

Adaptive Optics for CARS Microscopy

Travel Grant to Harvard

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Background

Non-linear microscopy has over the past ten years gained a significant strength in particular in life science research. Most of this work has been undertaken in a multiphoton configuration in which a fluorophore is excited using a femtosecond laser source. Inherently the method provides optically sectioned images of the sample and through the use of long wavelength near infrared light the method is less invasive than conventional optical confocal microscopes which use blue or ultra-violet light for excitation. The use of long wavelength light sources also reduces scattering and hence imaging can be undertaken at greater depths within the sample, which can be intact and living tissue. However, in multiphoton microscopy a fluorophore is required to be present to be excited by the light source. Eight years ago the group at Harvard lead by Prof Sunney Xie pioneered the excitation of molecular vibrational bands using Coherent Anti-Stokes Raman Scattering (CARS)¹ for microscopy. Again this is a non-linear process thus the method inherently provides optical sectioning, the complication being the requirement to use two wavelengths of laser light tuned to the molecular transition of interest and spatially overlapped.

However, as one images more deeply into the sample the optical properties of the sample disturb the image leading to a larger focal volume with the result of significantly lowered excitation rate, and a loss of spatial resolution. Using active optical elements, adaptive optics (AO) originally designed for astronomy, the Strathclyde team had previously demonstrated the advantages of correcting for tissue induced optical aberrations for improved in depth non-linear microscopy².

This brief project (two weeks in Harvard) was to demonstrate improvements in CARS microscopy through the integration of these leading methods in Adaptive Optics with the pioneers of CARS microscopy.

Key Advances and Supporting Methodology

- First practical demonstration of increase resolution in CARS microscopy from 8 microns to 1.5 microns 300 μ m into an argarose test sample
- Signal increase of 3.2 times at a depth of 700 μ m (depth limited by objective working distance)
- An intensity enhancement of 8 times, 250 μ m into muscle

The imaging system (Fig. 1) utilized an optical parametric oscillator (OPO) synchronously pumped by a frequency doubled Nd:YVO₄ laser with 7 ps pulses at 532 nm and a 76 MHz repetition rate. The OPO was based upon a temperature-tuned non-critically phased matched LBO (LiB₃O₅), providing collinear, temporally overlapped signal and idler output pulse trains. A stacked Lyot filter, positioned at

Brewster's angle in the cavity, provided fine wavelength tuning. The signal and idler beams were used as the pump and Stokes beams, respectively, for the CARS process. For all experiments, the pump and Stokes wavelengths were set to 920.0 nm and 1254 nm to target the symmetric CH₂ stretching frequency of lipids.

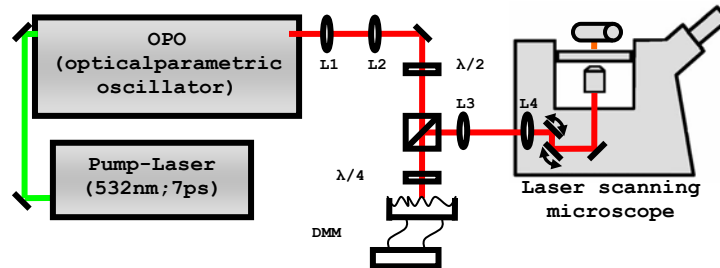


Fig 1: Adaptive CARS setup. The signal and idler pulse trains exit the OPO collinearly and overlapped in time. A set of achromatic lenses (L1, L2) expand and collimate the beam. The polarizing beam splitter cube and quarter-waveplate form a double-pass configuration such that beams deflected by the deformable mirror are redirected to the microscope.

The pump and Stokes beam were coupled into a modified inverted microscope (IX71/FV300, Olympus) optimized for near-IR throughput. A 20X 0.75NA objective (UPlanSApo UIS2, Olympus) with a working distance of ~ 0.6 mm was used for all imaging experiments. Due to low lateral and axial chromatic aberrations, this objective gives a very large CARS field of view, and is therefore ideal for tissue imaging, although a higher numerical aperture lens would give a smaller initial point spread function. The CARS signal was always collected in the forward direction to keep imaging conditions similar for all samples. All specimens were prepared with thicknesses less than 1 mm, and were sandwiched between two No. 1 coverslips for imaging. The total power level at the microscope focus was kept at approximately 150mW throughout the experiments.

The adaptive optic element was integrated into the beam line in a perpendicular geometry similar to that used previously for multiphoton imaging by the Strathclyde team, consisting of a polarizing beam splitter cube and a $\lambda/4$ -waveplate. In order to ensure a correct initial polarization, a $\lambda/2$ -waveplate was used before the beam splitter. The adaptive optic element used to shape the incoming wave front was a deformable membrane mirror (DMM) consisted of 37 electrostatic actuators mounted below a silicon nitride membrane (Flexible Optical BV/OKO Tech, The Netherlands). The shape of the membrane was altered by applying a voltage of up to 175 V to the individual actuators. The DMM was controlled via an in-house built electronics and software interface that used a random search optimization algorithm to determine the correct shape required to overcome the aberrations present. A figure of merit from the CARS image was fed into the optimization algorithm and the mirror shape was rapidly altered in order to optimize this value [3]. This optimization algorithm approach is well suited to CARS microscopy, since photobleaching does not occur and any signal enhancement leads directly to an enhancement in the resolution of the system, making signal intensity an ideal figure of merit. A control experiment verified that signal intensity increases were a result of aberration correction and not due to a beam size change at the objective back aperture that might alter the objective throughput.

The optimization algorithm was programmed to stop when it was unable to increase the figure of merit in the previous 2000 mirror changes. On average, ~ 3000 mirror changes were required to satisfy this criterion during a time period of 3-4 minutes.

The initial starting point for the DMM was set to correspond to the half way point for the maximum membrane deformation, and was equivalent to a adding a small quantity of defocus onto the incoming wave front. The position of Lens L2 (Fig. 1) was adjusted slightly so that collimated light entered the microscope objective. This mirror setting allowed for the equivalent of both push and pull motions on the membrane so that both positive and negative aberration corrections could be used.

As a standard test sample agarose-bead-samples were prepared using UltraPure™ Agarose (Invitrogen) mixed and heated with TAE Buffer to 1% concentration. Approximately four drops of aqueous Polybead® polystyrene suspension (Polyscience, Inc) were mixed with 15 g of agarose gel in its liquefied heated state to achieve suitable bead concentrations (≈ 100 beads per 500 μm stack). The agarose gel was sandwiched between two coverslips separated by a varying number of adhesive spacers, each with a thickness of 120 μm . The final thickness was measured on the microscope by taking a z-stack and correcting for the refraction index of the gel numerically.

To demonstrate the capabilities of aberration corrected CARS microscopy for deep tissue imaging, chicken breast muscle was used as a representative sample for tissue with low absorption and scattering properties. The tissue naturally contained adipose deposits with a high concentration of fatty acids as well as distinguishable structures at all tissue depths. The fresh, unfrozen tissue was embedded in agarose gel and sliced using a vibratome (Ultracut, Leica Microsystems).

The imaging system background was subtracted from all CARS images to solely display signal arising from resonant and non-resonant CARS. The main contributions of this background arose from the noise of the analog-to-digital converter (FV300, Olympus) and voltage offsets of the PMT pre-amplifier. Image post-processing was accomplished using ImageJ with the UCSD Fluoview ImageJ Plugins and IgorPro.

Results

To demonstrate the capability of adaptive optics to correct for system induced aberrations the optimisation algorithm was used to maximize the non-resonant CARS intensity from the top of a coverslip-air interface. The laser beams were parked on-axis whilst the optimization was performed. The images in Fig. 2 show the full field of view before and after correction with false colour mapping the intensity. By comparing the peak intensities of Gaussian fits through the centre of the field of view before and after aberration correction we observed an increase the in signal intensity of 2.2 times. Furthermore, applying the system aberration correction centred the field of view primarily due to centre parking of the beams during the optimization process. It should be noted that the field of view in a CARS microscope is typically non-uniform, with some ellipticity, due to a combination of lateral chromatic aberrations in the objective for the scanned beams at high angles and astigmatic excitation beams.

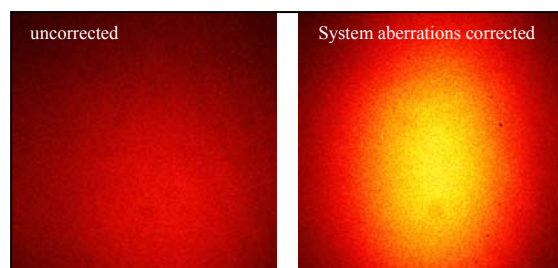


Fig. 2. Images of the field of view before and after the aberration correction for system induced aberrations were applied.

For imaging the agarose bead sample, the CARS resonance was tuned into the CH_2 -vibrational mode so that the signal was predominately produced by the non-resonant signal of water and resonant signal of the polysaccharide. The figure of merit for the optimization was the CARS signal from a point on the top agarose–coverslip interface after passage through the bottom coverslip and agarose and beads. Optimizations were taken using several samples of various thicknesses (120 μm , 213 μm , 311 μm and 496 μm) enabling the construction of a look-up table (LUT) of optimized mirror shapes for differing depths.

With a particle finding algorithm the bead images were extracted from the agarose-bead sample dataset to compare their mean intensity enhancement with the average optical section enhancement. Figure 3 clearly demonstrates the improvement gained when using adaptive optics to image a polystyrene bead at a depth of 400 μm with a CARS microscope.

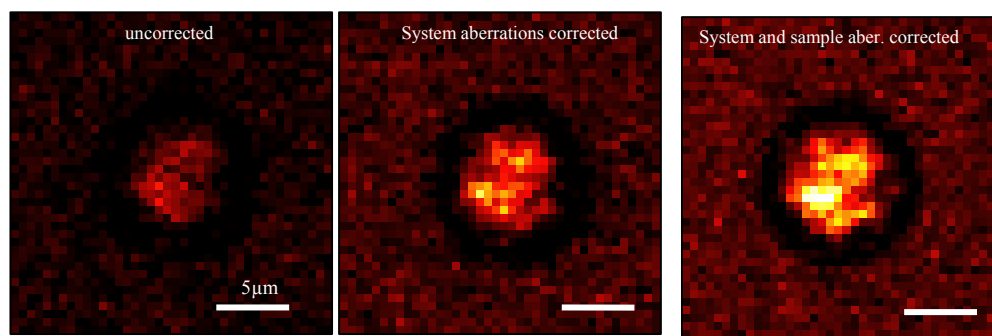


Fig. 3: CARS image of a polystyrene bead at 400 μm depth in agarose gel (10% off-set subtracted), showing the intensity improvement achieved with adaptive optics using the LUT approach.

To demonstrate deep tissue imaging a sample of adipose globular deposits in white chicken muscle was used with the Raman shift tuned to resonance with the CH_2 vibrational mode. Due to the high concentration of CH_2 -rich fatty acids within adipose globular deposits, as well as their relatively high frequency throughout the tissue sample, the globular deposits were chosen as a suitable feature from which the CARS signal could be optimized. When optimizing at greater depths into the sample, where the uncorrected signal is often very noisy, we used an incremental approach, starting the optimization from the system aberration correction or, if available, from a DMM shape obtained with a thinner sample. This technique provided the best improvement factor and the fastest convergence time.

Figure 4 shows an image of a typical fat deposit at a depth of 178 μm in the tissue. The 3 images of the same structure, and imaging parameters were obtained without aberration correction (left), with the system aberration correction LUT (middle) and with the sample and system aberration correction applied (the arrow shows the point at which the signal has been optimized) (right). Graphically the intensity enhancement can be seen from the linescans through the centre of the structure.

We achieved a maximum enhancement factor of approximately 8 times, at the position of optimization, indicated by the arrow. At a position 20 μm from the point of optimization the system and sample induced aberration correction gave a greater enhancement factor than the system induced aberration correction alone.

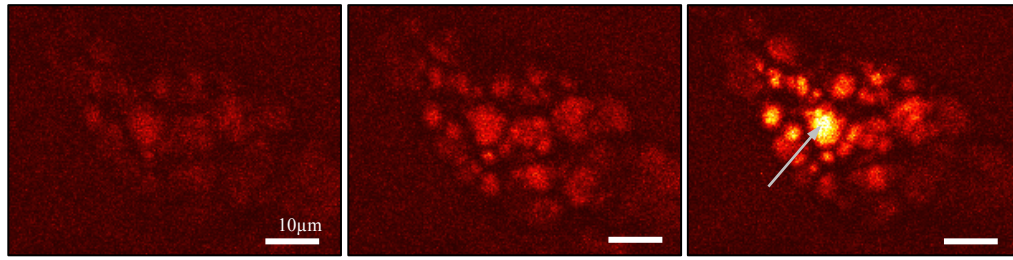


Fig. 4: Adipose globular deposit imaged with CARS microscopy at 178 μm depth in white chicken muscle (11 times zoom). Top: Comparison of image without correction, with the system aberrations corrected and with the system and sample induced aberrations corrected for the globule marked by the arrow. Bottom: Horizontal linescans through tip of the arrow, intensity-averaged over 3 adjacent lines

The practical work undertaken in this project has thus demonstrated that adaptive optic elements can significantly improve both the resolution and more importantly in this situation the signal from a CARS microscope in particular when imaging at depth inside a tissue sample.

Project Plan

The proposed plan worked well with the two week timescale being sufficient to undertake these initial experiments. With two post doctoral researchers from Strathclyde working in Harvard where the CARS and AO systems were integrated both optically, and in the software, quickly. Before this work Prof Girkin, prior to support on this grant, had visited Prof Xie's laboratory to determine exactly what was required for the work and this saved considerable time during the two weeks of experimentation.

The one part of the project which did not run quite as smoothly as it might have done was the publication of the results. This was partly because one of the Harvard researchers transferred to a new job after the experimental work and the complication of writing a joint paper using emails and telephone conversations. In hindsight a further brief visit by one of the Strathclyde team about a month after the experimental work had been completed, and the results analysed, would have speed up the paper production as two further outputs are in preparation. This was a minor complication.

Research Impact

The work has already led to an invited presentation and it is hoped that this work will increase the interest in CARS microscopy in the UK. The long term promise of CARS microscopy is only likely to be realised when the method can be applied at depth in living samples. The use of Adaptive optics offers one way to achieving the long term goal and realising the potential of CARS based imaging. The long term impact should therefore be high. The work further promotes the use of AO in microscopy a field currently being lead by teams in the UK, at Strathclyde and Oxford.

Expenditure

The funds supported two post doctoral researchers (Drs Amanda Wright and Simon Poland) from Strathclyde to spend two weeks in Harvard integrating the systems. Limited PI time was claimed for the project management. Equipment was all provided from previous grants included Basic Technology and EU projects. Using the state of the art CARS microscope built by the Harvard team (from NIH and NSF funding) enabled high quality research to be undertaken in a very timely and cost effective manner.

Further Research and Dissemination

A grant application is in the process of being written which will develop a fully integrated CARS and AO system. This work will be undertaken at Strathclyde but a close collaboration will be made with the Harvard team. Through funding available to the Harvard team they are expecting to spend time in Strathclyde to gain further understanding of the AO methodology while the Strathclyde team learn more about the CARS imaging process. Several groups of life science end users are keen to be involved including those based in Strathclyde, Glasgow University and the Beatson Cancer Institute. All believe that the true long term role for CARS microscopy is for deep, minimally invasive (particularly excluding exogenous fluorophores) imaging.

In a commercial context two collaborations are developing, though unfortunately not with UK companies who do not appear to be interested despite several attempts. A US company who develops and produces MEMS based adaptive optic mirrors, have approached us on routes to exploiting their technology in microscopy and they will be providing support in kind for future developments (a mirror has already been supplied to Strathclyde as part of this work). A second European company (€20M turn over in 2006) wishing to move slightly beyond its current product range has also approached the Institute of Photonics with the idea of developing the AO systems into a full product (possibly using the US company mirrors). The full commercial details of this work are currently subject to final contract negotiations but the core of the “deal” is agreed.

Two further papers are in preparation and these are aimed to be submitted in the next two months, along with two further presentations of the work in both the UK and US by the team. The Harvard collaborators are also actively promoting the work and both teams closely acknowledge the financial support from all sources and each others contributions to the work.

References

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