



A Flexible Chamber for Time-lapse Live-cell Imaging with Stimulated Raman Scattering Microscopy

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Abstract

Stimulated Raman scattering (SRS) microscopy is a label-free chemical imaging technology. Live-cell imaging with SRS has been demonstrated for many biological and biomedical applications. However, long-term time-lapse SRS imaging of live cells has not been widely adopted. SRS microscopy often uses a high numerical aperture (NA) water-immersion objective and a high NA oil-immersion condenser to achieve high-resolution imaging. In this case, the gap between the objective and the condenser is only a few millimeters. Therefore, most commercial stage-top environmental chambers cannot be used for SRS imaging because of their large thickness with a rigid glass cover. This paper describes the design and fabrication of a flexible chamber that can be used for time-lapse live-cell imaging with transmitted SRS signal detection on an upright microscope frame. The flexibility of the chamber is achieved by using a soft material – a thin natural rubber film. The new enclosure and chamber design can be easily added to an existing SRS imaging setup. The testing and preliminary results demonstrate that the flexible chamber system enables stable, long-term, time-lapse SRS imaging of live cells, which can be used for various bioimaging applications in the future.

SUMMARY:

We report a stage-top, flexible environmental chamber for time-lapse imaging of live cells using upright stimulated Raman scattering microscopy with transmitted signal detection. Lipid droplets were imaged in SKOV3 cells treated with oleic acid for up to 24 h with a 3 min time interval.

Keywords

Flexible chamber; live-cell imaging; time-lapse; stimulated Raman scattering; microscopy; lipid droplets; cancer lipid metabolism

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

INTRODUCTION:

Optical microscopy is used to observe the microstructures of samples. Optical imaging is rapid, less invasive, and less destructive than other technologies¹. Live-cell imaging with optical microscopy is developed to capture the dynamics of cultured live cells over a long period². Different types of optical contrasts provide distinct information about biological samples. For instance, optical phase microscopy shows the subtle difference in the refractive indices across the sample³. Fluorescence microscopy is widely used to image specific biomolecules or cellular organelles. However, the broadband excitation and emission spectra of fluorescence usually result in spectral overlapping when multicolor imaging is performed⁴. Fluorescent molecules are light-sensitive and can be bleached after long-term, periodic light exposure. In addition, fluorescence labeling may change the biodistribution of the molecules in cells⁵. SRS microscopy is a label-free chemical imaging technology⁶. The contrast of SRS relies on the vibrational transition of specific chemical bonds. The vibrational frequency of a chemical bond often exhibits a narrow spectral bandwidth, making it feasible to image multiple Raman bands in the same samples⁷. SRS microscopy is a unique tool for live-cell imaging, providing multiple chemical contrasts in a label-free manner⁸.

While SRS imaging of unstained cells has been used for many studies, long-term time-lapse SRS imaging of live cells has not been widely adopted. One reason is that commercial open chambers cannot be directly used for SRS imaging because of their large thickness^{9–12}. These chambers with a glass lid are mostly designed for brightfield or fluorescence imaging using a single high NA objective with a backward detection scheme. However, SRS imaging prefers transmitted detection using both a high NA objective and a high NA condenser, which leaves only a very short gap (typically a few millimeters) between the objective and the condenser. To overcome this problem, we designed a flexible chamber using a soft material to enable time-lapse SRS imaging of live cells using an upright microscope frame. In this design, the water dipping objective was enclosed in the soft chamber and can freely move in three dimensions for focusing and imaging purposes.

The optimal temperature for culturing most mammalian cells is 37 °C, while the room temperature is always 10° lower than this. Temperature higher or lower than 37 °C has a dramatic effect on cell growth rate¹³. Therefore, temperature control of the cell culture environment is required in a live-cell imaging system. It is known that temperature instability will lead to defocusing issues during long-term imaging¹⁴. To achieve a stable 37 °C environment, we built a large enclosure chamber to cover the entire microscope frame, including a thermal insulation layer underneath the microscope (Figure 1). Within the sizeable temperature-control chamber, the small flexible chamber helps to accurately maintain the physiological humidity and pH via the regulated air flow supplemented with 5% CO₂ (Figure 2). The temperature and humidity of the chambers were measured to confirm that the double-chamber design provided the optimal cell culture condition for cell growth under long-term, periodic SRS imaging (Figure 3). We then demonstrated the application of the system for time-lapse imaging and tracking lipid droplets (LDs) in SKOV3 cancer cells (Figure 4, Figure 5, and Figure 6).

PROTOCOL:

1. Build the microscope environmental enclosure

NOTE: This large microscope environmental enclosure is used to control the temperature of the microscope body and the imaging environment to be stabilized at 37 °C (Figure 1A).

1.1. Mark the locations of the feet of the SRS microscope frame and the motorized stage using a marker pen on the optical table. Mount two Iris Diaphragms in front of the Galvanometer scanner of the microscope and adjust to make the pump and Stokes laser beams pass through the center of the Iris Diaphragms.

1.2. Remove the microscope frame and the stage from the optical table.

1.3. Lay the silicone rubber sheet (size: 31 × 29 inches, thickness: 1/8 inch) on the optical table (Figure 1B).

1.4. Cut the silicone rubber along the marks using a knife, remove small rubber pieces, and place the square MICA ceramic sheets (size: 6 × 6 inches, thickness: 1/4 inch) into the same locations.

NOTE: MICA ceramic is an easy-to-machine material¹⁵. It is as hard as aluminum but is an excellent thermal insulator. MICA ceramic sheets were used to stop heat transfer from the metal microscope frame and stage to the stainless-steel optical table. A few through-holes should be drilled on the MICA sheets to allow the use of ¼–20 screws for mounting the feet of the frame and the stage.

1.5. Move the microscope frame and the stage back to the optical table and carefully align the feet onto the MICA ceramic sheets along the marker lines. Use ¼–20 screws to mount the frame and the stage on the table.

1.6. Realign the SRS optical path. Adjust the mirror mounts of Mirror 1 and Mirror 2 to make the laser beam pass through the center of the two pre-mounted Iris Diaphragms (Figure 2).

NOTE: Technical details of the lab-built SRS microscope used for the current live-cell imaging work is described previously¹⁶. The pulse width of the pump and Stokes beams are ~3–4 picoseconds with glass rod dispersion. The system is controlled by the ScanImage¹⁷ software.

1.7. On top of this thermal insulation foundation, assemble the environmental enclosure to cover the entire microscope frame using five pieces of large polycarbonate sheets (size: 31 × 29 × 28 inches, thickness: 0.25 inch).

NOTE: The size of the enclosure box is determined based on the dimensions of the microscope frame and the stage.

1.7.1. To assemble the enclosure, carry out simple machining work, including cutting, drilling, and tapping screw holes on each edge of the polycarbonate sheets. Cut two large

holes with a diameter of 2.6 inches on the right and the left sheets of the enclosure to fit the inlet and outlet tubing, respectively. Cut a small hole with a diameter of 5 mm on the back sheet to allow the laser beams to enter the enclosure.

1.8. Seal the edges and interfaces of the box using aluminum foil tape.

1.9. Connect the flexible duct hose to the inlet and the outlet ports of the enclosure box to allow circulated warm airflow pumped and controlled by the heater module. Place the thermal sensor of the heater module in the flexible chamber where the cells are cultured and imaged. Set the targeted temperature at 37 °C.

NOTE: A diffuser may be used to get a more uniform airflow distribution in the environmental enclosure.

2. Assemble the flexible chamber.

2.1. Mount the machined hollow cylindrical aluminum piece 1 (material: aluminum 6061) to the nosepiece of the objective using three set screws (Figure 2).

2.2. Mount the machined hollow cylindrical aluminum piece 2 to the sample holder using four ¼–20 screws (Figure 2).

NOTE: The sample holder must be modified to hold the 50 mm cover glass-bottom cell culture dish. Drill a through hole in the center of the sample holder using a 1–7/8 inches hole saw. Counterbore the hole using a 50 mm hole saw and keep the depth of the through hole around 1 mm.

2.3. Fit the sample holder with the aluminum piece 2 onto the motorized stage and mount it using screws.

2.4. Place the natural rubber film sleeve (thickness: 0.01 inch; glued with cyanoacrylate adhesive) between the two machined aluminum pieces and mount it using rubber bands at each end.

2.5. Connect the compressed CO₂ cylinder to the gas mixer module using proper tubing and connectors. Set the CO₂ input pressure at 20–25 psi. Use a built-in CO₂ sensor and a controller to ensure the air mixer module can regulate and mix 5% CO₂ into the airflow. Use inline filters to clean up the airflow.

2.6. Using proper tubing and connectors, guide the mixed air (with 5% CO₂) to the presterilized water bottle placed on the hot plate, and then guide the humidified air to the flexible chamber. Set the hot plate at 37 °C. Bubble the airflow in the warm water to increase the humidity of the airflow.

3. Preparation for time-lapse live-cell SRS imaging experiments

3.1. Wipe all parts of the flexible chamber with 70% ethanol, including the nosepiece, the water dipping objective, and the sample holder.

3.2. Decontaminate the entire enclosure system using a UV lamp placed in the enclosure for 20 to 30 min.

NOTE: Do not stay in the lab room during the UV disinfection process for safety.

3.3. Culture SKOV3 cells in a 50-mm glass-bottom Petri dish for 12 h under normal physiological conditions in a regular incubator.

NOTE: Start the SKOV3 cell culture¹⁸ in a standard biosafety cabinet.

3.4. Disconnect the machined aluminum piece 2 from the sample holder by removing the screws.

NOTE: This is the way to open the flexible chamber to load the cell culture dish.

3.5. Load the cell culture dish. Remember to add immersion oil on the top of the condenser before placing the cell culture dish. Remove the cover of the dish and immobilize the dish using the clamps.

NOTE: To avoid contamination, all the operations should be performed with gloves.

3.6. Lower the objective into the cell culture media for coarse focusing. Lower the aluminum piece 2 to enclose the flexible chamber and mount it to the sample holder using screws.

NOTE: A 2 mm silicone rubber cushion pad is attached to the bottom of the aluminum piece 2 to seal the gap tightly.

3.7. Set the air supply with 5% CO₂ and 19% O₂ for normal cell culture with an airflow rate of 200 cc/min.

NOTE: A lower airflow rate may be used. It depends on how well the flexible chamber is sealed.

4. Conduct time-lapse live-cell SRS imaging experiments.

4.1. Tune the laser wavelength to 805 nm to target the 2854 cm⁻¹ Raman shift, which is attributed to the vibration of CH₂ chemical bonds. Use low laser power to reduce photodamage to the cells. To follow this protocol, use ~15 mW average power of the pump laser and ~7.5 mW of the Stokes laser (fixed at 1,045 nm) for long-term live-cell imaging.

NOTE: Higher laser power will yield better SRS imaging quality. However, too high laser power will induce photodamage to live cells. There is a tradeoff between image quality and photodamage.

4.2. Adjust and focus the objective to achieve good SRS imaging of the cells using the FOCUS button on the MAIN CONTROLS panel of ScanImage. To perform rapid focusing, the pixel number is typically set to be 512 × 512 pixels with a pixel dwell time of 4.8 μs on the CONFIGURATION panel of ScanImage.

4.3. Set the lock-in amplifier input range (typically 5 mV) to be twice the maximal signal voltage. Set the low-pass filter to be the same as the pixel dwell time (4.8 μ s).

NOTE: Different lab-built SRS systems may use different data acquisition settings.

4.4. After achieving good focusing, set the imaging resolution to be $2,048 \times 2,048$ pixels for a 175 μ m square field of view for the acquisition of high-quality images. Check the SAVE function on the MAIN CONTROLS panel and check the SRS channel on the CHANNELS panel of ScanImage. Set the interval time between two frames to be 180 seconds (3 min) on the MAIN CONTROLS channel. Set the acquisition number to 480 for time-lapse imaging of 24 h.

4.5. Start automated imaging acquisition using the **LOOP function** on the MAIN CONTROLS panel of ScanImage.

NOTE: Check whether there is focal drift due to temperature instability in the first hour of imaging. The first-hour imaging may not be stable. Check focusing every 2–3 h during the time-lapse imaging session.

4.6. Process the collected images using ImageJ¹⁹. Two methods are used for LDs quantification: (i) the LD/cell body area ratio, and (ii) the mean SRS intensity of total LDs. The cell body area is measured by thresholding and zeroing the non-cellular pixels in the SRS images at 2854 cm^{-1} . The LDs area and intensity are measured by thresholding and zeroing the non-LD pixels in the SRS images. More details about SRS image processing were reported previously¹⁶.

REPRESENTATIVE RESULTS:

We fabricated and assembled the flexible chamber system for time-lapse SRS imaging (Figure 1 and Figure 2) and then evaluated the performance of the system. The temperature inside the microscope environmental enclosure reached the expected 37 °C within 1 h, which did not significantly affect the room temperature (Figure 3A). The temperature in the flexible chamber reached 37 °C in 1.5 h, and it was stably maintained at 37 °C for at least 24 h (Figure 3B). The relative humidity in the flexible chamber could reach 85% in 1 h and then be maintained for at least 24 h (Figure 3C). The measured temperature and humidity data confirm that this system can provide an optimal environment for long-term cell growth.

Live-cell imaging with SRS has been applied to many biological and biomedical studies^{20–24}. In particular, SRS imaging of label-free LDs in live cells to understand lipid metabolism in cancer has drawn much attention^{16,25,26}. Using the designed flexible chamber system, we first performed time-lapse SRS imaging of live SKOV3 cells for 24 h with a time interval of 3 min (Figure 4). The video data showed the rapid and active movement of intracellular LDs with a temporal resolution of 3 min. By the end of the 24 h imaging session, the cells still showed normal morphology and density, indicating that the cells were in a healthy condition. We then imaged SKOV3 cells treated with oleic acid (OA) and tracked the dynamic process of LD accumulation within 10 h (Figure 5A).

The LD amounts were quantified in the OA-treated SKOV3 cells in two ways (LD to cell body area ratio and total SRS intensity of the LDs) using ImageJ¹⁹. The results indicate that the amount of LDs (in size and number) kept increasing over 10 h (Figure 5B). We also demonstrated simultaneous forward-SRS imaging of LDs (pseudo color green) and backward two-photon fluorescence (TPF) imaging of lysosomes (pseudo color red) labeled with a fluorescence dye DND-189 (Figure 6). It is noted that SRS/TPF dual-modality imaging can be used to analyze the colocalization of two cellular compartments. In this experiment, a very low degree of colocalization of LDs and lysosomes was observed, which was indicated by the small yellow regions. Collectively, these results demonstrate that the flexible chamber system enables stable, long-term, time-lapse SRS imaging of live cells, which can be used for various imaging applications in the future.

DISCUSSION:

Time-lapse live-cell SRS microscopy is an alternative imaging technique for molecule tracking in a label-free manner. Compared to fluorescence labeling, SRS imaging is free from photobleaching, enabling long-term monitoring of molecules. However, to date, the live cell imaging system on an upright SRS microscopy is not commercially available. In this work, a live cell imaging system with a stable thermal-insulated microscope enclosure box and a flexible inner soft chamber was developed to enable transmitted SRS time-lapse imaging. In this setup, the large enclosure box maintains temperature stability at 37 °C, while the internal soft chamber supplies humidified air to establish an optimal cell culture environment. The flexible open chamber demonstrated in this work enables a simple workflow for long-term SRS imaging of live cells. Cells seeded in a glass-bottom dish can be first prepared and cultured in a regular incubator before transferring to the flexible on-stage chamber for imaging. An alternative solution to conduct live-cell SRS imaging is to use a closed flow cell, including the microfluidic and flow cytometry platforms^{27–30}. It is feasible to design a flow cell with a thin thickness. However, culturing cells in a perfusion chamber can be technically challenging³¹.

Focal drift is a common issue in live-cell imaging³². As a multiphoton process, SRS signal generation requires a tightly focusing of the laser beams and, therefore, SRS microscopy is highly sensitive to focal drift. Temperature instability is an essential factor in inducing focal drift. To improve thermal stability, the entire microscope was enclosed with thermal insulating materials. However, in some imaging sessions, we still experienced focal drift after 2–3 h of imaging. SRS microscopic imaging is also sensitive to vibration, which may destroy focusing. An anti-vibration optical table helps reduce vibration to achieve stable imaging. To solve the focal drift problem, in future experiments, autofocus technologies may be adopted³³.

The disinfection procedure of the imaging system is critical to avoid contamination of the cells, especially for the water immersion objective, which will directly contact the cell culture media. It is safe to use 70% ethanol for lens top cleaning. Because UV light can only effectively disinfect the surface of the objects, four UV lamps were mounted at different locations in the enclosure box to perform disinfection. However, the UV light may degrade the plastic components in the imaging system. In this case, one may use aluminum foil to

wrap and cover the plastic parts. For live-cell imaging, antibiotics (usually 100 units/mL of penicillin and 100 µg/mL of streptomycin in the culture media) are highly recommended.

We imaged and quantified the LDs in live cancer cells in these experiments. For these experiments, a 3 min time interval was reasonable. It is noted that the imaging time interval could be changed depending on the needs of the research project. For example, to track a single LD in a live cell, a less than 1 min time interval may be required. In contrast, a longer time interval of a few minutes is sufficient to track slow biological processes³⁴.

SRS imaging uses higher laser power for excitation than many other optical imaging technologies, which can be challenging for long-term time-lapse SRS imaging of live cells. SRS imaging of the native biomolecules such as lipids is even more challenging because of the tiny Raman cross-section of the chemical bonds³⁵. In these experiments, a 15 mW pump laser at 805 nm and 7.5 mW Stokes laser at 1045 nm were used, and no significant photodamage was observed in 24 h with a 3 min time interval. The use of sensitive Raman tags can reduce the laser power further³⁶.

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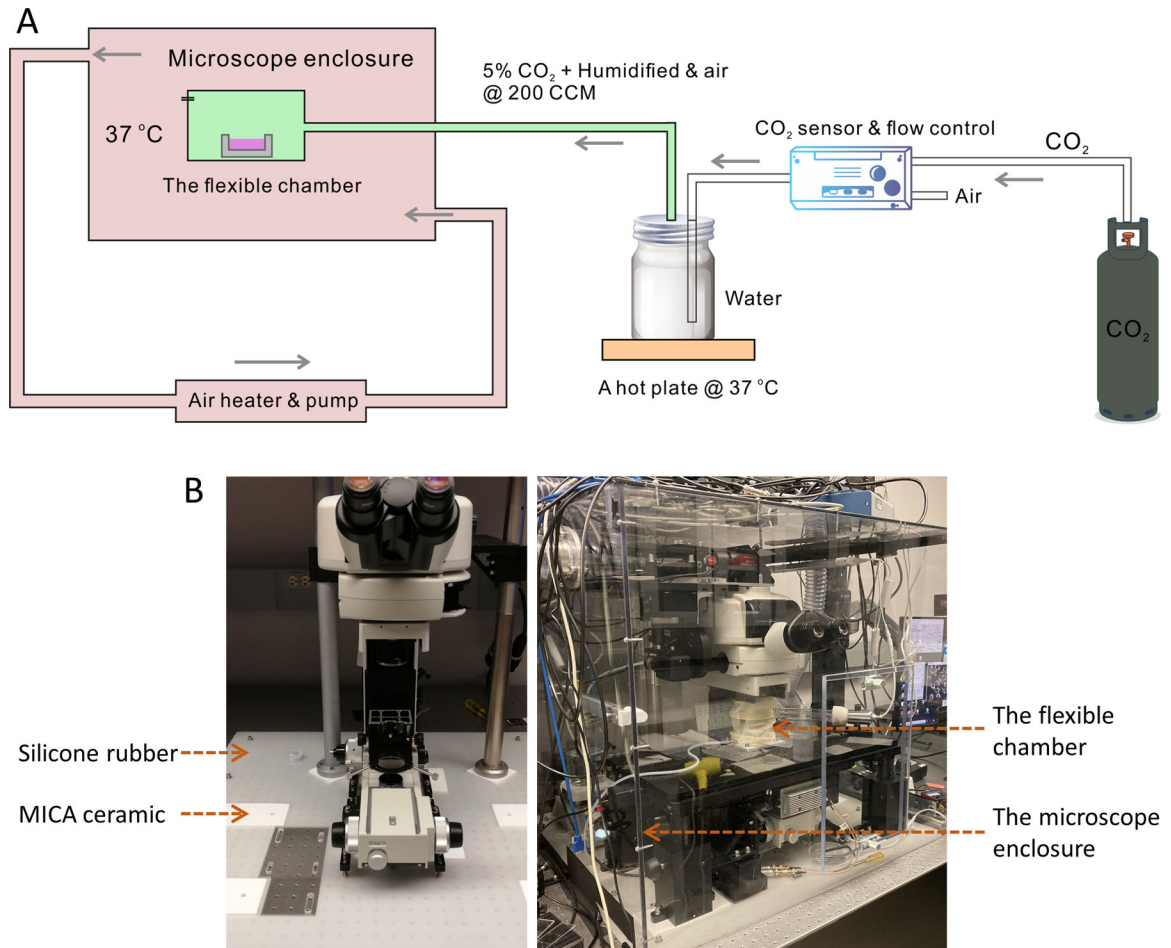


Figure 1: The flexible chamber system for time-lapse SRS imaging of live cells.

(A) Schematic of the flexible chamber system for time-lapse SRS imaging of live cells.

(B) The left image shows the thermal insulation layer using a silicone rubber sheet and MICA ceramic pads underneath the microscope frame and the stage. The right image shows the environmental microscope enclosure and the flexible chamber. Abbreviations: SRS = stimulated Raman scattering; CCM = cubic centimeters per min.

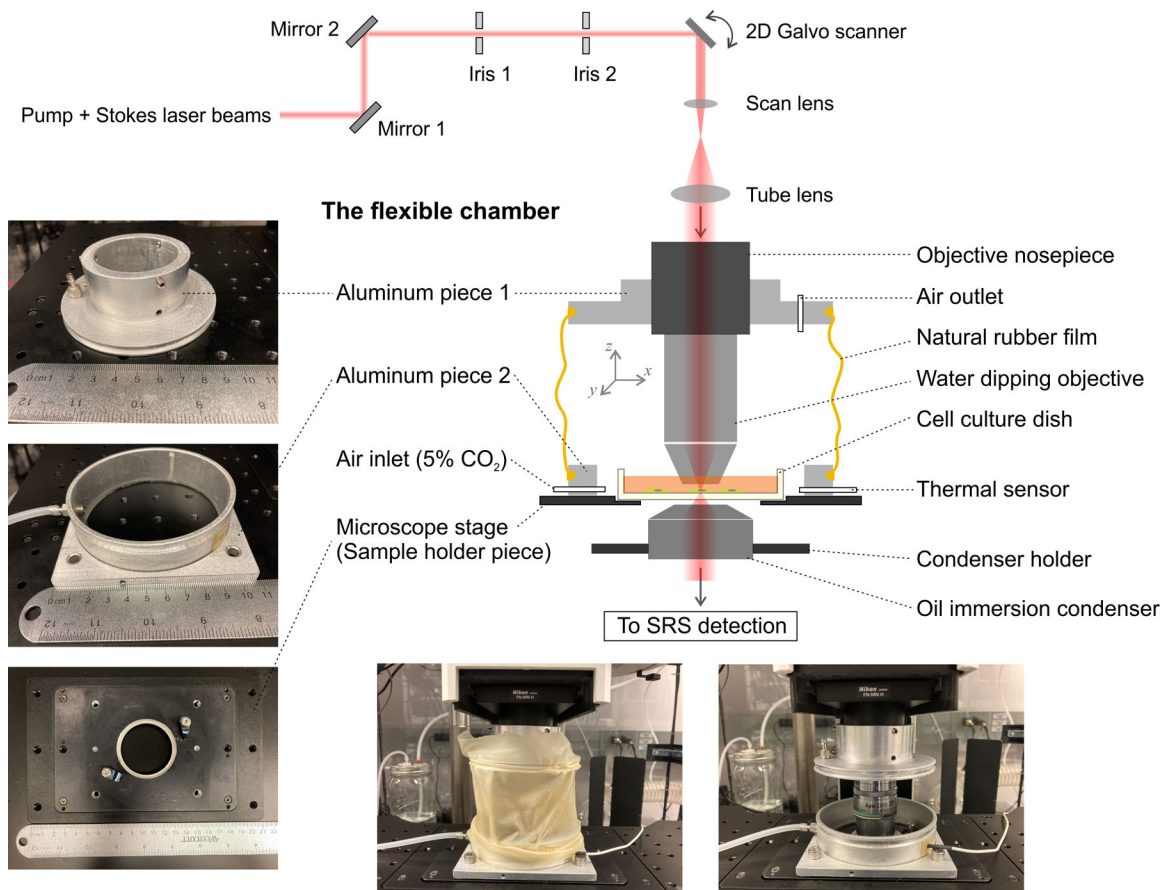


Figure 2: Schematic and images of the flexible chamber with the SRS optical path, allowing three-dimensional free movement of the water dipping objective for focusing and imaging. The left images show the two machined aluminum modules connected to a commercial objective nosepiece and a modified sample holder. The bottom images show the assembly flexible chamber system. Abbreviation: SRS = stimulated Raman scattering.

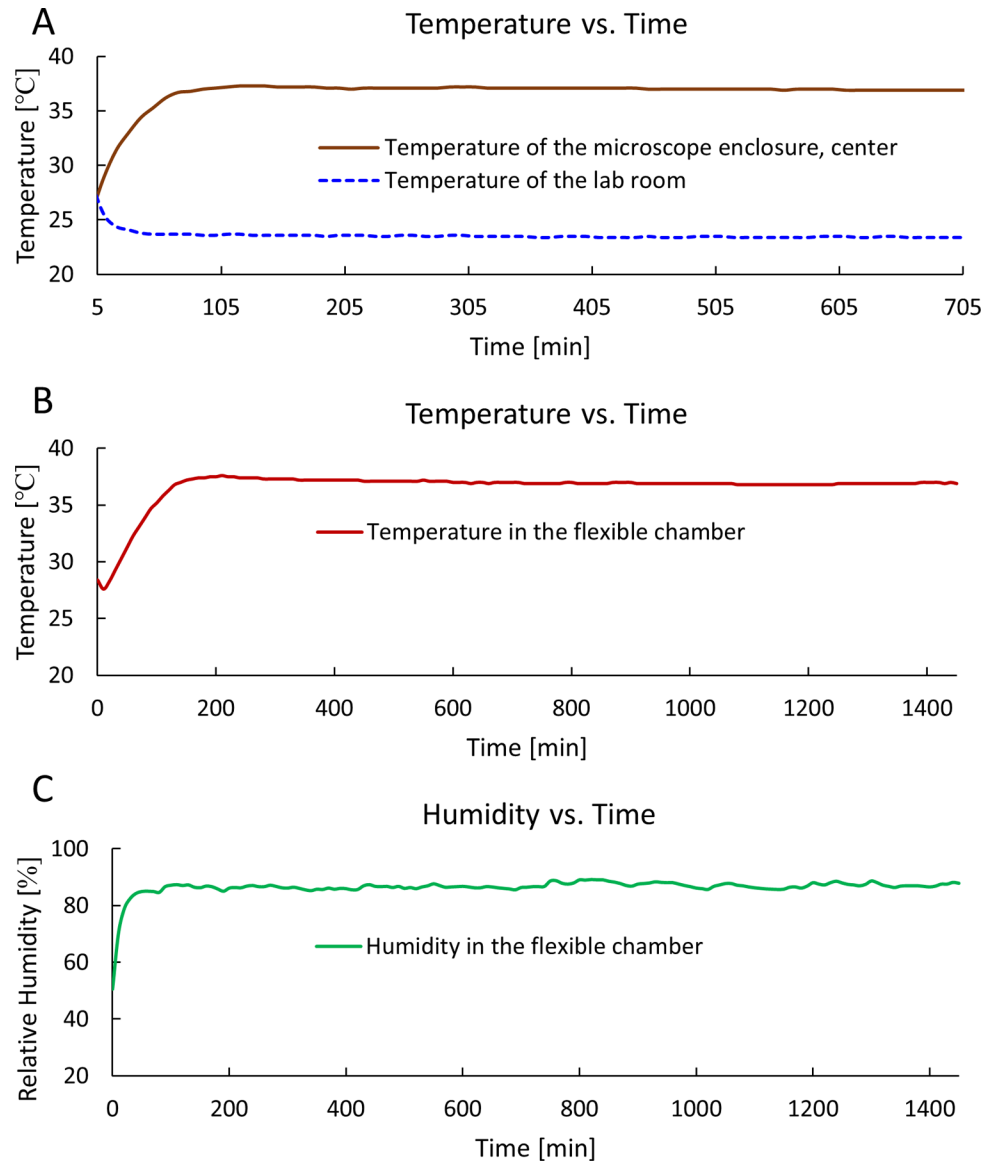


Figure 3: Temperature and humidity data of the microscope enclosure and the flexible chamber. (A) The measured temperature data of the microscope enclosure and the lab room temperature, up to 12 h. (B) The measured temperature data (average at 37 °C) within the flexible chamber, up to 24 h. (C) The measured relative humidity data (average at 85%) within the flexible chamber, up to 24 h.

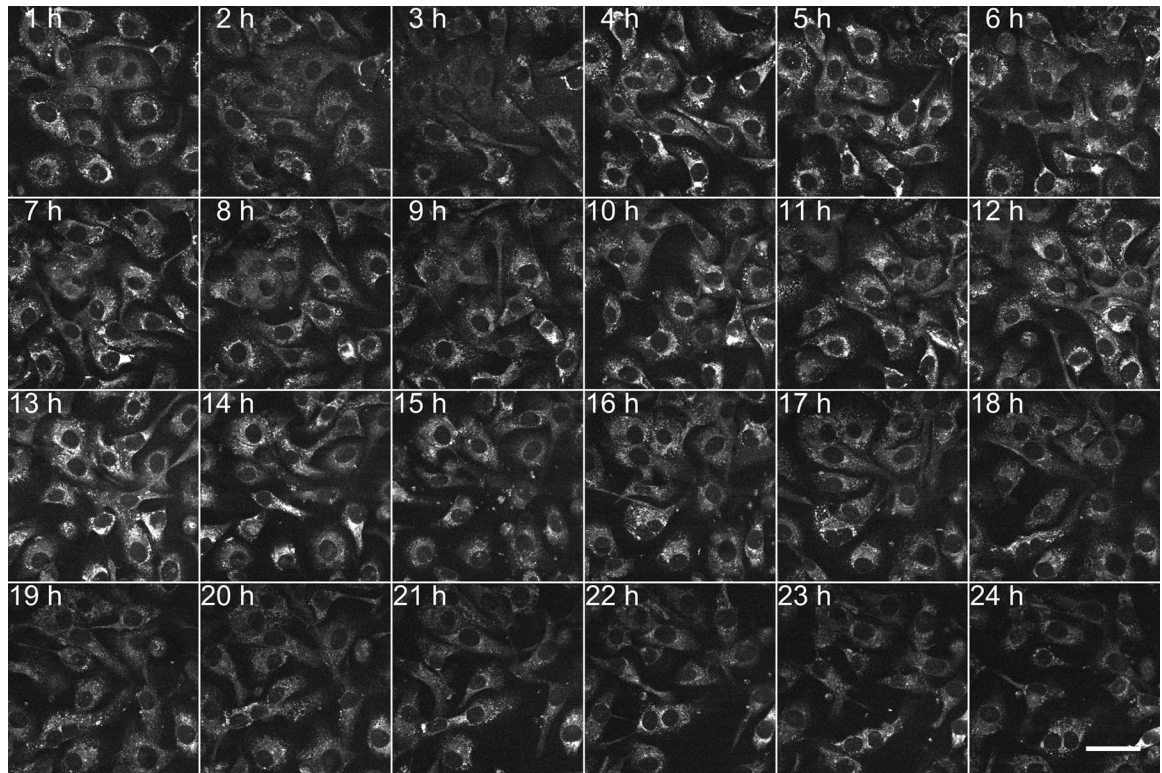


Figure 4: Representative 24 frames of time-lapse SRS imaging.

Time-lapse SRS imaging (at $2,854\text{ cm}^{-1}$) of live SKOV3 cells using the flexible chamber, up to 24 h. In this experiment, 480 frames were recorded with a fixed time interval of 3 min. Cells were cultured under normal conditions. Scale bar = $50\text{ }\mu\text{m}$.

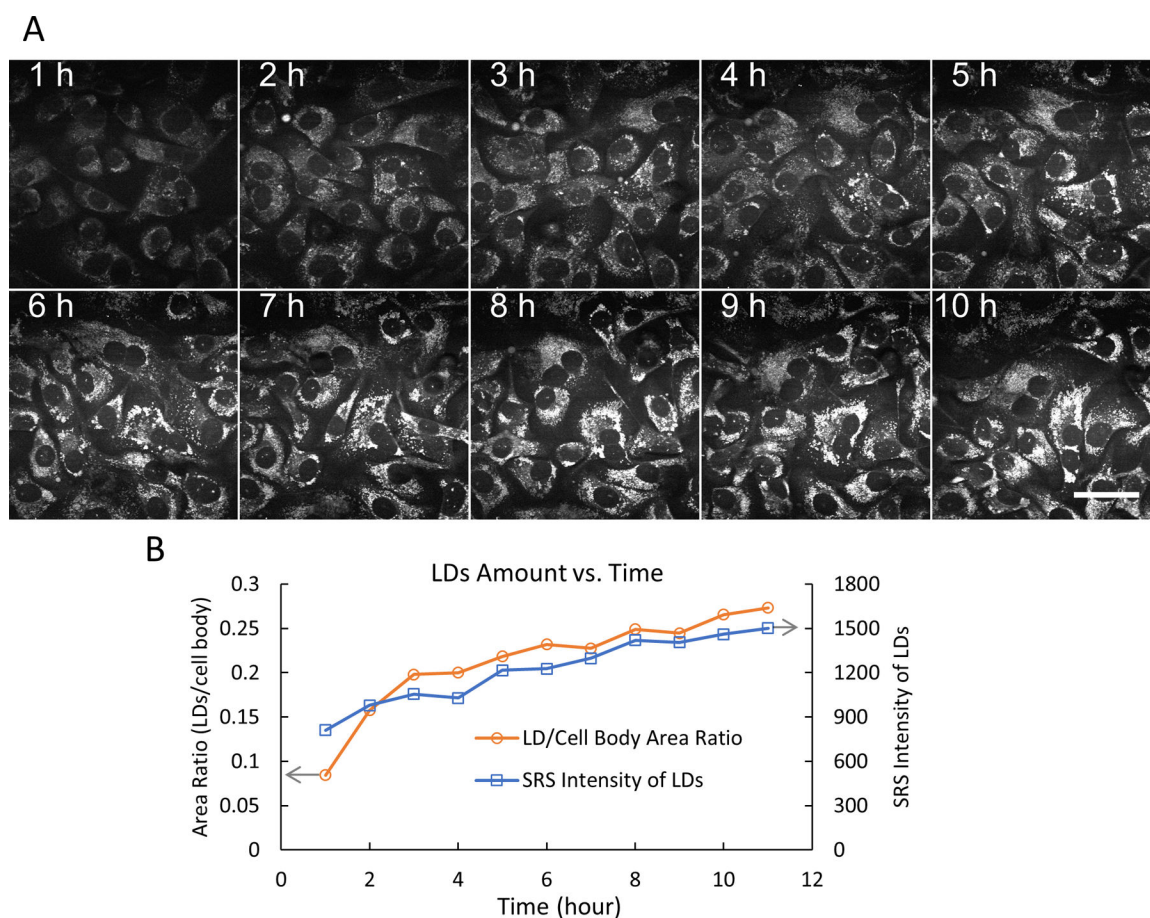


Figure 5: Time-lapse SRS imaging and lipid droplet quantification.

(A) Representative 10 frames of time-lapse SRS imaging (at $2,854\text{ cm}^{-1}$) of live SKOV3 cells treated with $500\text{ }\mu\text{M}$ OA, up to 10 h, using the flexible chamber. In this experiment, 200 frames were recorded with a fixed time interval of 3 min. Scale bar = $50\text{ }\mu\text{m}$. (B) The amount of LDs versus time (0–10 h) was quantified in two ways (LD/cell body area ratio and total SRS intensity of LDs) using the thresholding function and the particle analysis functions in ImageJ. Abbreviations: SRS = stimulated Raman spectroscopy; OA = oleic acid; LDs= lipid droplets.

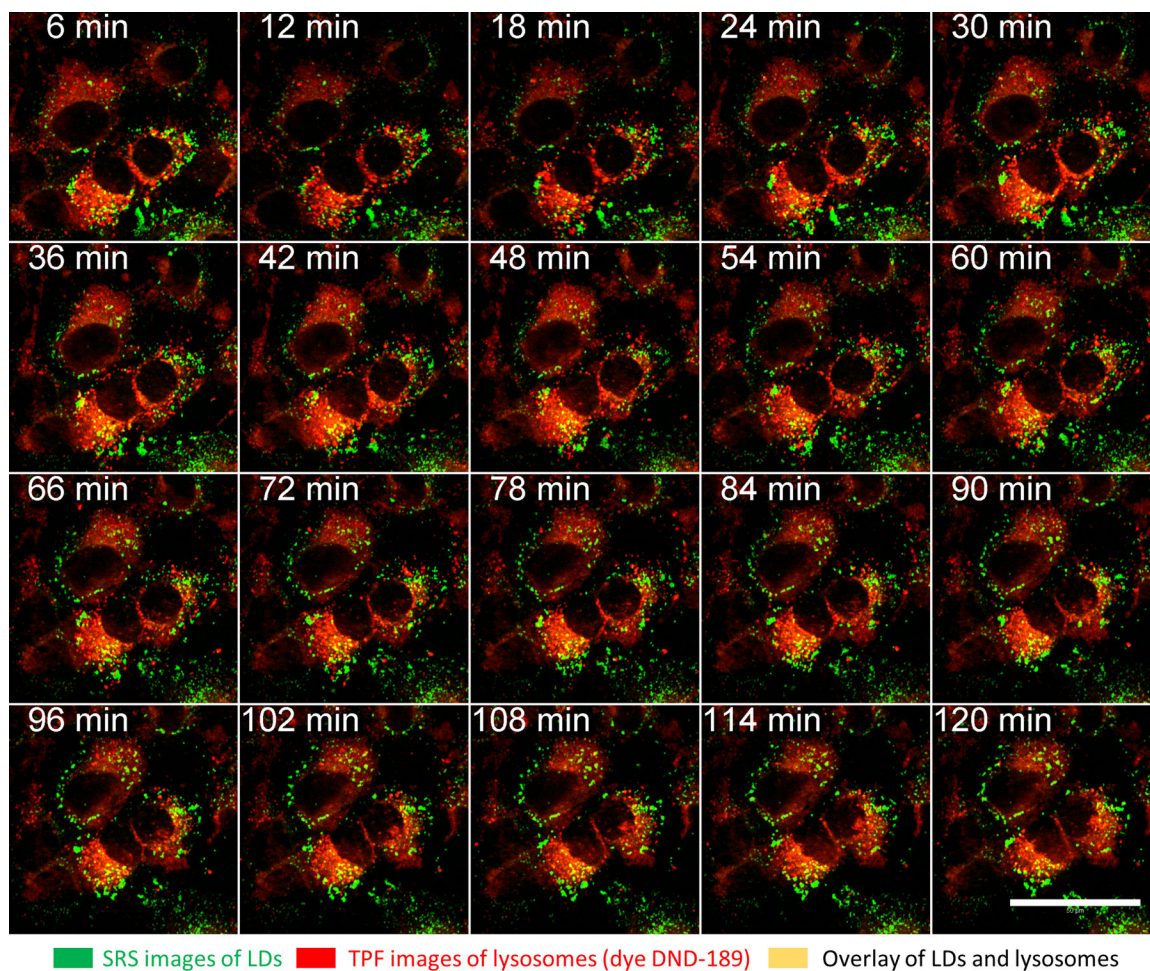


Figure 6: Time-lapse, simultaneous SRS and two-photon fluorescence imaging for LDs and lysosomes.

Time-lapse, simultaneous SRS (at $2,854\text{ cm}^{-1}$), and two-photon fluorescence imaging for LDs (pseudo color green) and lysosomes (red), up to 2 h. Cells were treated with a fluorescence dye (LysoSensor DND-189, $1\text{ }\mu\text{M}$) for 1 h before imaging. Images were taken every 3 min. Scale bar = $50\text{ }\mu\text{m}$. A low degree of colocalization of LDs and lysosomes were observed, indicated by the yellow color. Abbreviations: SRS = stimulated Raman spectroscopy; LDs= lipid droplets.