Supplementary Materials

I. Preferred pulse parameters for SRS

Achieving maximal sensitivity under biocompatible excitation conditions is a major challenge when designing laser sources for SRS microscopy. It is critical to consider the SNR for various laser parameters. For continuous wave (CW) lasers, the SRS signal is proportional to the product of the average power of the pump and Stokes beams, i.e. the SRS signal in nonlinear in the overall excitation power. It is thus advantageous to utilize pulsed lasers, which have high peak power but moderate average power to minimize heating due to linear absorption. For pulsed lasers with average power $\hat{I}_{p,S}$, pulse duration duration $\tau$ and repetition rate $R$, the SRS signal is propoertional to $\left( \frac{\hat{I}_p}{\tau \cdot R} \right) \cdot \left( \frac{\hat{I}_S}{\tau \cdot R} \right) \cdot \tau \cdot R = \left( \frac{\hat{I}_p \cdot \hat{I}_S}{\tau \cdot R} \right)$. The shot noise only depends on the average power of the pump beam and is proportional to $\hat{I}_p^{0.5}$. Consequently the $\text{SNR} \propto \hat{I}_S \cdot \hat{I}_p^{0.5} / \tau \cdot R$. For a fixed total average power $\hat{I}$ at the sample, it is thus advantageous to chose $\hat{I}_S = 2\hat{I}_p = \frac{2}{3} \hat{I}$ to maximize the SNR. The overall SNR is:

$$\text{SNR} \propto \hat{I}^{1.5} / \tau \cdot R.$$ 

To avoid the need for additional synchronization of the laser repetition rate and the pixel clock, the repetition rate should be $> 40\text{MHz}$ in high-speed microscopy (up to videorate). The pulse duration is limited by the time-bandwidth product, which states that a laser pulse of a given duration can only be achieved if it has a certain spectral bandwidth. Because the spectral resolution has to be narrower than a typical Raman line, this puts the limitation at $\tau > 0.7\text{ps}$.

It is further critical to consider the damage threshold of the sample. Optimization of the sensitivity requires design of the laser system to approach this threshold. While absolute quantification of photodamage is sample dependent, studies suggest that near-IR laser damage in biological samples is primarily due to nonlinear absorption phenomena with a scaling of $I_{ave}^\gamma / (\tau \cdot R)^{\gamma - 1}$ and nonlinear
scaling parameter $\gamma$ being in the range from 2.5 to 3.5\textsuperscript{1-3}. Our crude measurements carried out with $R = 80\,\text{MHz}$, $NA = 1.2$ and $\lambda_{\text{pump}} = 817\,\text{nm}$ and Stokes $\lambda_{\text{Stokes}} = 1064\,\text{nm}$, indicate that the sample shows morphological changes after a single scan (the most drastic form of damage) for $\hat{I} = 25\,\text{mW}$ at 180fs, $\hat{I} = 80\,\text{mW}$ at 1ps, and $\hat{I} = 280\,\text{mW}$ at 6ps. This suggests $\gamma \approx 3.2$, i.e. the photodamage is more nonlinear than the CRS SNR. The maximal permissable average power can thus be estimated by the experimental equation

$$\hat{I}^{\text{max}} \approx 25\,\text{mW} \cdot \left( \frac{\tau}{180\,\text{fs} \cdot \text{R/80MHz}} \right)^{\frac{3.2-1}{3.2}} \approx 25\,\text{mW} \cdot \left( \frac{\tau}{180\,\text{fs} \cdot \text{R/80MHz}} \right)^{0.7}.$$  

With these assumptions, we have performed simulations to determine the best pulse properties for SRS ($\tau = 1\,\text{ps} - 10\,\text{ps}$ and $R > 40\,\text{MHz}$) and found that the SNR hardly varies within these limits (Fig. S1). However, in order to achieve the same signal with a 10ps rather than 1ps laser pulse, the average power requirement of the laser system is ~5x higher.

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**Figure S1 Pulse parameters for CRS.**

**a,** Estimated SNR as a function of pulse duration at a fixed repetition rate of 60MHz and normalized to SNR for excitation with 80HMz, 6ps pulses (solid-state OPO system). This assumes excitation with maximal average power that does not cause photo-damage and neglects effects due to limited Raman linewidth.  

**b,** Average power requirement to achieve maximal SNR.
II. Interpretation of the ANSI standard

For clinical use, there is a large body of laser safety data in skin and eyes known as the ANSI standard\(^4\). For pulsed lasers, the maximum permissible exposure (MPE) is limited by one of three rules:

**Rule 1 - single pulse limit:** The exposure from any single pulse in a train of pulses shall not exceed the MPE for a single pulse of that pulse duration. The single pulse MPE for a 1ns laser pulse is \(0.02 \times 10^{2-(\lambda-0.700)} \text{ J/cm}^2\), i.e. 30mJ/cm\(^2\) at 800nm and 100mJ/cm\(^2\) at 1050nm. There is no specific data for picosecond lasers so it is recommended that the peak irradiance be limited to the MPEs applicable to 1ns pulses. This rule leans on the side of caution and the MPE has to be reduced by the ratio of the pulse duration over 1ns. The single pulse MPE for 1ps pulses is thus 30µJ/cm\(^2\) at 800nm and 100µJ/cm\(^2\) at 1050nm. For repetition rates >10MHz and for 1s exposure, this corresponds to >300W/cm\(^2\) at 800nm (or >1000W/cm\(^2\) at 1050nm).

**Rule 2 - average-power limit:** The exposure from any group of pulses (or sub-group of pulses in a train) delivered in time T shall not exceed the MPE for time T. For wavelengths from 700nm to 1050nm and exposure times from 10s to 3×10\(^4\)s, the average power MPE in skin is \(0.2 \cdot 10^{2-(\lambda-0.700)} \text{ W/cm}^2\), i.e. 0.3W/cm\(^2\) at 800nm (or 1W/cm\(^2\) at 1050nm).

**Rule 3: Repetitive-pulse Limit:** Only applies if the laser repetition rate is less than the critical frequency (55kHz for the wavelength range from 400nm to 1050nm), which is much smaller than the repetition rate used for a high-speed SRS imaging.

The overall MPE is limited by the smallest out of the three rules. For lasers in the regime relevant to SRS imaging the MPE this is average power (Rule 2) and the MPE is 3W/cm\(^2\) at 800nm and 1W/cm\(^2\) at 1050nm.

To convert from power density to in-focus power, the ANSI standard defines the limiting aperture as the diameter of a circle over which irradiance or radiant exposure is averaged for purposes of hazard
evaluation and classification. It can be interpreted as the area over which heat diffuses quickly and was determined experimentally to be 3.5mm in skin. The standard states that when the laser beam is smaller than the limiting aperture, the MPE is determined by averaging the beam energy over the limiting aperture. As the critical aperture is much larger than the laser focus or the field of view, this sets the MPE to 30mW at 800nm (or 100mW at 1050nm).
III. Additional detail of the balanced detector design

Figure S2 shows a detailed schematic of the voltage-subtraction auto-balanced detector. The design of the detector can be divided into three sub-systems: (1) pre-amplifiers, (2) auto-balancing loop, and (3) subtraction circuit.

To detect the laser beams, we used large-area (1cm x 1cm) Si photodiodes from OSI Photonics to accommodate for the high laser power and applied high back bias (48V) to reduce their capacitance for high speed operation. The photocurrent of each photodiode is first separated into DC and AC currents. The DC current is converted to voltage signal by a resistor and used for auto-balancing. The AC current is sent through a band pass filter around 10MHz to remove the interference from the laser repetition rate and then amplified with a trans-impedance amplifier (LMH6624) with a trans-impedance gain of 2.2k Ω to convert current to voltage and keep the noise at low level.

In the auto-balancing circuit, the DC and AC voltages from signal and reference arm are all amplified in a four channel variable gain amplifier (VGA chip model: AD8264). The gain for AC and DC of the reference arm is set to a constant of 12dB, which is the middle of the tuning range of the VGA. The gain of DC in the signal arm is controlled by a feedback loop so that the DC levels of both arms are kept the same at the outputs of the VGA. As the gain of the AC levels of the signal arm is tied to that of the DC level, this creates the auto-balancing. An important parameter is the speed of the PID feedback loop. We tested the feedback speed with 10% of variation in the reference arm, and found the bandwidth for the feedback loop is about 500kHz, which is enough to achieve pixel by pixel transmission compensation in current imaging condition 1.1s/frame (2 μs per pixel).
Figure S2 Schematic of the voltage-subtraction auto-balanced detector. Back-biased large-area silicon (Si) photodiodes convert the optical signals of the signal and reference arm into electric signals. The AC and DC portion are then separated by bias-T and band pass filter. The AC portion is pre-amplified by a trans-impedance amplifier. Both the AC and DC portions of each arm are then amplified by a 4-channel variable gain amplifier (VGA). In the VGA, the gain of reference AC and DC is fixed. The gain of signal diode is controlled by a PID loop that feedbacks on the difference of the DC signals from the signal and reference arm. The phase of the AC signal is precisely matched by voltage-controlled phase shifters. The balanced signals are then subtracted by a differential amplifier.

In the last part of the auto-balancing detector, AC signals from the signal and reference arms are sent though phase-shifters and then subtracted by a differential amplifier. The phase shifter is voltage controlled to generate a phase shift from 0 to 180 degrees. The phase shifters guarantee the perfect phase match between signal and reference arms and are necessary to achieve high cancelation ratios. Alternatively, phase shifting could be implemented by an optical or electrical delay line. The phase shifter is a narrow bandwidth device (8-12MHz) and therefore introduces different dispersion on signal and reference arm, which, in turn, limits the noise cancellation bandwidth. Yet, in current imaging condition 1 frame/s (512 by 512 pixels) the bandwidth is not an issue.
Figure S3 shows the relative intensity noise of the pump beam of our fiber laser as well as the solid-state OPO system. The OPO is shot noise limited at high frequency (>4MHz). The fiber laser is about 27dB above the shot-noise at 10MHz.

![Figure S3](image)

**Figure S3 Comparison of the relative intensity noise of our fiber laser and the solid-state OPO.** The dashed line shows the theoretical shot noise at 28 mW. The red curve is the noise spectrum of the OPO, which is shot-noise limited above 4MHz. The blue curve is the noise from our fiber laser, which is about 27 dB higher than the shot noise.

Figure S4 shows the imaging test results of the auto-balanced detector. In the test, we used 1.1μm polystyrene beads. Figure S4a was taken with the auto-balanced detector under fully balanced conditions. Figure S4b was taken with the reference beam blocked and all auto-balancing function turned off, i.e. this image is equivalent to the image taken with simply a photodiode. The SNR improves about 10.6 times from 0.5 in (a) to 5.3 in (b).

Figures S4c and d show the background noise of an SRS image to demonstrate the ‘auto’-balancing function by blocking the Stokes beam. When the ‘auto’-balancing function is turned off (Fig S4d), one can see that the edges of the beads are noisier due imperfect balancing cause by sample scattering. When the ‘auto’-balancing function is on, such variation in transmission is compensated and the laser noise is always cancelled (Fig. S4c).
**Figure S4 Demonstration of the auto-balanced detector.** a and b, SRS image of 1.1μm polystyrene beads with (a) and without (b) the auto-balanced detector. c and d, Background image with blocked Stokes beam with (c) and without (d) ‘auto’-balancing function.
IV. The stability of the fiber laser system

Clinical applications of SRS require the laser system to work under a variety of ambient conditions (e.g. temperature and humidity) and without optical tables. Fiber lasers are better suited for such environments because light guiding by the core and permanently combining different fiber-optic elements with fusion splicing minimizes the potential for misalignment, compared to free-space lasers. To realize this potential, we have developed an all-fiber laser system. Even at this early stage of prototyping (no professional packaging and non-polarization-maintaining components), our system has proven to be intrinsically stable:

1. Figs. S5 shows operation of the fiber laser system on a breadboard placed on a wooden table (no vibration isolation) at the Invenio facilities. The room did not have dedicated humidity or temperature control. Nevertheless, all spectra and cross-correlation measurements in Fig. 2, as well as hyper-spectral imaging data (Figs S5B and C) were obtained under these conditions.

![Figure S5 Operation of the fiber laser system on a wooden table.](image_url)
2. Sup. Video 2 shows performance of the laser system to mechanical shock. The output of the fiber oscillator, the core of the laser system, shows no intensity fluctuations when it is shaken vigorously.

3. Sup. Video 3 shows long-terms SRS imaging of polystyrene beads over 44 hours with no adjustment of the laser. No degradation of the SRS signal is observed over time. We remark that the image quality is reduced due to compressing the video files. Another evidence of long time stability is that a fiber oscillator in Prof. Kieu’s lab has been running continuously for more than 3 years.
SUPPLEMENTARY VIDEOS:

Sup. Video 1: Z-stack of a sebaceous gland at (A) 2850cm\(^{-1}\) and (B) 2950cm\(^{-1}\). The pixel dwell time was 4\(\mu\)s, and the z-step size was 1\(\mu\)m over a total imaging depth of 100\(\mu\)m. The sampling was 512 x 512 pixels.

Sup. Video 2: Robustness of fiber-laser source to mechanical shock.
Sup. Video 3: Long-term stability of fiber-laser based SRS microscopy. SRS images of polystyrene beads were acquired at 2950 cm\(^{-1}\) over a total duration of 44 hours. The pixel dwell time was 2 \(\mu\)s, and an image was taken every minute. The original sampling was 512 x 512 pixels, but the videos were resized to 320 x 240 pixels.
References