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A versatile CARS microscope for biological imaging

Andrew Downes,* Rabah Mouras and Alistair Elfick

We have constructed a novel, versatile Coherent anti-Stokes Raman scattering (CARS) microscope for biological imaging. The system employs a Nd:YVO₄ laser as Stokes (6 ps, 1064 nm), and a tunable optical parametric oscillators (OPOs) (5 ps, 700–1000 nm) as pump excitation, resulting in an observable range of $\sim 600\text{--}4000\text{ cm}^{-1}$. A second OPO can be used as a replacement pump for low wavenumber excitation (down to $\sim 100\text{ cm}^{-1}$). Pump and Stokes excitations are focused onto a single-mode fibre for ease of alignment, which is connected to the input of a confocal inverted optical microscope. The ep-detected CARS signal is filtered using a spectrometer, and the photons counted with an avalanche photodiode. This detection is preferable to using bandpass filters and photomultipliers, as it allows all wavenumbers to be imaged and ensures rejection of unwanted fluorescent and scattered light. Our microscope comprises a heater stage and perfusion cell for imaging live cells, and allows complementary biological imaging techniques (differential interference contrast (DIC), second harmonic generation, sum frequency generation, one- and two-photon fluorescence, fluorescence lifetime imaging). In addition, our system features an atomic force microscope (AFM) which enables CARS imaging at 10-nm resolution. The details and advantages of the modular imaging system are described more fully, and the latest results are presented. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: CARS; coherent anti-Stokes Raman; live cell imaging; atomic force microscopy

Introduction

Optical microscopy is without doubt invaluable to biomedical research. However, optical microscopy suffers from two principal drawbacks – the lack of chemical information and the limit of resolution which restricts the size of observable features. Raman spectroscopy of cells and tissue delivers significant information on cell viability,^[1,2] disease^[3–5] and chemistry. Such spectra can be acquired in a typical time of 1–60 s on live mammalian samples,^[6–8] but this is normally too long to enable Raman microscopy – the acquisition of a spectrum at each imaging pixel. Coherent anti-Stokes Raman scattering (CARS) microscopy^[9–12] gets around the inefficiency of Raman spectroscopy by enhancing the signal level by 4–6 orders of magnitude.^[10,13,14] This brings the image acquisition time down to seconds, so processes in live cells, such as mitosis, can be followed in real time.

In order to circumvent the second drawback of optical imaging – that of resolution – atomic force microscopy can be carried out with gold- or silver-coated tips. Optical spectroscopy and imaging has been performed on the scale of around 10 nm.^[15–18] CARS microscopy combined with atomic force microscopy has been performed by one group^[19–21] to enable chemical imaging of DNA nanoclusters.

In much of this paper, we will describe the design considerations behind the construction of a CARS microscope coupled to an atomic force microscope (AFM), for biological imaging. CARS microscopy images will be most useful when combined with other optical imaging modes, so the remit was to provide a highly versatile microscope with many imaging modes capable of live cell imaging. The clearest advantage of this hybrid microscope over a CARS microscope is that it offers an extremely wide range of imaging modes to provide complementary information on biological systems. The advantage of this system over a straightforward AFM is that this wide range of optical imaging

modes can all be mapped with the same $\sim 10\text{-nm}$ resolution as topography.

Experimental

Choice of laser sources

Live cells undergo photodamage when exposed to high levels of continuous wave (CW) or pulsed laser power.^[22,23] Owing to the absorption of light by a combination of water, lipids and haemoglobin, tissue is best imaged with light within the range 700–1100 nm.^[24] In this wavelength range, photodamage depends quadratically on laser power, showing that two-photon absorption^[22,23,25] is responsible. This is far more pronounced with femtosecond pulses; hence longer picosecond pulses are preferred for biological imaging as greater powers can be employed, resulting in substantially higher CARS signal levels. However, the efficiency of the CARS process drops as pulse length increases,^[26] so the optimum pulses should be 1–10 ps in duration. Two-photon absorption is also more pronounced at shorter wavelengths, as increasing numbers of compounds can be excited, and this is accompanied by more autofluorescence.

Two types of pulsed laser source emerge as candidates. The first are Ti:sapphire oscillators, and the second are neodymium-pumped optical parametric oscillators (OPOs). In the first case, two Ti:sapphire sources with pulse widths of around 2 ps and repetition frequencies of around 80 MHz are linked electronically to limit the 'jitter' between sources. These sources are tuneable

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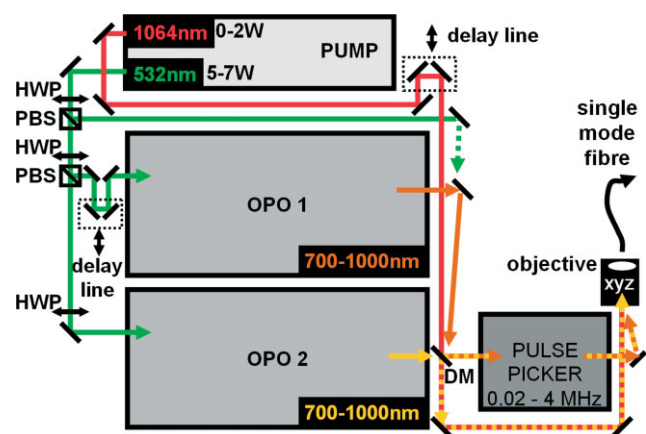


Figure 1. Schematic of illumination sources. The Nd:V pump produces the Stokes pulse (1064 nm, 6 ps) as well as a frequency-doubled 532-nm beam (5 ps). This green beam is launched into either optical parametric oscillator (OPO, 5 ps), or into both – the proportion is set by a half wave plate (HWP) and polarizing beamsplitter (PBS). For normal CARS imaging the 1064-nm beam (red, power ~2 W) is combined with the output of OPO 2 (yellow, power ~1 W) by a dichroic mirror (DM). Tipenhanced CARS imaging requires both OPO outputs (yellow and brown) to be guided through a pulse picker to reduce average power. In addition, the 532-nm beam, or any single beam can be launched into the fibre.

over the range ~700 to over 900 nm with a good amount of power. Sadly, we were unable to identify a picosecond Ti:Sapphire source with computer control; manual tuning between wavelengths limits the usability of these sources.

The alternative picosecond source is a diode-pumped Nd:vanadate source, providing a wavelength of 1064 nm, which is frequency doubled to 532 nm. Our chosen source (High-Q Picotrain, Hohenems, Austria) provides over 7 W of 532 nm (pulse width 5–6 ps) and over 12 W of 1064 nm (pulse width 6–7 ps) with a repetition rate of 76 MHz. These longer duration picosecond sources generate significantly less non-resonant CARS than with femtosecond excitation. This non-resonant signal occurs in the absence of any sample and must be removed from the total CARS signal from femtosecond-based systems.^[27] The illumination system is drawn schematically in Fig. 1. The relative amount of power of 532 and 1064 nm beams is computer controlled by a motorized half-wave plate and polarizing beamsplitter. The 1064-nm beam acts as the Stokes pulse in the CARS process, and the pump pulse comes from an OPO (APE Levante Emerald, Berlin, Germany) seeded by the 532-nm output. The OPO output is computer tuneable from 700 to 1000 nm, with powers of around 1 W. The OPO also provides 'idler' wavelengths above 1140 nm which can be combined with the lower wavelength ('signal') output, and are inherently synchronised. However, the minimum achievable wavenumber shift between the signal and idler is then around 1200 cm⁻¹, which is too high for biological material, whose spectra start above 600 cm⁻¹. Instead, the 'signal' output (700–1000 nm) is combined with the 1064 nm source with a dichroic mirror (Chroma, Rockingham, USA) to enable imaging of wavenumbers in the range 605 to over 4000 cm⁻¹. The pulses are synchronised in time by adjusting a micrometer-driven delay stage.

Modification to confocal microscope

Both pulses are focussed onto a 2-m long single mode fibre (Thorlabs SM980-5.8–125, single mode from ~780 nm to >1064 nm)

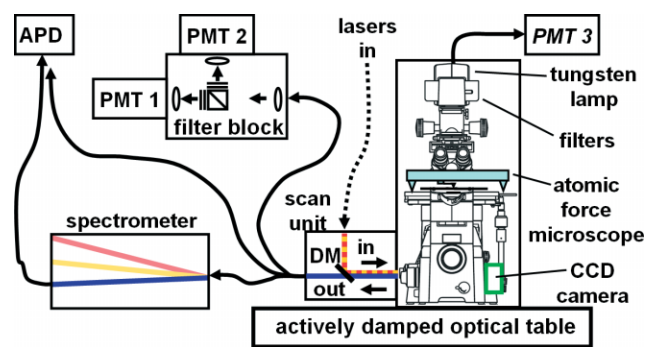


Figure 2. Schematic of the CARS microscope and detection. The illumination sources connect to the scan unit (dotted line). A suitable dichroic mirror reflects these wavelengths towards the objective lens, and removes most of the backscattered laser light from the required signal (CARS, SHG, SFG, or fluorescence). A multimode fibre passes this signal to the photomultipliers (PMT1 and PMT2), directly to the avalanche photodiode (APD), or via the spectrometer. The forward-scattered signal is detected by another photomultiplier unit (PMT3) via another multimode fibre after passing through the appropriate filters.

with high transmission in the near infrared (over 60%), and transferred to a laser scanning confocal microscope (Nikon BV 'C1', Amsterdam, Netherlands) on a separate optical table. The microscope and detection schemes are depicted in Fig. 2. A laser scanning microscope is required rather than a sample scanning stage, for fast imaging. A number of manufacturers produce similar laser scanning microscopes, but for the greatest flexibility a single-mode fibre input and multimode fibre output is required. Various wavelengths of custom-made long pass dichroic mirrors (Chroma, Rockingham, USA) are required within the scan unit, in order to separate the anti-Stokes (CARS signal) from the longer wavelength pump and Stokes (inputs).

Since both sources are inherently linked, a lengthy superposition of both focal spots before each imaging session is not required. Full alignment of the system is performed by optimizing the amount of power emanating from the single-mode fibre, of each beam in turn. No chromatic dispersion within the fibre is observed, so the delay stage does not need to be adjusted when changing Stokes wavelength. Owing to this linking of both Stokes and pump lasers into one effective point source – a single-mode fibre – an objective lens with extremely low chromatic aberration (axially) must be found to ensure that both spots are superimposed at the focus within the sample. A water immersion objective (Nikon BV Plan Apo VC 60×, Amsterdam, Netherlands) with numerical aperture (NA) 1.2 demonstrated large axial aberrations (almost 1.5–2 μm), whereas the oil immersion objective with NA 1.4 in the same range showed only ~200 nm aberration over a wavelength range of 150 nm. This is significantly smaller than the axial size of the focus spot (around 1 μm). Thus signal levels are not greatly affected, but flexibility and ease of use are greatly improved.

Since both epi-scattered (backscattered) and forward-scattered CARS images contain complementary information, a system is required that enables both signals to be collected into separate multimode fibres. In the backscattered (epi-) direction, as well as due to losses in the objective and scan unit, only 25% of the descanned light directed toward the fibre is transmitted through it. The CARS signal represents only a small part of the scattered light – the remainder being mostly scattered laser light – and so several short pass or bandpass filters are required to remove all of these excitation lasers. In biological samples, two-photon

autofluorescence, second harmonic generation (SHG) and sum frequency generation (SFG) are all present, which all need to be separated as signals from each other. A spectrometer acts as a variable filter, in order to enable spectroscopy, which requires automated scanning of wavelengths.

Live cells can withstand around 2 mW without significantly affecting their constitutive processes^[22] when scanned with pulses of around 170 fs duration. Photodamage depends on the square root of the pulse width,^[22] so the maximum useable power for live cell imaging is around 10 mW with a pulse width of 5 ps, repetition rate around 80 MHz, wavelength of 780 nm, and NA of 1.4. For fixed cells, tissue sections and skin, up to 100 mW can be used without visible photodamage.^[10] In order to achieve around 30 mW of each beam at the sample, far more power needs to be produced because of the losses in the fibre, scan unit and objective lens. After replacing a collimating lens in the confocal scan unit to now slightly overfill the objective, 30 mW can be achieved at the sample throughout the whole range of the OPO, as well as for the 1064 nm beam.

Detection

For a Stokes wavelength of 1064 nm and pump wavelength of 770–1000 nm (corresponding to a wavenumber range of 605–3600 cm^{-1}), the anti-Stokes signal is produced at 943 nm down to 603 nm. Although red-sensitive photomultiplier tubes (Hamamatsu R3896, Shizuoka, Japan) are supplied with the laser scanning microscope, their efficiencies drop off rapidly above 800 nm. The quantum efficiency at 700 nm is 10%, at 800 nm is 6%, at 850 nm is 0.7% and at 900 nm is 0.01%. Hence wavenumbers in the important fingerprint region ($\sim 600\text{--}1500\text{ cm}^{-1}$) above 800 nm will be significantly harder to detect than those in the C–H stretch region (around 2900 cm^{-1}) measured at $\sim 660\text{ nm}$. Photomultipliers also suffer from dark currents, which limit the minimum achievable photon flux when compared to photon counting. So photon counting from an avalanche photodiode (Perkin Elmer SPCM-AQR-14, Vaudreuil, Canada) is preferred, given its low dark counts (around 100/s) and greatly improved quantum efficiency – rising from 25% at 943 nm (605 cm^{-1}) to 65% at 800 nm (2445 cm^{-1}).

A photon counting \bullet PCI card was acquired (Becker & Hickl SPC-140, Berlin, Germany), which is piloted by pixel, line and frame clock signals ('TTL' pulses) emanating from the laser scanning microscope. This unit can also perform gated detection, which reduces the dark counts further by sampling only in a nanosecond window around the laser pulse arrival time, thereby rejecting the noise in most of the 50 ns dead time between detected pulses. The card can also be piloted by Labview to achieve spectroscopy.

Multi-modal imaging

In addition to CARS imaging, a great deal of flexibility is offered in bio-imaging modes on this microscope. A 20 \times phase contrast objective allows us to find groups of cells on a cover slip, and differential interference contrast (DIC) on a 60 \times 1.4 NA objective imaging provides good-contrast white light images. Confocal fluorescence imaging is performed with a 532 nm source (or another simple diode laser). Alternatively, using filters in front of the 100 W tungsten lamp, widefield fluorescence is available with excitation down to 400 nm. But for reduced bleaching, superior resolution and background rejection, two-photon excited fluorescence (2PEF) is performed with either the OPO output (from

700 to 1000 nm, and above 1140 nm) or from the 1064 nm source; so we are able to excite virtually all available dyes. By either one- or two-photon excitation, fluorescence lifetime imaging (FLIM) can be performed by coupling the avalanche photodiode into the photon counting card. Both fluorescence- and CARS-correlation spectroscopy are also achievable with the same flexible photon counting card. Finally, SHG and SFG imaging can be measured either with photomultipliers or the avalanche photodiode. All these imaging modes can be acquired in the forward as well as the 'epi-' direction. SHG highlights non-centrosymmetric structures, especially collagen; SFG is sensitive to surfaces and interface (where inversion symmetry is broken) and offers spectroscopy; and 2PEF images fluorescence with low photodamage and photobleaching with good sectioning ability.

Results

Example images are shown in Fig. 3 of a 20- μm thick section of cancerous breast tissue donated anonymously by patients from the Edinburgh Royal Infirmary. The biopsies were embedded in paraffin wax to enable easy sectioning. The sample was then dewaxed with xylene washes to remove the paraffin, leaving holes in the tissue. Images in Fig. 3 were acquired in 20 s and averaged three times, and were collected in the backscattered (epi-) direction, with the exception of the DIC image (1 s, transmitted light). The CARS wavenumber in Fig. 3(b) was set to 1662 cm^{-1} to image the amide I band of proteins, which is stronger than the often-reported C–H stretch (around 2800–2950 cm^{-1}), as lipids have mainly been removed from the sample by the dewaxing process. The DIC image Fig. 3(a) maps changes in refractive index, and so highlights the fibres within the tissue – collagen and elastin. The 2PEF image Fig. 3(c) maps mainly the autofluorescent elastin fibres, and both SHG Fig. 3(d) and SFG Fig. 3(e) highlight collagen fibres.

Although picosecond pulse widths are far less efficient than femtosecond pulses (of the same average power) for SHG, SFG and 2PEF imaging, we are able to use significantly more power with picosecond sources. Photodamage occurs at far higher powers with a picosecond source, and hence imaging rates for two-photon microscopy (SHG, SFG and 2PEF) are quite similar for picosecond and femtosecond sources.^[24] \bullet PMTs were used to acquire all these images, with a variety of short-pass and bandpass filters required. This was slightly faster than the spectrometer- \bullet APD combination at this wavenumber, but is significantly slower at wavenumbers below $\sim 1200\text{ cm}^{-1}$. We then acquired a stack of 81 CARS images separated by 0.25 μm , with an acquisition time of 1 s per frame (averaged five times) giving a total stack acquisition time of 405 s. Using this data we constructed a three-dimensional model of Fig. 4 with the open source software Biolume XD. High-resolution CARS images of strong Raman peaks for fixed cells were also acquired in only 1 s with the PMTs, or in 20 s with the spectrometer/APD combination (indicating significant losses through the spectrometer). The CARS resolution was measured as 350 nm laterally and 1100 nm axially.

To maximize the potential of CARS microscopy – both the fast imaging and no requirement for labelling – live cells rather than fixed samples should be imaged. In order to achieve this, the sample temperature should be maintained at 37 $^{\circ}\text{C}$ (or slightly lower for yeast); so a heated microscope enclosure or sample heating stage is required. For cells to grow, essential nutrients should be supplied in a growth medium pumped slowly across

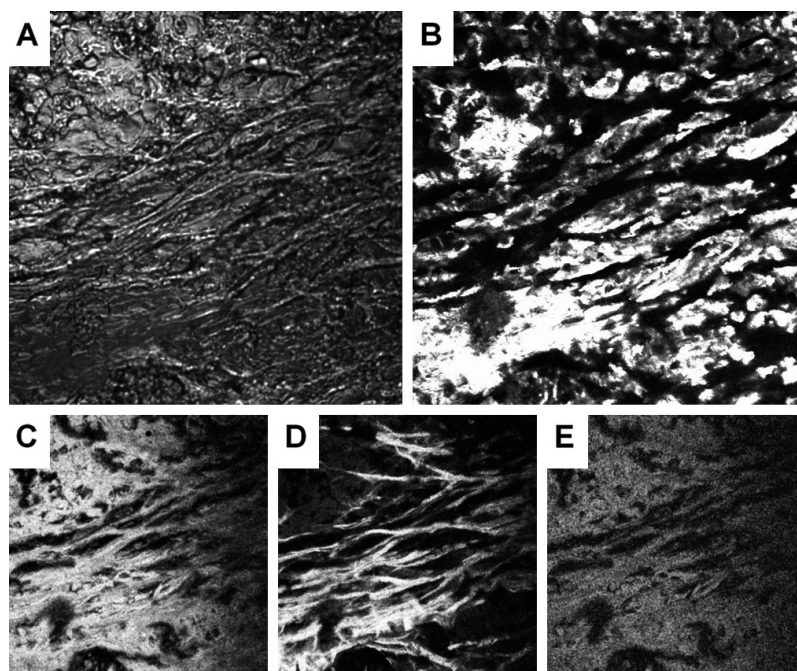


Figure 3. Complementary epi-detected confocal images from the same $212 \times 212 \times 20 \mu\text{m}^3$ region of a $20 \mu\text{m}$ thick sample of cancerous breast tissue. (a) Differential interference contrast (DIC), (b) CARS tuned to 1662 cm^{-1} , (c) two-photon fluorescence (2PEF), (d) second harmonic generation (SHG), (e) sum frequency generation (SFG). Images (b) and (e) were acquired simultaneously, with 11 mW of 1064.4 nm and 16 mW of 904.4 nm (measured at the sample), all other images were acquired sequentially with illumination only at 904.4 nm . All pixel dwell times were $61 \mu\text{s}$ (except 'a': $1.7 \mu\text{s}$), and all images were 512×512 pixels. The scan unit dichroic mirror ('DM' in Figure 2) was at 870 nm , the dichroic mirror in the filter block (PMT unit) was at 670 nm . Further shortpass filters were applied before 'b' (800 nm) and 'c', 'd', 'e' (660 nm). Bandpass filters were at 800 nm (width 30 nm) in 'b', 535 nm (width 30 nm) in 'c', 460 nm (width 50 nm) in 'd', 480 nm (width 30 nm) in 'e'.

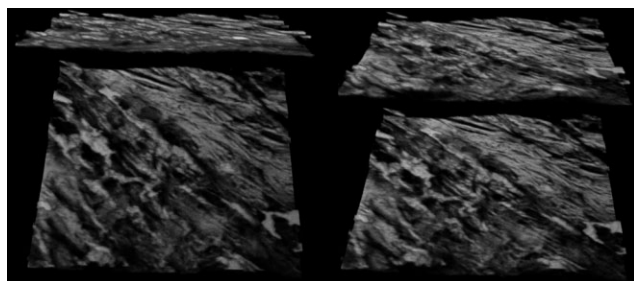


Figure 4. Views of a three-dimensional rendering of a stack of 81 epi-CARS images of cancerous breast tissue at 1662 cm^{-1} . The total image volume is $212 \times 212 \times 20 \mu\text{m}^3$. Powers measured at the sample were 11 mW of 1064.4 nm and 16 mW of 904.4 nm , pixel dwell times were $1.6 \mu\text{s}$ (1 second per image, averaged 5 times), and images were 512×512 pixels.

the sample. Our system uses a cell biology kit (Veeco, Santa Barbara, USA) comprising a heated sample stage and a miniature liquid perfusion cell. The same perfusion cell is connected to a $5\% \text{ CO}_2/\text{air}$ mix, a necessary constituent of many pH buffers used in the culture of mammalian cells.

Atomic force microscope

Atomic force microscopy has been used extensively in cell biology, mainly for topographic imaging with a lateral resolution of 10 nm . Although the AFM can measure forces, it does not deliver images with chemical contrast. By focussing light onto a metal-coated AFM tip, a significant optical enhancement can be achieved directly beneath the tip apex. This optical enhancement has been reported as high as 10^7 for Raman spectroscopy,^[17] but

Raman imaging at 10 nm resolution has only been performed on carbon nanotubes, which are strong Raman scatterers. Biological material has too low a Raman cross section for the acquisition of a spectrum at each AFM imaging pixel; individual spectra are acquired in a matter of seconds adding up to hours for an image. This is despite the massive enhancement under the tip and may be explained by the limitation of excitation power imposed due to tip heating^[28]; typically, $100 \mu\text{W}$ can be focussed into the tip region^[19,29,30] compared to tens of milliwatts used in standard Raman spectroscopy.

Fluorescence is greatly quenched by a gold or silver tip, giving only net enhancements of the order of 10, but fluorescence imaging has been performed at 10-nm resolution.^[18] Both tip-enhanced Raman and fluorescence techniques are affected by the background signal, which can be recorded without the tip present, and subtracted from the tip-enhanced image. This is acceptable for sparsely populated surfaces but can pose serious problems for densely packed surfaces. However, background removal is not an issue for multi-photon excitation – the intensity of illumination is then only sufficient for excitation in close proximity to the tip apex. Tip-enhanced two-photon fluorescence^[31] has been performed with a massively reduced background, reduced bleaching and improved enhancement.

CARS excitation offers a clear route for chemical imaging on the scale of 10 nm , when combined with AFM. Tip-enhanced CARS microscopy has been reported only by one group.^[19–21] Employing $30 \mu\text{W}$ at 786.77 nm and $15 \mu\text{W}$ at 879.25 nm , they were able to image a vibration in an adenine molecule at 1338 cm^{-1} in a DNA cluster. Enough CARS signal was acquired in 100 ms per imaging pixel, which is slower than standard topographic imaging. A gold or silver tip is required for enhancement of the

electromagnetic field to the scale of ~ 10 nm, as a surface plasmon is efficiently excited with red or near-infrared light in these metals.

The average laser power needs to be reduced to the microwatt range to achieve stable tip-enhanced CARS imaging. The repetition rate of the laser excitation was reduced by Ichimura *et al.*^[19] from 80 MHz to 800 kHz by employing a pulse picker (APE, Berlin, Germany) to select 1 pulse in every 100. This reduces the CARS signal only by a factor of 100 in the far field, whereas lowering the energy of every pulse with an attenuator or filter would reduce the CARS signal by a factor of $10^{[6]}$ because of the non-linear dependence of the signal on illumination intensity. The enhancement of CARS processes under the tip apex, given that it is a four-photon process, should be far higher than a Raman process involving just two photons – indeed, the ‘far field’ CARS signal without the tip present disappears into the noise.^[19] For tip-enhanced CARS, the two OPOs are focussed onto a polarization-maintaining fibre (Thorlabs NL-PM-750, single mode from ~ 650 nm to >1550 nm).

In Fig. 5, we used low laser power to image the scattering from a gold-coated AFM cantilever (Veeco NPG, Santa Barbara, USA) in air. When the 30–60 nm radius tip apex is in focus, it scatters a large amount of light – comparable to the reflection from gold-coated cantilever. The inset spectrum shows that there is a good amount of enhancement from 532 nm up to 820 nm. For gold tips of a typical radius of 20–40 nm in water, the enhancement has a peak close to 700 nm^[32–34]. The pump pulses employed should be around this value, and the Stokes pulse will be up to 800 nm for the ‘fingerprint’ region of biological molecules ($600–1700$ cm⁻¹), with the anti-Stokes signal occurring down to 625 nm. For this reason, we require two OPOs to provide both Stokes and pump pulses, which are tuneable from 700 to 1000 nm. Silver tips have peaks closer to 600 nm^[34] and have been shown to give less enhancement than gold at 647-nm excitation^[34] and will doubtless be even less suitable at 700 nm and above. In

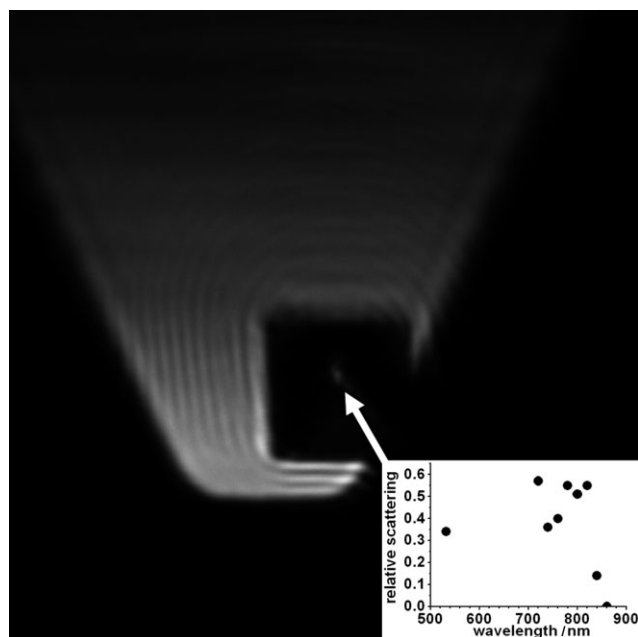


Figure 5. Confocal image of scattered light from an atomic force microscope (AFM) cantilever, illuminated at a wavelength of 720 nm. Image size $20 \hat{1}1/4 \text{ m} \times 20 \hat{1}1/4 \text{ m}$. Inset: graph of scattering from the gold tip apex normalized to the reflection from the surrounding gold-coated silicon cantilever.

detection, a spectrometer is required to filter out two-photon-induced fluorescence originating from the metal tip.^[35] A small amount of four-wave mixing also originates from the tip, emitting at the same wavelength as the anti-Stokes (CARS) signal.

An AFM with sample scanning is required, contrary to most AFM designs that scan the tip, as the metallized tip should be fixed in the laser spot. Alignment of both laser spots onto the end of the tip is difficult – manual adjustment of a mirror can rarely achieve a good alignment. As both beams are inherently linked with the confocal scan unit, they can simply be raster-scanned over the tip in three dimensions and the position of the tip defined by the position of maximum scattering. The AFM we selected (Bioscope II, Veeco, Santa Barbara) scans the sample laterally, but the tip moves up and down in the axial direction. In order to compensate for the tip moving out of the focal spot while following large topographic features, a piezo-driven objective (Physik Instrumente PIFOC, Karlsruhe, Germany) moves the focal spot by the same amount, in synchronisation with the AFM. The AFM and optical microscope are placed on a separate actively damped optical table to prevent unwanted vibrations from the water-cooled lasers affecting the AFM images.

The AFM controller is able to count TTL pulses from the avalanche photodiode detector, which correspond to individual photons, enabling the simultaneous acquisition of topographic and tip-enhanced CARS images. The AFM can also pilot the photon counting card with pixel and line clock signals, to achieve tip-enhanced FLIM and gated detection.

Conclusion

We have designed and constructed a highly versatile and powerful microscope for biological imaging. A number of considerations and constraints restrict the choice of equipment and methods of illumination and detection. The picosecond pulse width was chosen to be optimum for CARS imaging. In addition, we have enabled the collection of images of fluorescence, 2PEF, FLIM, SHG, SFG, DIC and phase contrast, as well as Raman spectra, from live cells. Such complementary datasets can often reveal far more information than only one type of imaging. All images can be acquired in both forward- and epi-detected paths; and all signals can be acquired in diffraction-limited resolution, or at around 10-nm resolution with metal-coated AFM tips. Some example CARS images are presented, which were acquired at high speed, in addition to some tip-enhanced Raman spectra.

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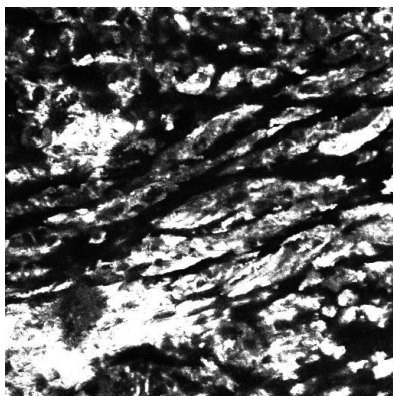
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'Probably the best microscope in the world!': We describe a versatile microscope designed for CARS imaging of live cells, with further capabilities of 2PEF, SHG, SFG, FLIM and DIC imaging. Using an atomic force microscope, all these properties can be mapped on the scale of ~10 nm.



A versatile CARS microscope for biological imaging

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A. Downes,* R. Mouras and A. Elfick

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