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TITLE:

Implementation of a Nonlinear Microscope Based on Stimulated Raman Scattering

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SUMMARY:

In this manuscript, the implementation of a stimulated Raman scattering (SRS) microscope, obtained by the integration of an SRS experimental set-up with a laser scanning microscope, is described. The SRS microscope is based on two femtosecond (fs) laser sources, a Ti-Sapphire (Ti:Sa) and synchronized optical parametric oscillator (OPO).

ABSTRACT:

Stimulated Raman scattering (SRS) microscopy uses near-infrared excitation light; therefore, it shares many multi-photon microscopic imaging properties. SRS imaging modality can be obtained using commercial laser-scanning microscopes by equipping with a non-descanned forward detector with proper bandpass filters and lock-in amplifier (LIA) detection scheme. A schematic layout of a typical SRS microscope includes the following: two pulsed laser beams, (i.e., the pump and probe directed in a scanning microscope), which must be overlapped in both space and time at the image plane, then focused by a microscope objective into the sample through two scanning mirrors (SMs), which raster the focal spot across an x-y plane. After interaction with the sample, transmitted output pulses are collected by an upper objective and measured by a forward detection system inserted in an inverted microscope. Pump pulses are removed by a stack of optical filters, whereas the probe pulses that are the result of the SRS process occurring in the focal volume of the specimen are measured by a photodiode (PD). The readout of the PD is demodulated by the LIA to extract the modulation depth. A two-dimensional (2D) image is obtained by synchronizing the forward detection unit with the microscope scanning unit. In this paper, the implementation of an SRS microscope is described and successfully demonstrated, as well as the reporting of label-free images of polystyrene beads with diameters of 3 μm. It is worth noting that SRS microscopes are not

commercially available, so in order to take advantage of these characteristics, the homemade construction is the best possible option. Since SRS microscopy is becoming popular in many fields, it is believed that this careful description of the SRS microscope implementation can be very useful for the scientific community.

INTRODUCTION:

In life science applications, SRS microscopy has emerged as powerful tool for label-free imaging. The basic idea of SRS microscopy is to combine the strength of vibrational contrast and its ability to acquire images in a few seconds.

SRS is a process in which the frequency difference between two laser beams frequencies (pump signal and stokes signal at different frequencies) matches the molecular vibration of an investigated sample, causing stimulated Raman scattering and a significant increase in the Stokes signal. Unlike linear Raman spectroscopy, SRS exhibits a nonlinear dependence on the incoming light fields and produces coherent radiation. SRS has two fundamental advantages: 1) speed, which makes images less sensitive to artefacts arising from sample movement or degradation, and 2) an excellent signal-to-noise ratio (SNR). In addition, SRS exhibits a spectrum identical to the spontaneous Raman, and the SRS signal is linearly proportional to the concentration of the chemical bond excited[1-5].

In our microscope, a femtosecond (fs) SRS experimental set-up is integrated with an inverted optical microscope equipped with a fast mirror scanning unit (**Figure 1**)[6-8]. Two pulsed laser sources are used to implement this microscope. The first is a fs-Ti:Sa with a pulse duration of approximately 140 fs, repetition rate of 80 MHz, and emission wavelengths in the range of 680–1080 nm. The second, used as probe beam and pumped by Ti:Sa, is a femtosecond synchronized optical parametric oscillator (SOPO), with a pulse duration of approximately 200 fs, repetition rate of 80 MHz, and emission wavelengths in the range of 1000–1600 nm. It should be noted that the minimum photon energy difference between the Ti:Sa and SOPO beam is 2500 cm^{-1}. Therefore, using this combination of laser systems, only the high frequency C–H region (2800–3200 cm^{-1}) of Raman spectra can be explored[6-8].

In order to set up an SRS microscope, there are three crucial issues to consider, which are described in the successive paragraphs. The first is the implementation of a high-frequency modulation transfer method (see **Figure 2** and step 2.1 of the protocol for a description). In an SRS experimental investigation, a crucial parameter is the sensitivity of the system. An SRS signal is detected as a small change in the intensity of excitation beams; therefore, it can be corrupted by laser intensity noise and shot noise. This issue can be overcome by integrating this system with a high-frequency modulation transfer method (see **Figure 2** and step 2.1 of the protocol for details). In this method, an electro-optic modulator (EOM) is used to modulate the pump. The modulation transferred to the probe beam can then be detected by a PD after blocking the pump beam with a stack of optical filters [stimulated Raman gain (SRG) detection mode-. The PD output is connected by a low pass filter to a lock-in amplifier (LIA), which demodulates the measured signal. By increasing the modulation frequency of the beam to frequencies above 1 MHz, the intrinsic limit of PDs can be obtained.

The second issue to consider is the installation of a mechanical mount which permits to carry out forward detection and at the same time to preserve microscope observation in brightfield. In addition, it has to reduce the noise due to mechanical vibration during the generation of images and to allow the precise repositioning of detection system (see **Figure 3** and step 2.2 of the protocol).

The third is the synchronization of the signal acquired by the phase-sensitive detection scheme, with the beam positioned onto the sample monitored by the scan head of the microscope. In order to realize images, the SMs require three TTL signals that are made available by the microscope controller connected to the scan head unit: pixel clock, line sync, and frame sync. The synchronization is achieved by controlling using a PCI card, the three TTL signals, and the acquisition of a voltage signal at the output channel of LIA[6-8]. A homemade software has been developed and described previously[6-8], while the hardware of the synchronization system is reported in **Figure 4**.

A fundamental procedure when carrying out SRS imaging is microscope alignment. It is realized over the course of four steps, which are described in the successive paragraphs. The first is the spatial overlap of two beams (see step 3.1 of the protocol). In this experimental set-up, the two beams were spatially collinearly combined by a dichroic mirror. The preliminary step is the alignment of OPO and Ti:Sa so that each reaches the microscope. Then, considering OPO as a reference beam and taking advantage of a position sensitive detector, the Ti:Sa is spatially overlapped to OPO.

The second crucial aspect is the temporal overlap of two beams (see step 3.2 of the protocol). Even if the pump and OPO beams are perfectly synchronized[9], since they follow slightly different beam paths inside the OPO housing, at the OPO exit they have a time delay of about 5 ns and spatial difference of 5 cm. Therefore, Ti:Sa and OPO require being re-timed optically to ensure temporal overlap at the sample. This is typically accomplished with a finely tunable optical delay line, which in this case is inserted between the Ti:Sa and microscope (see **Figure 1**). In order to obtain the temporal overlap of two beams, two techniques are used. The first is carried out using a fast PD and oscilloscope, while the second is based on auto- and cross-optical correlations. Using the first technique, a rough overlap of two beams is obtained (uncertainty of 10 ps), while an accurate temporal overlap of two beams is obtained using a cross-correlator (resolution of 1 fs).

The third crucial aspect is alignment of the two beams inside the microscope (see step 3.3 of the protocol). A preliminary white light observation of sample allows to individuate the desired field of view (FOV). Afterwards, laser beams, entering the microscope by a side port of microscope, are aligned in order to reach the PD mounted on the upper part (**Figure 3**). However, for a correct image acquisition, setting a number of parameters is required (for example, pixel dimension and pixel dwell time). The sampling frequency must respect the constraint imposed by Nyquist's theorem in order to preserve all information in an image, while for a correct correspondence between the spatial coordinates of pixels and SRS value measured

in each pixel, the integration time of LIA should be equal or comparable to the pixel dwell time.

In the final step of microscope alignment, numerous tests are carried out to optimize the spatial and temporal alignment (see step 3.4 of the protocol). A number of transmission images (TI) for both Ti:Sa and OPO are acquired in order to optimize spatial overlap. In a TI, a single beam is used, and the transmitted beam intensity from the sample is measured by a PD. In the case of TI realized by OPO, the PD output signal is directly connected to PCI card, while in the case of TI realized by Ti:Sa, the PD output signal is connected to LIA and analog output of LIA is connected to PCI card. The transmission images are very useful to optimize the FOV, the illumination, the focal position of microscope objectives and to check if the two beams are spatially overlapped[6-8].

The optimization of the pump and probe beam's temporal overlap is obtained by scanning the delay line with steps of 0.001 mm corresponding to a 3.3 fs time-shift and carrying out a SRS measurement in a single point of a polystyrene bead sample 3 µm in diameter. The amplitude of an SRS signal measures values from LIA, as a function of the probe-pump delay, and provides a maximum corresponding with exact temporal overlap of the two beams[6-8]. Before concluding, it should be noted that all discussed steps are mandatory to obtain a high quality image.

PROTOCOL:

1. Starting up the laser system

1.1. Check if the temperature of chillers is maintained at or below 20 °C.

1.2. Check if the humidity control unit is working properly and humidity is maintained at a value around 40%.

1.3. Turn on the Ti:Sa laser, strictly following the instructions in the manual.

1.4. Set the wavelength to 810 nm.

1.5. Turn on the OPO and the connected mini-computer. Run the application that controls the OPO laser.

1.6. Select **bypass** if 100% of the Ti:Sa laser output is required at the exit of the OPO box.

1.7. Deselect **bypass** if 20% of the Ti:Sa laser output and 80% of the OPO laser output are required at the exit of the OPO box.

1.8. **Open** the shutter of Ti:Sa and release the Ti:Sa beam to the OPO input.

1.9. Release the two laser beams at the OPO exit by clicking **signal-out** and **pump-out**.

1.10. Wait until both lasers Ti:Sa and OPO are stabilized (around 45–60 min).

1.11. Verify the beam spot at the exit of the OPO box for both Ti:Sa and OPO using a paper detector card and check the power using a power meter.

1.12. Tune the OPO laser wavelength to 1076 nm.

1.13. Reduce the power to ~10 mW for each laser beam to perform alignment.

2. Setting up the microscope

2.1. Implementation of high frequency modulation transfer method

2.1.1. Carry out the optical alignment procedure of the modulator such that the Ti:Sa beam enters and exits without any distortion.

2.1.2. Turn on the function generator and generate a TTL signal (square wave with amplitude = 5 V, offset = 2.5 V, frequency = 5 MHz).

2.1.3. Divide the TTL signal into two parts using a T junction; one for EOM and the other for the lock-in amplifier (LIA) (see **Figure 2**).

2.1.4. Check all signal levels with an oscilloscope.

2.1.5. Turn on the LIA and connect the generator output channel to the reference channel of the LIA.

2.1.6. Connect the generator output channel to the high voltage power amplifier of EOM.

2.1.7. Turn on the amplifier and set the voltage to almost the maximum level. Monitor the beam power at the exit of EOM.

2.2. Integration of mechanical mounting to fix PD and assign x and y relative motion

NOTE: With the microscope, an external mount is introduced, equipped with a micrometer that has motion control in x and y directions.

2.2.1. Mount two travel translation stages to allow movements along the x and y directions (see **Figure 3**).

2.2.2. Fix the stage on a Ø1.5" post of appropriate height.

2.2.3. Mount the PD to an external mount.

2.2.4. Maximize the beam intensity at PD, adjusting the PD positions (x and y coordinates) using the micrometers attached to the mount (see **Figure 3**).

3. Alignment of microscope

3.1. Spatial overlap of the beams

NOTE**:** Considering the OPO beam as a reference and taking advantage of a position sensitive detector, the Ti:Sa should be overlapped to OPO according to the following procedure:

3.1.1. Carry out the alignment for the OPO laser beam in order to reach the microscope. Carry out the alignment for Ti:Sa laser beam to reach the microscope.

3.1.2. Place the laser beam position sensors detectors in two positions in between DM1 and M4, the first located close to DM1 and the second close to M4. Detect, for each position, the x and y coordinates of the OPO beam (following **Figure 1**).

3.1.3. Verify that the x and y coordinates of the Ti:Sa laser beam are the same OPO in both positions of the sensors detectors. If in some positions the coordinates of Ti:Sa and OPO are not coincidental, tune the tilt of the adjacent mirror to compensate the difference (follow **Figure 1**).

3.1.4. Follow the same procedure to align the Ti:Sa beam positions with respect to OPO for the path in between M4-M5 (follow **Figure 1**).

3.2. Temporal synchronization of the beams

3.2.1. Use of the fast photodiode plus oscilloscope:

3.2.1.1. Stop propagation of the Ti:Sa and OPO beams and place a fast detector in front of the OPO beam (in between M6 and M7).

3.2.1.2. Connect the trigger signal provided by the Ti:Sa laser box with an oscilloscope in channel 2.

3.2.1.3. Connect the detector cable with the oscilloscope in channel 1 and visualize the OPO temporal profile.

3.2.1.4. Record the time (abscissa) measured by the oscilloscope corresponding to its maximum value, namely t1.

3.2.1.5. Stop the OPO beam and release the Ti:Sa beam.

3.2.1.6. Visualize the Ti:Sa temporal profile and record the time (abscissa) corresponding to its maximum value, namely t2.

3.2.1.7 Minimize the difference between t1-t2 using the delay line to overlap the two beams. In our case, the minimum measurable difference is 10 ps.

3.2.1.8 Remove the fast detector in between M4 and M5.

3.2.2. Use of the autocorrelator:

NOTE: In the schematic diagram shown in **Figure 1,** an autocorrelator is installed without interfering with optical paths of the beams. In addition, an additional mirror is introduced and mounted on a flip-flop mount (referred to as FFM/AM) in between M6 and M7 to divert the beam into the autocorrelator.

3.2.2.1. Flip the AM to direct the beam into the autocorrelator.

3.2.2.2. Stop the Ti:Sa and release the OPO.

3.2.2.3. Set the beam distance adjustment screw micrometer of the autocorrelator to **Normal position** (8.35 mm).

3.2.2.4. Power on the autocorrelator controller and start the software application on the personal computer controlling it.

3.2.2.5. Project the OPO beam from FFM/AM to the input mirror into the autocorrelator.

3.2.2.6. Control the reflection spot (using a paper detector card) of the beam on the alignment window of the autocorrelator.

3.2.2.7. In the case of no-beam or low-beam intensity, adjust the position and orientation of FFM/AM to the optimum extent, and try to adjust the input mirror (mounted on the autocorrelator) to maximize the laser pulse signal. The autocorrelator signal is obtained as shown in **Figure 5a**.

3.2.2.8. Stop the OPO and projection of the Ti:Sa beam from FFM/AM to input mirror into the autocorrelator. Repeat steps 3.2.2.6 and 3.2.2.7. The autocorrelator signal is obtained as shown in the **Figure 5b**.

3.2.2.9. Set the beam distance adjustment screw micrometer to **Cross position** (7.30 mm).

3.2.2.10. Release both beams.

3.2.2.11. Scan the delay line to obtain the two beams OPO and Ti:Sa overlapped. The cross-correlator signal is obtained as shown in the **Figure 6**.

3.2.2.12. Flip the mirror FFM/AM so that the beams can reach M7 and scan head of the microscope.

3.3. Microscope alignment

3.3.1. Perform white light microscopic observation:

NOTE: Prior to microscopic observation, ensure that the microscope is properly aligned.

3.3.1.1. Prepare the test sample, which consists of a phosphate buffer solution in which polystyrene beads with diameters of 3 µm are dispersed. The solution is placed inside a sandwich of two glass slides.

3.3.1.2. Turn on the microscope and power supply of white light. Follow the manual for observation under white light.

3.3.1.3. Use a condenser to illuminate the sample. Use objective of 60x to collect light. Place the sample onto the stage. Optimize the focal position of the 60x microscope objective.

3.3.1.4. Select the FOV of interest. Take a CCD image of the sample (**Figure 7**).

3.3.1.5. Turn off the power supply of white light.

3.3.2. Microscope alignment with femtosecond laser beams: OPO and Ti:Sa

3.3.2.1. Remove the condenser using the **escape** button to temporarily retract the 60x microscope objective lens. Move the 60x microscope objective lens off the optical path, rotating the nosepiece.

3.3.2.2. Mount the detector to the upper part of microscope using the external mechanical mount. Connect the detector output through a low-pass filter of 50Ω to oscilloscope and monitor the OPO signal.

3.3.2.3. Turn on the processor that controls the scanner head. Project the OPO beam into the scanner head of the microscope.

3.3.2.4. Check the position of the beam inside the microscope, make sure that the location of the beam is in the center or near the center.

3.3.2.5. Check the position of the beam inside the head of PD and check that the location of the beam is in the center or near the center of the microscope.

3.3.2.6. Maximize the power measured by the detector using an x-y translator.

3.3.2.7. Switch the beam from OPO to Ti:Sa and verify that they are both almost at the same position. THis indicates that both beams are well-aligned.

3.3.2.8. Finalize the beam alignment, introducing the 60x microscope objective lens and rotating back the nosepiece.

3.3.2.9 Use the **refocus** button on the microscope to regain the finalized focus to the 60x microscope objective lens.

3.3.2.10 Place the objective with magnification 40x in place of the condenser without touching or disturbing the sample.

3.4. Optimization of spatial and temporal synchronizations of beams

3.4.1. Temporal synchronization

3.4.1.1. Set the power of Ti:Sa and OPO measured before the microscope to 30 mW for both beams. Set the wavelength of OPO to a different value with respect to the previous one so that the pump and probe are not in resonance with the vibrational frequency of the beads.

3.4.1.2. Release both beams (Ti:Sa and OPO) so that they enter the microscope.

3.4.1.3. Run the scanning delay line computerized translator and record the measured intensity by LIA for each position of the delay line. Wait until the delay line scanning is complete. The obtained temporal profile is visualized in **Figure 8a**.

3.4.1.4. Set the wavelength of OPO to 1076 again so that pump and probe are in resonance with the vibrational frequency of the beads. Repeat step 3.4.1.3 (the obtained temporal profile is visualized in **Figure 8b**).

3.4.1.5. Set the obtained overlap beam position in the delay line to acquire SRS images.

3.4.2. Spatial synchronization of the beams

NOTE: The transmission images are useful to optimize the FOV, illumination, and focal position of the microscope objectives, and to check if the two beams are spatially overlapped.

3.4.2.1. Transmission image acquisition of OPO

3.4.2.1.1. Stop the Ti:Sa beam and reduce the OPO power to 8 mW.

3.4.2.1.2. Connect the detector readout to the data acquisition card.

3.4.2.1.3. Run the data acquisition program along with microscope scanning console.

3.4.2.1.4. Save the file and process the data to get the image. The raw image appears as shown in **Figure 9a**.

3.4.2.2. Transmission image acquisition of Ti:Sa

3.4.2.2.1. Stop the OPO beam and reduce the Ti:Sa power to 2.5–4.5 mW.

3.4.2.2.2. Connect the detector with LIA and LIA readouts with the data acquisition card.

3.4.2.2.3. Repeat steps 3.4.2.1.3 and 3.4.2.1.4. The raw image appears as shown in **Figure 9b**.

4. SRS image acquisition

NOTE: A dedicated algorithm has been realized in order to store data. It supports the following image formats: 512 px x 512 px and 256 px x 256 px, with acquisition times of 16 s, 8 s, 4 s, and 2 s.

4.1. Introduce a stack of filters in between the 40x objective and PD to remove the pump pulses (Ti:Sa) and acquire only the Stokes signal (OPO).

4.2. Set the pump signal to 810 nm with a focused power of 8 mW and the probe signal to 1076 nm with a focused power of 8 mW to investigate a typical C-H bond of polystyrene (Raman shift of 3054 cm^{-1}).

4.3. Connect the detector with the LIA and LIA readout to the data acquisition card.

4.4. Set the image acquisition pixel format as per requirement (we have supported formats of 512 x 512 pixels and 256 x 256 pixels) and set the acquisition time.

NOTE: There are acquisition time options of 16 s, 8 s 4 s, and 2 s.

4.5. Run the program that controls the microscope controller.

4.6. Run the dedicated algorithm program that acts as synchronization between the microscope controller, detection system, and DAQ (see **Figure 4**).

4.7. Save the matrix file once the acquisition is completed.

4.8. Import the raw data file and save the image in the required format (typically saved in .tif format) using ImageJ software. The image is shown in the **Figure 10**.

REPRESENTATIVE RESULTS:

An example of SRS measurement (i.e., SRS measurement in a single point of the sample) is

reported in **Figure 7**. When the beam is not overlapped in time or space, the obtained result is reported in **Figure 7a**. In off-resonance, the amplitude of signal measured by LIA is zero, while the phase of signal measured by LIA jumps between negative and positive values. In resonance, (i.e., when the beams are overlapped in space, moving the delay line in an appropriate range), the obtained results are reported in **Figure 7b**. The signal measured by LIA increases and reaches its maximum when the beams are perfectly overlapped, while the phase starts to achieve a fixed value during the time at which the beams are overlapped.

The absorption images obtained using a single beam (Ti:Sa or OPO) of the same polystyrene beads are represented in **Figure 9a,b** with scale bars of 6 µm. In order to acquire the SRS images, the delay line is set to the position achieved in **Figure 7b**, a typical SRG image is shown in **Figure 10** with a scale bar of 6 µm.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic layout of the f-SRS microscope system. OPO = optical parametric oscillator; Ti:Sa = Ti:Sapphire laser; M1-M4 = femtosecond mirror; M5, M6, M7, FFM/AM = broadband mirror DM1, DM2 = dichroic mirror; FFM/AM = flip-flop mirror/autocorrelator mirror; ACF = autocorrelation function, EOM = electro-optic modulator; FG = function generator; GM = Galvo mirror; Obj1, Obj2 = objectives 1 and 2; PD = photodiode; DAQ = data acquisition system; PC = personal computer.

Figure 2: Schematic of electronic scheme for phase detection system. Inset figures: “before sample” represents the two lasers beams before interaction inside the sample; “after sample” represents the modified probe due to interactions of the probe and pump inside the sample. Time is represented in ns, and laser output is represented in mW.

Figure 3: Representation of photodiode mount with mechanical mounting system.

Figure 4: Schematic of data acquisition system. PD = photodiode, LIA = lock-in amplifier, DS = detection system, MC = microscope control, DAQd= Data acquisition system, PC = personal computer.

Figure 5: Autocorrelator signal with OPO and Ti:Sa. (a) OPO signal on the autocorrelator and (b) Ti:Sa signal on the autocorrelator.

Figure 6: Raman peak of the autocorrelation function (ACF).

Figure 7: Peak with lock-in amplifier (LIA).

Figure 8: CCD image of the sample polystyrene beads. Scale bar = 6 µm.

Figure 9: Absorption images of (a) OPO and (b) Ti:Sa of polystyrene beads. Scale bar = 6 µm.

Figure 10: SRS image of polystyrene beads. Scale bar = 6 µm.

DISCUSSION:

SRS microscopy has taken label-free imaging to new heights, especially in studies of complex biological structures such as lipids, which are fundamental to cells and cellular architecture. Lipids are involved in multiple physiological pathways such as production of biological membranes, and they serve as biosynthetic precursors and signal transducers[10]. Lipids are packaged into specialized intracellular organelles, also called lipid droplets (LDs). Their diameters vary from few tens of nanometers to tens of micrometers[11,12]. LDs not only participate abundantly in adipose- and steroid-producing cells but are also present in other cell lines. LDs cooperate in a several physiological processes such as lipid storage. They are featured prominently in common pathologies (e.g., altered cholesterol metabolism)[13,14].

Traditionally, visualization of lipids is achieved using fluorescence microscopy and neutral lipid-specific dye-labeled fixed cells[10]. It should be noted that as lipids are smaller sized in comparison to proteins and DNA, structural and functional changes and unwanted artifacts can occur when adding fluorophores[15-16]. SRS has been shown to be powerful for studying lipid-rich structures. Lipids are abundant in C-H_2 groups. Therefore, the relatively isolated peaks associated with C-H bond vibrational states at 2845 cm^{-1} in their Raman spectra provide a unique signature for lipids inside a cell. Unfortunately, since the differentiable vibrational signatures are finite, it is rather difficult to distinguish a target biomolecule from the other related species inside cells that share similar chemical bonds. However, it is possible to add tiny Raman-active vibrational probes (e.g., alkynes and stable isotopes) to obtain specificity for imaging of small biomolecules[17].

For biological and biomedical in vivo applications, simultaneous mapping of various chemical species in a given sample is necessary for investing the co-distribution and dynamic correlations between pairs of biomolecules[18,19]. Therefore, many efforts have been made to obtain multiple chemical contrasts. In the simplest option of multicolor imaging, to image different Raman modes of a sample, the frequency of the pump beam or Stokes beam are tuned in sequential scans[18]. However, using the wavelength tuning approach may cause loss of co-localization information of different Raman modes, especially when the sample is in a dynamic environment[18].

As a consequence of nonlinear excitation, SRS offers intrinsic 3D resolving capabilities of the selected chemical bond within biological samples[20]. Volume reconstruction of the selected chemical bond and its spatial distributions can be simply achieved by collecting SRS images at different focal plane along the z-axis. Since the images are acquired with high spatial and temporal resolutions, other pieces of key information (i.e., 3D structure, chemical composition, etc.) about the biological sample can be obtained.

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DISCLOSURES:

The authors declare no conflicts of interest.

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Figure1

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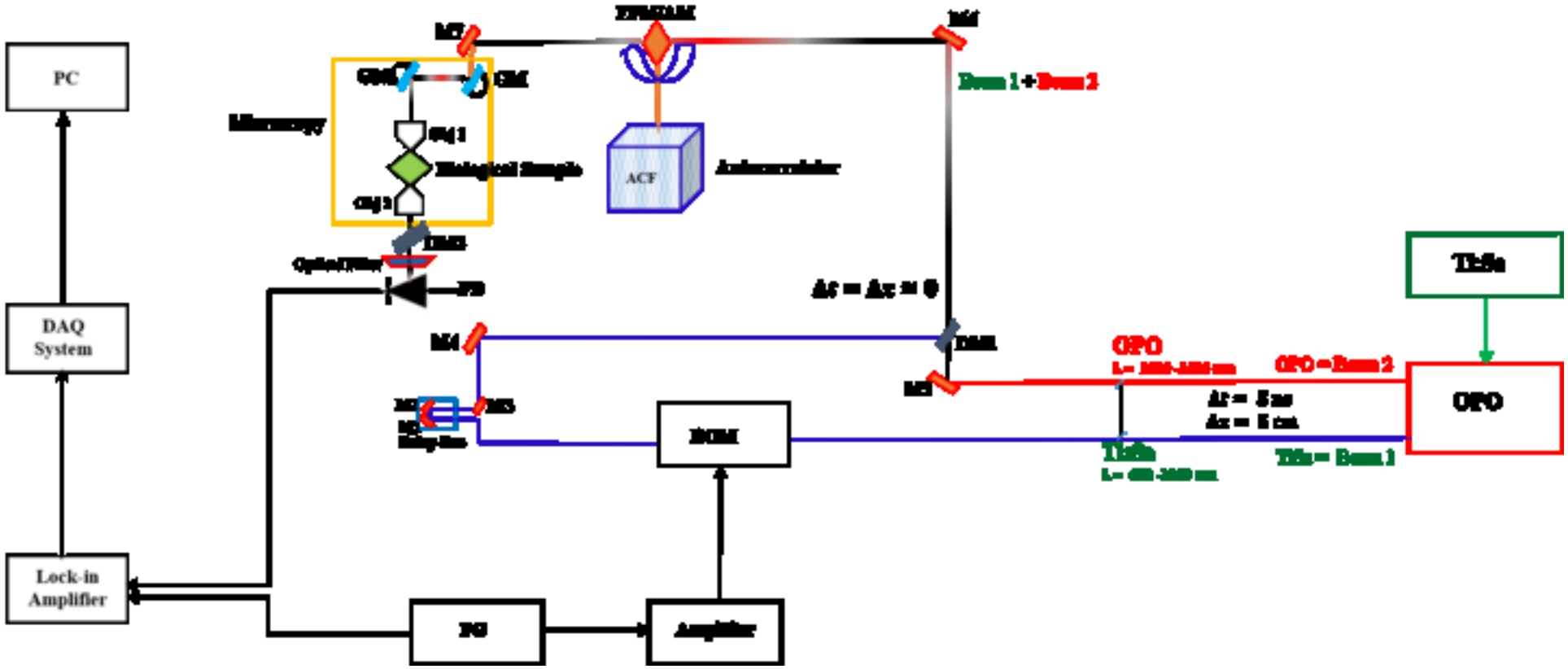

Figure2

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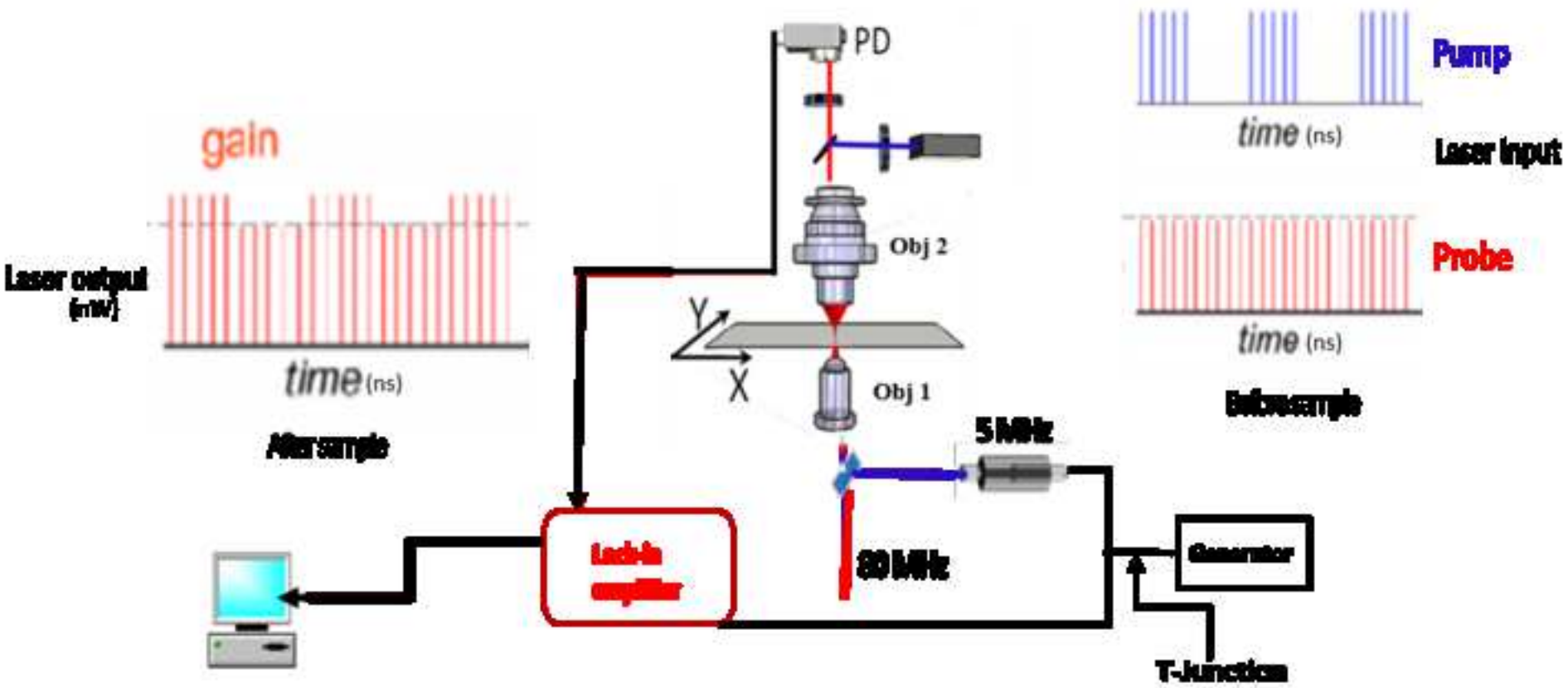

Figure3

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Figure5

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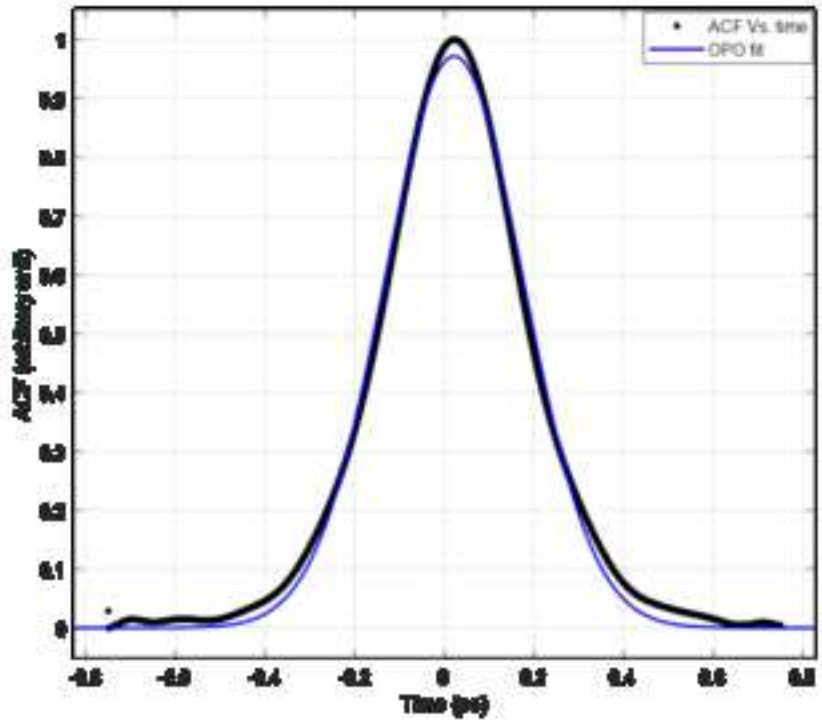

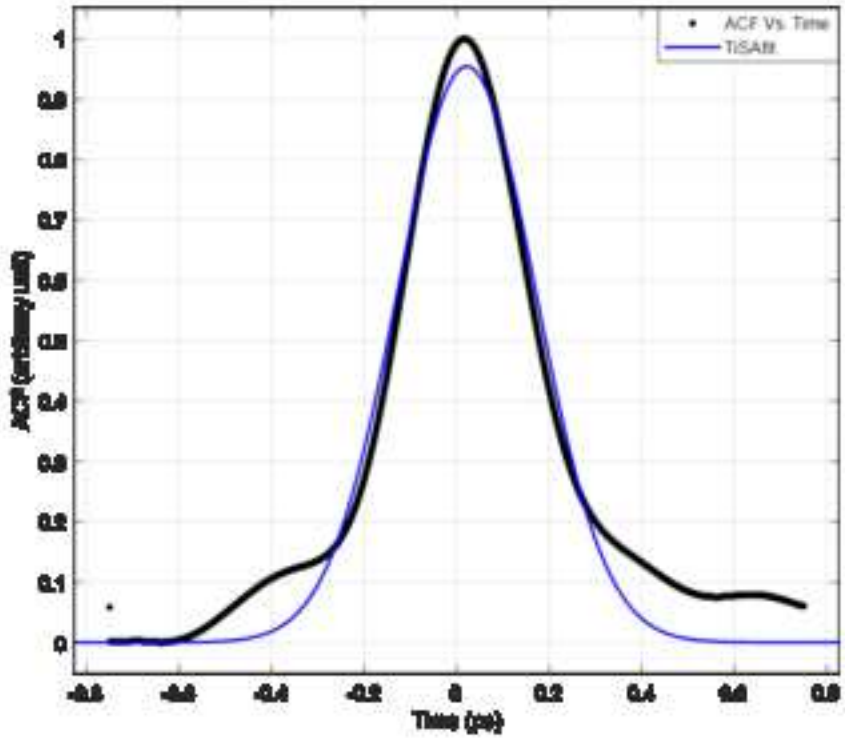

Figure6

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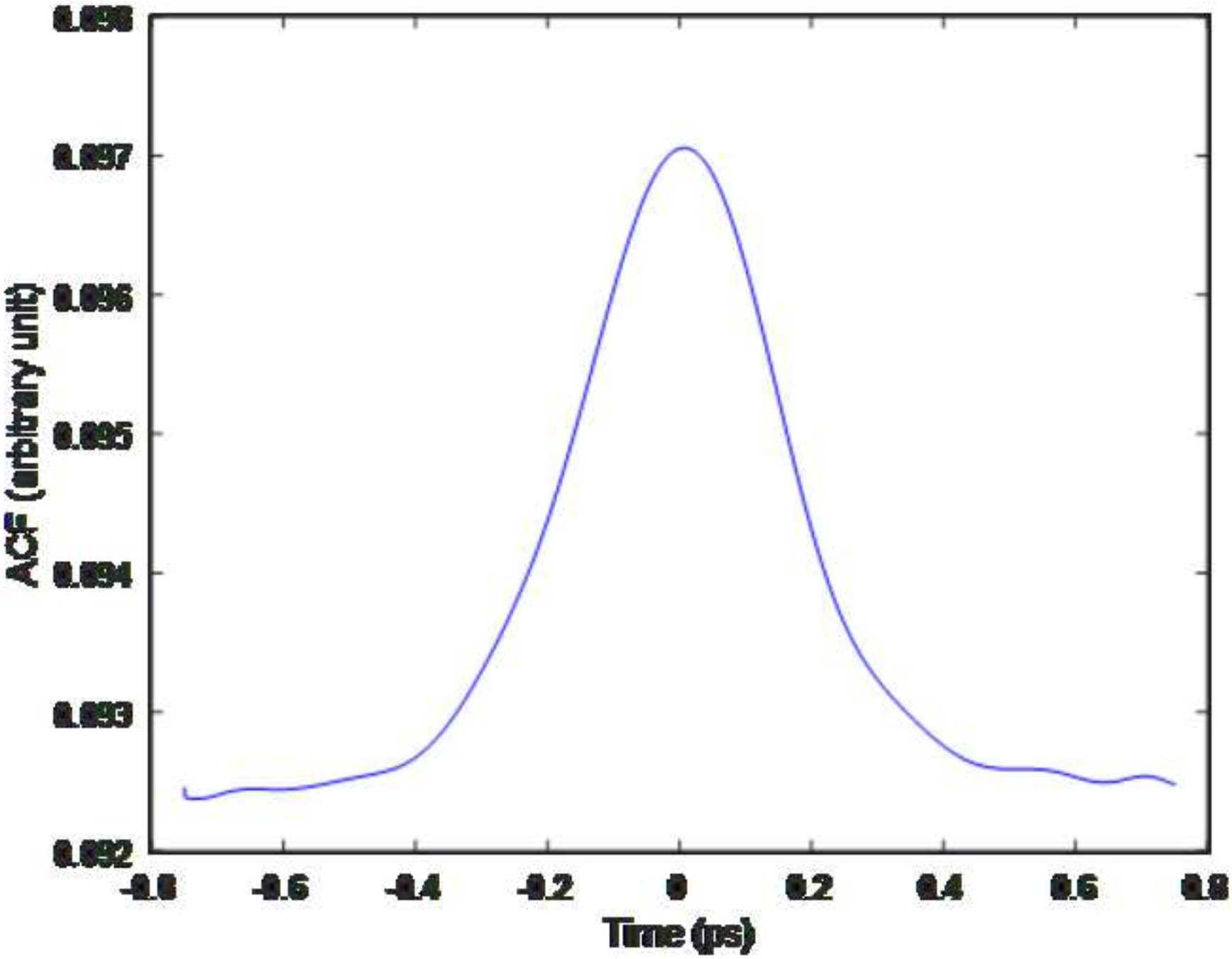

Figure7

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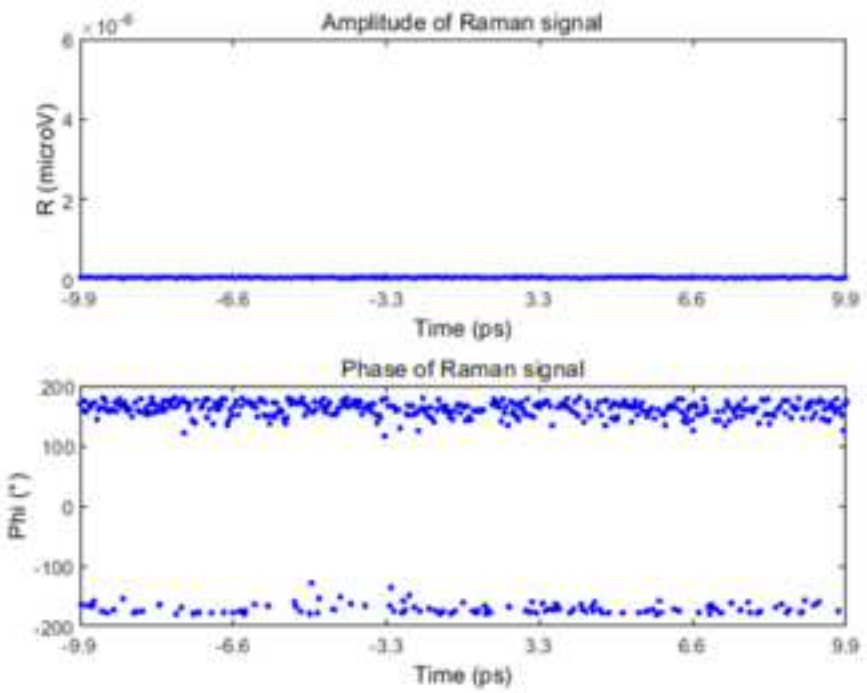

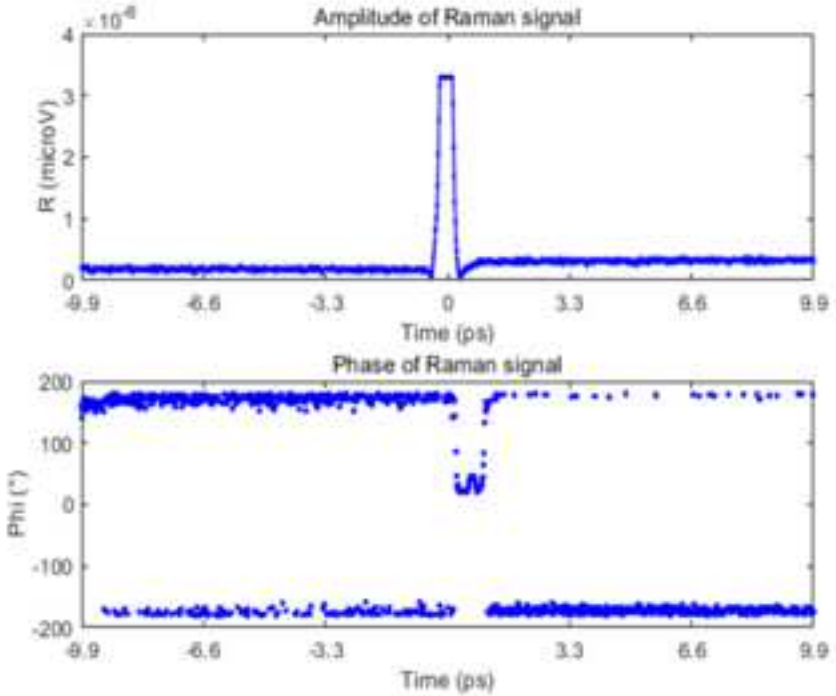

Figure8

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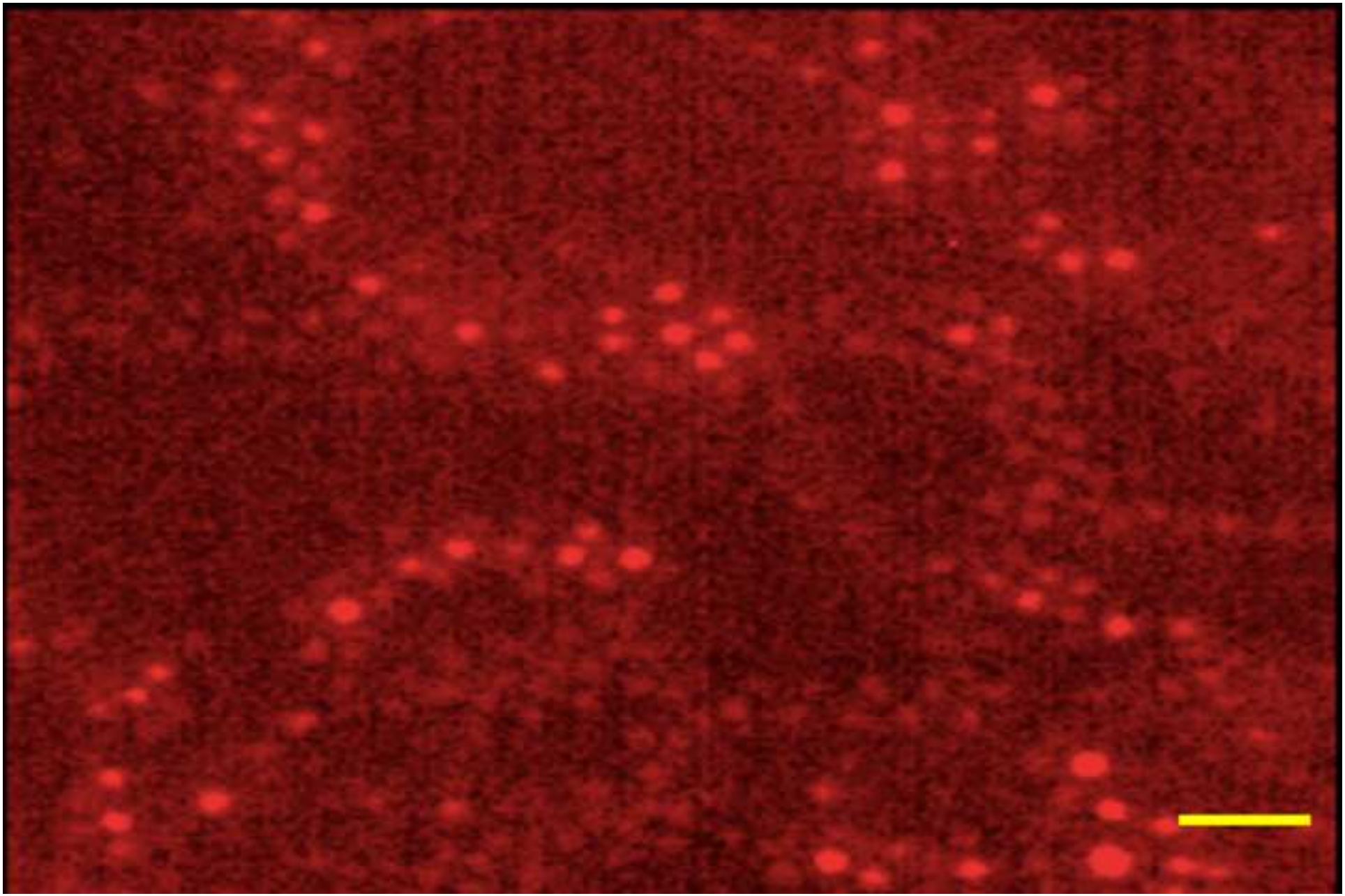

Figure9

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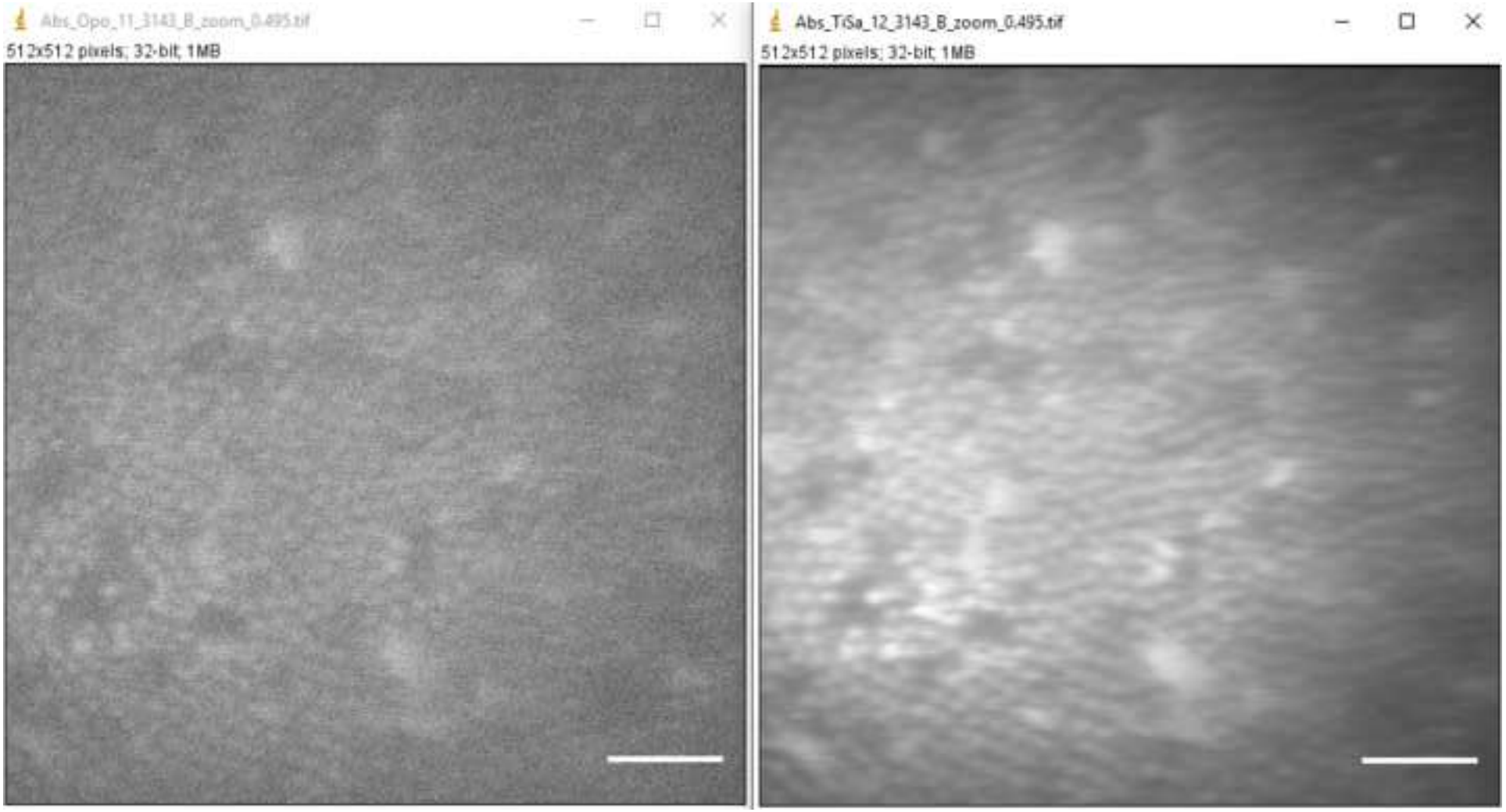

Figure10

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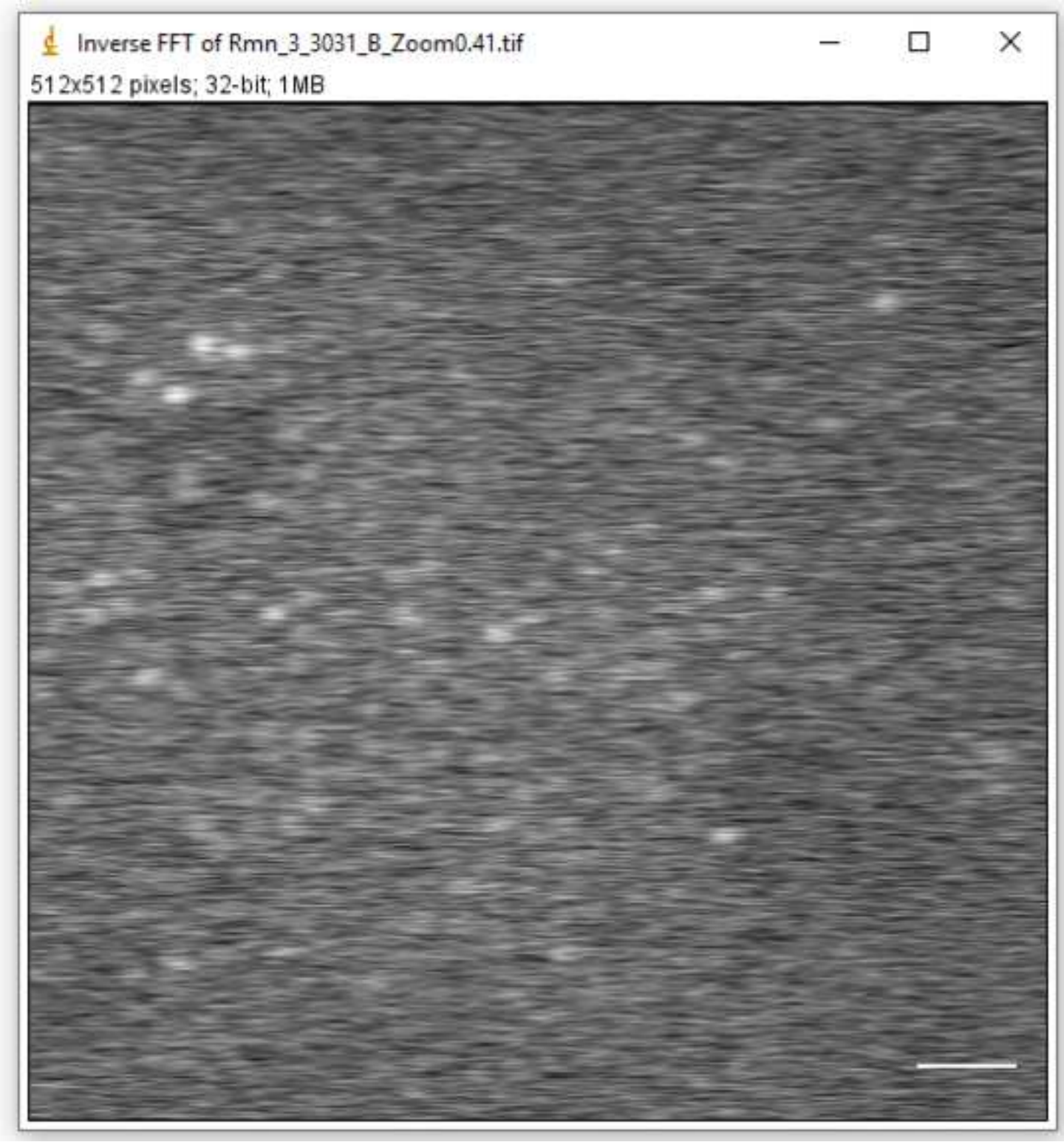

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Acquisation tool	Nikon	Nikon C2Tool
APE Pulse link control software	APE-	APE Pulse link control software
Autocorrelator	APE	APE PulseCheck USB 50
Detector	Thorlabs	Thorlabs DET10A
Detector card	Thorlabs	Thorlabs VRC
Dichroic mirror	Semrock	Semrock FF875-Di01-25X36
Dichroic mirror	Semrock	FF875-Di01-25x36
EOM	Conoptics	(EOM CONOPTICS 3350-160 KD*P).
Fast detector	Thorlabs	Thorlabs DET025AL/M
Fast mirror scanning unit	Nikon	C2
Femtosecond laser Ti:SA	Coherent	Coherent Chameleon Ultra II
Function generator	TTi	TG5011 AIM – TTi
Inverted optical microscope	Nikon	Eclipse TE-2000-E, Nikon
Lock-in Amplifier	Standford Research System	SR844-200 MHz dual phase
Notch filter,	Semrock	NF03-808E-25
Optical delay line	Newport	Newport M-ILS200CC
Optical Parametric Oscillator	Coherent	Coherent Compact OPO
Oscilloscope	WaveRunner	640Zi 4GHz OSC/LeCroy
PCI Card	National instrument	NI PCIe 6363
Position Sensors Detectors	Newport	Newport Conex PSD9
Power meter head	Coherent	PowerMax PM10,
Translation Stages	Thorlabs	Thorlabs PT1/M

Comments/Description
Acquisation supported tool
software control
Autocorrelator
Photodiode
IR detector Card
Dichroic mirror
Dichroic mirror
Pockels cell
Photodiode
Microscpe scanning head
Chameleon Ultra II
Function generator
Eclipse TE-2000-E, Nikon
A lock-in amplifier from Stanford Research Systems
Notch filter
Tunable optical delay line
Coherent Compact OPO
Digital Oscilloscope
Data acquisation card
Position detector sensor
Laser power detector
Meachnical Translation Stage with Standard Micrometer

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Institution:	National Research Council
Title:	Researcher

Signature:	L. Sirleto	Date:	December 26, 2018

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Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
• **Textual Overlap:** Significant portions show significant overlap with previously published work. Please re-write lines 20-26, 99-102, 137-139, 414-419, 425-431, 440-445 to avoid this overlap.
Following the suggestion we amended the same in revised manuscript.

•**Protocol Language:**Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

1) Examples NOT in imperative voice : 2.2.1, 2.2.2, 2.2.4, 2.3.1-2.3.8, 4.1,4.2, etc.
Following the suggestion we amended the same in revised manuscript

•**Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:

1) 1.2: what is the humidity limit? See line 172
2) 1.11: Mention wavelengths tuned to. See line 175 and 185
3) 2.1: Please describe in detail how this is done.
4) 2.2: Mention wavefrom shape, frequency, amplitude, pulse width etc. See line 191 and 192
5) 2.7: What is the proper voltage level? See line 199
Following the suggestion we added in the revised version of paper all required details.

•**Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations.
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Following the suggestion we amended the same in revised manuscript

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• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

In order to comply with these requirements in the discussion of revised version we added two new paragraphs (line 442-459).

• **Figures:** Please remove the embedded figures from the manuscript. Figure legends, however, should remain within the manuscript text, directly below the Representative Results text.

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Reply to Reviewers:

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript reports the implementation of a stimulated Raman scattering (SRS) microscope obtained by the integration of an SRS spectroscopy set-up with a laser scanning microscope. This SRS microscope is based on two femtosecond (fs) laser sources: a Ti-Sapphire (Ti:Sa) and a synchronized optical parametric oscillator (OPO).
The developed system appears to be very important for bio chemical application and of course for the research in Material science.
It is worth noting that SRS microscopes are not commercially available, so in order to take advantage of their characteristics, the home-made realization is the only possible option.
Paper sounds well, the experimental part is clearly reported and discussed allowing to develop the system in other labs.
I have highly appreciated this work.

We sincerely thank the reviewer and appreciate for time you spent reviewing our manuscript. We also like to thanks for the positive comments and appreciation of our work.

Reviewer #2:

Manuscript Summary: The manuscript submitted by Ranjan et al. presents the implementation of a stimulated Raman scattering (SRS) microscopy by combining SRS spectroscopy with a laser scanning microscope system (LSM). Since no commercial SRS microscope system is available at the moment, if efficiently presented, this manuscript could bring a detailed guide of how to set up such a system on an existing LSM, and become useful to the imaging community.

We thank the esteem reviewer for his appreciation of our work.

However, I found many major weaknesses of this manuscript.

We would like to thanks reviewer for his constructive suggestions. We highly acknowledge the reviewer for highlighting the major and minor concerns about the manuscript. We are replying the raised issue point by point.

Major Concerns:

Overall, I found the writing requires much rework, and ask JOVE to require a major revision from the authors, before considering it for publication.

In details, I find many sentences and paragraphs need to be better arranged and organized. Also the English should be used with more care and in the professional style common in science community. References are not used effectively. Reference 1-9 are all together used in the "Discussion" while no reference was used in the "Introduction".

Following the comments, we have thoroughly revised the English of our manuscript. Additionally, we have included more reference in the introduction section of the manuscript.

1) Line 19: What is the "problem of other coherent Raman techniques,"? Name a few.

Coherent Antistokes Raman scattering suffers from nonresonant background. Due to a nonresonant background, CARS spectrum is different from the corresponding spontaneous Raman spectrum. This background complicates spectral assignment; it causes difficulties in image interpretation and limits detection sensitivity[7]

In the revised version we substitute 'other coherent Raman techniques' with 'coherent antistokes Raman scattering' (see line 18).

2) Line 22 and Line 33 stated about the availability of a commercial system. Since there isn't any yet available, state that and indicate the significance of this work.

We rewrote the abstract, according to the suggestion of reviewer (see line 34-42).

3) From Line 40-141 (Introduction): The "Introduction" is the part I find to need most revision. In the introduction, the readers would expect a certain level of introduction of the field, the history of the technical developments, the current status and limitations of the systems available, what the authors achieved (improvements/breakthroughs) or how the authors overcame the limitations, and the significance of the work (academically, financially or etc.). However, the "Introduction" of this manuscript barely state any of these aspects. I indeed found the earlier paragraphs in the "Discussion" (Line 409-424) are more appropriate to be placed in the "Introduction". Without such content, the current "Introduction" looks more likely a method section of a paper. I understand that these descriptions are necessary, and due to the format of JOVE, they are too long to be put in "Protocol section." Thus if it is inevitable to put the technical details in the "Introduction," they have to be better arranged in a more logical flow, with a smooth transition from the regular introduction content (Line 409-424).

We move the line 408-424 to the beginning of introduction according to the suggestion of reviewer (see line 44-57).

4) Line 44-46: The first laser stated is the Ti:Sa (pump) laser, but it is stated as "Beam2" in Fig. 1. Try to state the two lasers in the same order as in the Figure.

We have revised and reproduced the figure accordingly.

5) Line 53-59 summarizes three issues of the SRS microscopy, and the following three paragraphs give detailed descriptions of those issues (Line 60-88). This arrangement is not indicated or stated anywhere, so the readers would get confused initially, also because Line 53-59 state elsewhere of the manuscript (2.1~2.3). For a better flow, indicate this way of flow by inserting "(and will be described in the following paragraphs)" at the end of Line 52.

We inserted the suggest sentence suggested by reviewer at the end of line 68 of revised version.

6) Line 52: "some important issues" -> avoid using words like "some." State exactly what these issues are in a proper category.

In the revised paper we substitute ‘some important issue’ with ‘three crucial issues’ (see line 68).

7) On the other hands, Line 53-59 do not deliver the essential idea of each issue. For example, Line 55-56 stated about the mechanical arrangement, but skips why this became an issue (which was stated later in Line 70-75, the sensitivity and noise problems). The summary of each issue should be concise and compressive at the same time. Put lines between paragraphs. Currently it is hard to identify different paragraphs.

We have inserted a part of line 70-75 in the summary of the issue according the suggestion of reviewer.

8) Start the three paragraphs (Line 60-88) by restating the issues stated in Line 53-59, so that it would read clearly that these paragraphs are describing the issues in details. For example, Line 76 should start with a sentence including the word, “synchronization” Cite the proper figures in any paragraph describing the system details.

We have rearranged the revised version of paper according to the suggestions of reviewer (see line 91-93)

9) Line 62: the “Therefore” seems not right. To deliver the message logically, the sentence should be like “We overcame this noise issue by implementing a high frequency modulation…”

Following the suggestion, we amended the same in revised manuscript (see line 74)

10) Line 63: “According to” seems not right. “this process” should mean the previously stated “high-frequency modulation transfer method, but this method was never explained in detail. Therefore, instead of "According to this process,” “In this method” should be used.

Following the suggestion, we amended the same in revised manuscript (see line 75)

11) Line 72: “white light” -> does this mean the brightfield imaging?

Yes. We substitute white field for brightfield into the text (see line 88).

12) Line 82: What is “Nikon controller”? According to Fig. 3 it should be what is called “Microscope controller” but no detail of what it is described.

We substitute “Nikon controller” with ‘microscope controller connected to the scan head unit’ (see line 101).

13) Line 95-125: These are the details of the four steps stated in Line 91-94. Again, the readers should be guided properly for those paragraphs. Put “(and will be described in the following paragraphs)” at the end of Line 90. Also start each paragraph of Line 95-125 by restating the according step stated in Line 91-94.

We inserted ‘’will be described in the following paragraphs” (see line 108 of revised version).

14) Line 99: “Recently, OPO technology has allowed perfect synchronization…” -> Can a reference be placed here?

Yes, we added the reference 9 in revised paper.

D.T. Reid, J. Sun, T.P. Lamour, and T.I. Ferreiro. Advances in ultrafast optical parametric oscillatorsLaser Phys. Lett., 1–8 (2010) / DOI 10.1002/lapl.201010085

15) Line 101: “5 cm” -> is this correct? This looks too big spatial difference.

Yes

16) Line 117-118: “To realize the image… software is used” -> This is not relevant to the “steps” topic (Line 93). This sentence can be placed in a separate paragraph in the end of “Introduction”.

We revised it and address the issue (see line 164-165).

17) Line 126: Avoid a value word like “some”. In fact, these tests are stated in Line 128-140, but this flow is not well indicated. Thus, state it by "we carry out the tests described below, …"

Following the suggestion, we amended the same in revised manuscript (see line 148).

18) Line 133: "The only difference" -> What difference to what? The sentence including this phrase is describing the difference between the procedures of obtaining transmission images between OPO and Ti:Sa, but this sentence can be very confusing. Restate this in a clear way.

We revised it and address the issue (see line 151-155).

19) Line 185 as well as Figure 3: Figure 3 is too simple, and the visualization of section 2.3 is very weak by Figure 3. Either by putting more words or graphical descriptions in Figure 3, support the idea of 2.3. Then describe each component of Figure 3 (especially the "Detection System" and "Microscope controller" well in the description (from Line 188-206). Currently it is not very clear which parts are describing what. "Note" can be used to divide parts in Line 188-206, so that readers would know what is described in Fig. 3.

We note that Figure 3 describes the hardware of synchronization system, while paragraph 2.3 describe the basic idea of our ‘in house software’ realized to obtain the synchronization. In the revised version of figure 3.a, we add graphical descriptions and new words and also change the name of paragraph 2.3. The new one is Synchronization software.

20) Line 190, 192 and 194: Put quotation marks around the TTL signal, so “frame sync”, “pixel clock” and “line sync”

Following the suggestion, we amended the same in revised manuscript (see line 100-101).

21) Line 192-193: This sentence is very confusing. Make it simple and clear.

We substitute the sentence with a new one (The basic idea of **Syncronization software** is the following: the frame sync controls the start acquisition in post trigger synchronism mode, line sync manages the memory area and the image realization, while the pixel clock is used as SRS signal acquisition sampling rate. A dedicate algorithm in LabVIEW12 has been realized in order to store data. Here, we describe the main steps), (see line 208-212).

22) Line 208, 220 and many other places: State “Figure 1” in the note or steps. The readers should know what Figures to look at when reading the steps.

Thanks for suggestion. We have amended the manuscript accordingly.

23) Line 221: The title is strange. It should be like “Procedure using fast photodiode plus an oscilloscope”

We have implemented this change in the subtopic (see line 248).

24) Line 238: Put the “AM” and “autocorrelator” in the Fig. 1

We have reproduced the figure 1 following suggestion.

25) Line 242-263: No visualization is given. Video can include this part, or photos can be placed in the manuscript.

We have already Figure 4a (Line 279), 4b (Line 283) and figure 5 (287) which are dedicated to this section.

26) Line 242: What is “ACF”? It was never introduced.

We remove it from the text

27) Line 244: What is “APE application”? APE is a company, so either state the software name, or just state it like software #1 and put the details in the materials list. Same for "pulseLink."

APE application is the support and control software for the Autocorrelator.

28) Line 246, 257 and many other: Avoid using the word, “now”

We revised the manuscript following the suggestion.

29) Line 262 and several other places in steps: this should be written in the imperative tense.

We revised the manuscript following the suggestion

30) Line 271: What manual? The microscope manual?

Yes.

31) Line 276: Avoid the word “you” but instead state like “FOV of interest”

Thanks for suggestion we amended it (see line 302).

32) Line 282-303: Visualization is very weak. Adding some photos will help.

We additionally added the picture of the upper section of microscope where the photodiode is mounted with mechanical mount (see figure 3a).

33) Line 311: What to do with Figure 7(a) and (b)? Repeat the work until (b) is obtained?

We rewrote the paragraph 3.4.1. and we repet the work until (b) is obtained (see line 331-345).

34) Line 349-360: SRS images of beads are obtained, but what is the useful and additional information we can extract out of SRS image of beads compared to the conventional brightfield or epi images? Also can there be a biological sample images obtained by this SRS microscope?

Being the beads, a homogeneous sample, we don’t expect additional information we can extract out of SRS image compared to the conventional brightfield or epi images. Concerning biological sample, in order to elucidate this point we added two new paragraphs in discussion (line 442-459).

35) Figure 1 & Figure 2: I suggest combining Figure 1 and 2 since they are very much related and can help readers to understand if combined. Color code should be consistent. Figure 1 put green color for the Beam 2 (pulse laser) but Figure 2 uses blue color. Also indicate each objective differently (like Obj1 and Obj2), since Figure 1 and 2 have objective in the opposite orientation.

In the revised version of figures 1 and 2, color code are consistent and focused and collected objective are indicated in the same way (Obj1 and Obj2). However, we don’t prefer to combine them.

36) Figure 2: indicate where the “T-junction” is.

We have indicated in figure 2 where the T-jucntion is.

37) Figure 3: put more details as stated before. Also the arrows and figures should be better aligned (also for Figure 2).

Following the suggestion we amended the same in revised manuscript.

38) Line 409-424: as stated before. Those should be moved into the “Introduction”.

In revised our manuscript we move line 409-424 at the beginning of ‘Introduction’.

39) Line 409: “is a great opportunity” is a too vague statement. State the advantages specifically.

We revised our manuscript and we introdude the sentence , SRS microscopy of biological samples is one of the most important player for future challenges of label free imaging.

40) Line 410-413: The sentence is too long.

Following the suggestion, we have shortened it (see line 44-47).

41) Line 425-438: Each paragraph should deliver a single idea, but this one has multiple stories. For example, Line 430-432 describes a limitation of SRS microscopy, but it doesn't serve any role. Try to make each paragraph to deliver a single idea, and if otherwise, separate it into several.

We have amended it.

42) Line 450: "very expensive" give a rough estimate of the cost.

Roughly estimate figure is about € 400 KEURO

43) Line 446-447: This is the main selling point of this manuscript, so it should be put into the "Introduction" as well as "Abstract"

We rewrote the abstract section of the manuscript according to the suggestion of reviewer (see line 34-42).

44) Line 451-452: This sentence should be written in a more professional way, like "becoming popular in varying fields."

We rewritten the sentence (sse line 40).

45) Line 455: delete "really"

Deleted.

Reviewer #3:

Manuscript Summary:

The manuscript provides a good amount of detail about the construction and implementation of a stimulated Raman scattering microscope system based on a Nikon microscope frame. Methods in beam alignment and signal acquisition conditioning are discussed wherein an SRS image of polystyrene is lastly demonstrated.

We thank reviewer for the constructive inputs about our manuscript.

Major Concerns:

1. The article's abstract describe the construction of an SRS spectroscopy setup however it was not clear that spectrometers were used or any kind of spectroscopy was performed. The pump and Stokes beams were described to have pulse durations of 140 fs and 200 fs, respectively. I presume that the bandwidths of the laser beams span several nm or wavenumbers and tuning the wavelengths of the lasers at 809 nm and 1073 nm not only excites the 3054 cm^{-1} but more likely a large span of the CH vibrational region. Without any sort of dispersion mechanism for the two beams, this setup prevents vibrational spectroscopy with a reasonable value for spectral resolution. Is there by any chance a dispersion mechanism is inserted somewhere in the setup, if so, what would be the spectral resolution of the system? Otherwise, the use of the term ""spectroscopy", should be refrained from the manuscript as it may confuse readers.

According to the suggestion of reviewer, we refrained from the manuscript the term 'spectroscopy'

Minor Concerns:

The article is written with great detail and may be very helpful for a lab with the aim of building an SRS microscope or even something similar. However, there are numerable inconsistencies and vagueness in the article draft which are listed below:

1. line 65 mentions PD which I assume stands for photodiode. Please include manufacturer information and model.

Yes, PD stands for Photodiode. Acronyms PD is also defined in line 31 of revised version. The manufacture is Thorlabs and the model is DET10N/M. Following the guidelines and policies of JOVE we can't add the commercial details of the products, but we have described all the details in material list.

2. line 74: in order to avoid small oscillation of detection system --> does this mean mechanical vibration?

Yes. This mechanical vibration may generate from the mechanical mounting. In the revised paper we substitute small oscillation with mechanical vibration (sww line 82).

3. Please correct typographical errors in optic elements:

3.1 Line 96: Typo in Semrock FF875-Di01-2536, should be FF875-Di01-25x36.

3.2 Line 103: Typo in Newport MOD MILS200CC, should be Newport M-ILS200CC

We have corrected and added the commercial information(s) of all the products from the manuscript following the guidelines and policy of JOVE in the material list.

4. Ti:Sa and OPO are often mentioned in the article and they clearly mean pump and Stokes. Please revise as suited.

We have revised the same as suited.

5. Line 192: The pixel clock adjusts the reading by the buffer of the voltage values to be referred as pixel, acting as a sample clock ---> Confusing. Please rephrase.

We have rearranged it (see line 217-218 of revised version).

6. "the peak overlap of two beams is obtained with an approximation of 10 ps" ---> Seems vague, what does approximation of 10 ps mean? Is this device resolution?

It is the uncertainty of our measurements (we specify inside the text see line 126)

7. Line 213: I could not find a Newport SI-PSD9, perhaps this is the Newport CONEX-PSD9? If so, please revise.

Yes, it is CONEX-PSD9. We have added the commercial information(s) of all the products from the manuscript following the guidelines and policy of JOVE in the material list.

8. Line 213 to Line 217: The pump beam (Ti:Sa) does not seem to pass through M1 so the placement of laser beam position detector at M1 would only be useful in detecting the Stokes beam. How would the placement of the sensor at M1 allow the detection of the pump beam?

Following the suggestion, we amended the same in revised manuscript. We rewrote the 3.1 paragraph (see lines 239-249).

9. Line 242: The abbreviation ACF was not previously mentioned, please clarify what ACF means.

ACF indicates the autocorrelator signal. We remove the abbreviation from the text.

10. Line 283: Typo in "Photodiode DET10A". I believe this should be Thorlabs DET10A.

Thanks for the figuring out the typo. We have corrected it. We have added the commercial information(s) of all the products from the manuscript following the guidelines and policy of JOVE in the material list

11. In sections 3.2b and 3.3b, laser beams are incident on detectors (autocorrelator and photodiode) and the use of large powers may saturate or damage these detectors. A short mention of the laser powers may provide additional guidance to the readers.

We have mentioned the laser power in revised manuscript (see line 335 and 377)

12. Line 349: An example of SRS spectroscopy measurement --> remove "spectroscopy" if no spectroscopy was actually demonstrated.

We remove ‘spectroscopy’ in revised version.

13. Figure 1: Since there are already a lot of details included, a placement of optical filters may somehow add usefulness to the figure.

We added optical filter into the figure1.

14. Figure 9 shows a streaky and noisy image. From your research group's other publications, e.g. D'Arco et al. Biomed. Opt. Express 7, 1853-1864 (2016), Ranjan et al. Opt. Express 26, 26317-26326 (2018), shown are higher quality images of polystyrene beads. Perhaps using a similar image with better quality may improve the impact of this paper to future readers. If possible, try to explain why streaks appeared on the first image vs. the new. Explain steps to minimize image streaks/noise.

In submitted version of paper, we inserted a rough image (without using image processing technique), while in reference cited by the reviewer an image processing was used. In revised version we improve the quality of image, using a simple filtering process.

15. Line 440 - 441: distinguish a target biomolecule from the sea of the other related species... I assume "sea" is a typo, please revise.

No sea is not a typo. It is right. This paragraph describes the difficulty in identifying a particular type of biological species from the large amount of species which share same chemical bonds.

16. The manuscript seems to mention some jargon on the image acquisition details (sec. 2.3). I would suggest, though not heavily required, about adding a figure related to the image acquisition system in order to clarify concepts and terms.

We have revised figure 3 in order to clarify concept a terms

17. Reference #3 is about CARS and not SRS. Adding this to the list of references in relation to its citing paragraph (line 424) does not seem to be appropriate. Please revise accordingly.

We have removed reference 3 and updated our list of references.

[A1] Please thoroughly review the grammar in your article. Several scattered errors are present which hinder comprehension. I have flagged some (not all) of them.

[R1] We have revised it and corrected the same wherever it required.

[A2] Please reduce this SUMMARY to 300 words.

[R2] We reduce the abstract and now it is only 291 words.

[A3] I edited this, please check if it is correct.

[R3] Yes, it is correct, but the sentence has been removed in order to reduce the abstract.

[A4] Please clarify, you mean the transmitted light is collected correct? Please avoid field-specific jargon as much as possible.

[R4] Yes it's correct. We try to avoid jargon.

[A5] Filters?

[R5] Yes.

[A6] by synchronizing.

[R6] Yes.

[A7] Something is missing here.

[A8] Unclear what is being said here.

[A9] Tensing errors.

[R7], [R8]. [A9] the sentence has been removed in order to reduce the abstract

[A10] Fields?

[R10] Yes.

[A11] Awkward phrasing.

[R11] We revised it.

[A12] Awkward phrasing.

[R12] We revised it.

[A13] Awkward phrasing.

[R13] We revised it.

[A14] Avoid bullet points as much as possible in the introduction.

[R14] We had address it.

[A15] Needs grammar revisions.

[R15] We revised it.

[A16] Step 2.1 of the protocol?

[R16] Yes.

[A17] Awkward, needs revisions.

[R17] We revised it.

[A18] To?

[R18] yes.

[A19] This whole sentence needs revision.

[R19] We restructured the sentence.

[A20] Step 2.2 of the protocol?

[R20] Yes.

[A21] Please revise.

[R21] We did it.

[A22] Please revise.

[R22] We did it.

[A23] This whole sentence needs revision.
[R23] We revised it.
[A24] revise
[R24] Done.
[A25] ??
[R25] the sentence has been removed.
[A26] revise
[R26] Done.
[A27] step 2.3 in the protocol?
[R27] Yes.
[A28] Due to the presence of numerous grammatical errors, I am no longer flagging errors in the rest of the manuscript. Please assume that the entire manuscript needs grammatical revisions and proofing by a proficient English speaker.
[R28] We have revised the entire manuscripts and corrected the errors and grammars.
[A29] Please remove the bullet points and use proper sentence punctuation.
[R29] We had remove the bullet points.
[A30] I have edited the protocol structure to meet JoVE's requirements.
[R30] Thanks.
[A31] Laser output?
[R31] ***bypass*** itself represent the laser output, but for better clarification we have added **output**
[A32] What should the power level be for Ti:Sa and OPO?
[R32] We add the value at point 1.13
[A33] The current highlight (~5 pages) exceeds the limit (2.75) pages; please trim it while retaining continuity and completeness.
[R33] We reduced it within the limit.
[A34] Sentence is incomplete. Please re-write in the imperative voice
[R34] We rewrote it, following the suggestion.
[A35] This section lacks filmable content; I recommend unhighlighting it.
[A36] Please avoid commercial names in the manuscript. Please add this to the table of materials.
[A37] Please edit the step numbers and re-write all steps in the section in the imperative voice.
[R35, R36, R37] These paragraph has been delete because not suitable with the aim of protocol paragraph
[A38] Please provide exact distances and positions if relevant. Define what DM1, M4, M5 are.
[R38] It's not relevant. DM1 is Dichroic Mirror, M4, M5 are the mirrors; also described in the figure1 as well as in the material lists.
[A39] Please clarify; I cannot understand what is meant here.
[R39] It's means that we need to verify the positions considering one beams as a reference beams. If we considered OPO beam as a reference beam at position P1 (i.e. in-between DM1 and M4) the X and Y is (X1.xx, Y1.yy) µm then we have to align the Ti:Sa beam at the position P1 in that manner so that it's must be (**X1.xx ± 0.1, Y1.yy±0.1) µm.**
[A40] Perhaps either 3.2.1 or 3.2.2 can be highlighted instead of both.

[R40] 3.2.2 highlighted.
[A41] Update
[R41] Updated.
[A42] update.
[R42] Updated.
[A43] update
[R43] Updated.
[A44] update
[R44] Updated.
[A45] add scale references (with units) for time and output
[R45] Added.
[A46] Add a title to fig 3.
[R46] Added.
[A47] Add a title
[R47] Added.
[A48] Add a title
[R48] Added.
[A49] ?
[R49] Reference.
[A50] check
[R50] Checked.

[1] Please rewrite lines 55-58, 80-87, 481-513 using original text to avoid overlap with previously published works.

Reply We have addressed these issues. Revised line are as follow:
Line 55 – 58: Revised Lines: 60 – 63
Line 80 – 87: Revised Lines: 91 – 97
Line 841 – 513: Revised lines: 503 – 512
: 528 – 538
: 548 - 555