerical Simulations**

421 All the simulations of the CARS-RIM experiments were performed by calculating Eq. (1) and
422 using the image formation technique described in [19]. For simplicity, we approximated the
423 anti-Stokes susceptibility tensor by $\chi_{aS}^{(3)} \approx \chi_{aS,1111}^{(3)}$, implying that we only considered x-polarized
424 light in excitation and detection. The pump and Stokes speckled beams were generated using
425 two independent masks placed at a conjugated image plane with random phases which were
426 uniformly distributed in $[0, 2\pi]$ and random amplitude in $[0, 1]$. Each wide-field speckle-CARS
427 image was obtained through the averaging over 800 distinct pump speckle patterns for one fixed
428 Stokes pattern. Then, 400 speckle-CARS images were generated for 400 different Stokes speckle
429 patterns. The numerical aperture (NA) of the excitation and detection objectives were set to 0.9
430 and the Stokes, pump and antiStokes wavelengths to 1030nm, 800nm and 654nm, matching our
431 experimental conditions.

433 **4.2. CARS-RIM reconstruction procedure**

434 All the CARS-RIM super-resolved images were obtained with the reconstruction code algoRIM
435 which is accessible at <https://github.com/teamRIM/tutoRIM>. The different steps of the recon-
436 struction procedures are detailed in the supplementary information 1 part 2. AlgoRIM requires
437 the knowledge of diffraction-limited g_{aS} and g_S with the experimental parameters NA_S , λ_S and

438 NA_{aS} . The tuning of Tikhonov parameters required by algoRIM for dealing with the signal to
439 noise ratio of the speckled images was performed manually.

440 4.3. *Experimental setup*

441 The CARS-RIM setup is shown schematically in Fig. 3. The output of a Yb-based amplified
442 solid-state laser (Light Conversion, PHAROS; 150 fs, 20 W, 515 kHz, 1030 nm) is split into 2
443 parts. Part 1 serves as the Stokes beam to drive the CARS four-wave mixing process. The second
444 part is coupled into an optical parametric amplifier (OPA; Light Conversion, ORPHEUS-HP)
445 whose emission at 650 – 900 nm is used as the pump beam for the CARS generation. The
446 pump beam goes through the outer edge of a 2" diameter fast-spinning (≈ 5 Hz) optical diffuser
447 (Thorlabs, DG20-220). The Stokes beam is centered onto a spatial light modulator (SLM, BNS,
448 HSPDM256-700-900) and recombined as well as temporally superimposed with the pump pulses
449 using a dichroic mirror (Thorlabs, DMSP900L) and a mechanical delay stage. The illuminated
450 spots at the diffuser and SLM are imaged by a 10x telescope (Thorlabs LA1509-B-ML; Nikon,
451 MBL71105, effective NA 0.9 under water immersion) onto the sample. The power at the sample
452 was approximately 250mW per color dispersed over a field of view larger than 80 μ m. To obtain
453 3D images, the sample is moved along the optical axis using a motorized translation stage (PI,
454 M-110.12S). Speckle CARS radiation is collected in forward direction by a 40x objective lens
455 (Nikon, PLAN, NA = 1.15, immersion: water), separated from the excitation wavelength by
456 dielectric filters (Thorlabs, FESH0700) and imaged by a second tube lens (Thorlabs, AC254-200-
457 B-ML) onto a CMOS camera (Hamamatsu, ORCA-fusion, C14440). A Matlab-based control
458 software is used to switch between random phase-mask pattern of the SLM which is synchronized
459 to the trigger of the camera's readout.

460 4.4. *Sample preparation*

461 Preparation of the glass beads in oil: glass beads (diameter 30 μ m, ThermoFisher) were deposited
462 on a standard glass object older (thickness 1mm) and a droplet of oil (IMMOIL-F30CC, Olympus)
463 was added. The sample was sealed using a standard coverslip (thickness 170 μ m) but no spacer
464 was used to avoid floating of the beads along the direction of gravity.

465 Preparation of the lipid vesicles: The multilamellar vesicles were prepared following the in-
466 structions outlined in [29]. Briefly, the lipids (dissolved organic carbon (DOC), Avanti Polar
467 Lipids) are dissolved in chloroform within a beaker creating a 10mM solution. The organic
468 solvent is slowly evaporate using a stream of dry nitrogen. The resulting layer of lipids at the
469 bottom of the beaker is suspended in cyclohexane. Dry ice is added to freeze the mixture of
470 lipids and cyclohexane. The solidified mixture is placed under high vacuum to remove the
471 cyclohexane. Multilamellar vesicles are obtained by dissolving the remnant lipids in an aqueous
472 buffer (Phosphate Buffered Saline, Sigma-Aldrich) for 30-60 minutes at room temperature.

473 Preparation of the multi-component sample: polystyrene beads (diameter 60 μ m, Fluka Analyti-
474 cal) and polypropylene (PP, Sigma-Aldrich) powder were mixed and placed on a glass object
475 holder. To match the refractive index and immobilize the sample, the mixture of powders were
476 immersed in a solution of 1% of agarose (thermoscientific, TopVision Agarose) in deuterium
477 oxide (Sigma-Aldrich, deuteration degree min. 99.9%). A 100 μ m thick spacer and a standard
478 coverslip has been used to seal the sample.

479 Preparation of the tissue sample: 20 μ m thick unstained tissue sections from a surgery of a human
480 breast were provided by the public bio-bank hospital of La Timone hospital (Marseille, FR). A
481 droplet of de-ionized water was added on top of the tissue matching its refractive index in order to
482 reduce linear scattering of the sample. To avoid draining of the water and a potential contamina-
483 tion of the condenser lens, the tissue sample was sealed using a 100 μ m thick spacer and a cover slip.

484

485 **Data Availability**

486 The data that support the findings of this study are available from the corresponding authors upon
487 request.