Bioimaging has enabled us to visualize biomolecules in action. However, current development of fluorescent reagents mainly relies on trial and error, because the rational design is hindered by limited mechanistic knowledge. The stimulated Raman technology, particularly on the ultrafast timescales, has provided new insights into bioimaging, but the operation of a sophisticated optical setup requires expertise. We present key application notes for implementation of tunable FSRS across disciplines.

Introduction

As the old saying goes, seeing is believing. In the past few decades, scientists and engineers have made tremendous progress in imaging the small, and tracking life processes in action. Among the enabling advances, green fluorescent protein (GFP) stands out as one of the revolutionary forces due to its ubiquitous genetic encodability and engineering versatility. In order to improve the imaging capabilities of FPs from increased brightness to decreased bleaching, we aim to visualize the actual imaging processes of these FPs and FP-derived biosensors before their fluorescence. To achieve this unique line of inquiry, femtosecond stimulated Raman spectroscopy (FSRS) [1-4] is a powerful table-top optical technique which by itself has the potential for cellular imaging at video rate [5]. This article presents the essential procedures to experimentally acquire and analyze FSRS data.

Ultrafast Raman Methods

Raman spectroscopy has enjoyed immense growth since Raman scattering was first discovery by C. V. Raman, et al. in the 1920s [6]. With the advancement of laser technology, there has been an increased interest in Raman applications ranging from optical telecommunications (e.g., stimulated Raman scattering, all-optical amplification), atmospheric and geosciences, carbon and nanomaterials, and life sciences (e.g., sensing pathophysiological conditions, cell imaging) where non-destructive, microscopic, sensitive chemical analysis and imaging are needed for a wide range of sample states. Our goal is to extend the equilibrium study into the
dynamic regime where the structural change and chemical reactivity can be monitored in real time by ultrafast stimulated Raman spectroscopy [7].

**Laser Source and Overlap Issues**

The conventional FSRS setup comprises a sequence of ultrafast laser pulses irradiating the sample of interest, typically photosensitive molecular systems in solution.

The common laser source is a commercially available Ti:sapphire laser regenerative amplifier with 800 nm center wavelength and >1 mJ pulse energy at 1 kHz repetition rate. The three beams can be grouped into (1) the initiation pulse, which is a femtosecond ($10^{-15}$ s) actinic pump that prepares the system in the electronic excited state, and (2) the probing pulse pair, which consists of a picosecond ($10^{-12}$ s) pump coincident with a femtosecond probe to complete the stimulated Raman process. The noncollinear geometry with a crossing angle of $<10^\circ$ between all three beams is used to find the balance between scattering reduction and interaction length, which requires a pinhole after the sample to select the transmitted probe beam carrying the heterodyne-detected FSRS signal [3,8-10]. The beam collimation at the sample spot is crucial to achieve optimal spatial overlap, and a thin BBO crystal can help find the time zero before using sample cell. The power of laser pulses can be tuned while checking FSRS signal from a standard solvent such as cyclohexane. To avoid thermal effect or the pulsed laser peak-density-induced optical damage, low laser intensity is desirable and the typical actinic pump and Raman pump power is set below 500 µW and 4 mW, respectively. Because the Raman probe is a supercontinuum white light generated in a 2-mm-thick sapphire plate, its stability with a relatively flat profile in the spectral region of interest requires low power (in the 50 µW range). We recently found that a 2-mm-thick flowing water film can generate white light with more photons below 450 nm, which broadens the overall detection window from the near UV to near IR. Additional pinholes in the probe beampath before the spectrograph can help select
the central region which can lead to higher FSRS signal strength due to the reduction of noise, scattered light, or fluctuations of the outer region of the probe beam. The synchronization of the CCD array camera (e.g., PIXIS 100F; Princeton Instruments), optical chopper in the Raman pump beampath, and data collection software (typically a LabVIEW suite) with the laser system ensures the efficient collection of FSRS spectra in the mixed time-frequency domain without complication from mixing three femtosecond pulses at early time (starting from the Franck-Condon region) of a photochemical reaction [11].

**Wavelength Selection and Sample Conditions**

Sample concentration needs to be in the nanomolar range or higher, depending on the Raman cross-section and resonance Raman conditions for the solute [12-14]. An effective way to avoid thermal effect or pulsed-laser-induced sample degradation is to provide a fresh sample spot for each laser shot. For sufficient sample volume (e.g., >1 mL) a liquid flow cell with 1–2 mm pathlength and 0.5–1 mm thickness quartz windows can be used; but for smaller sample volume, a motor-driven miniature magnetic stir bar or a piece of steel staple wrapped by a thin layer of parafilm (particularly in acidic solutions) can be used to constantly stir the sample during measurements. Scattering from proteins typically limits the detection window above ~500 cm\(^{-1}\), but for smaller solutes such as photoacids [14-16] or metal-containing complexes [17-19], a filtered sample solution can yield low-frequency modes below ~200 cm\(^{-1}\) which include the H-bond stretching mode that plays a key role in gating excited state proton transfer [14,20]. Figure 1 shows a schematic of tuning the incident laser pulse wavelengths to achieve pre-resonance enhancement of transient molecular species from reactant to product [10,14], which helps to uncover the multidimensional reaction coordinates responsible for fluorescence modulation inside functional biosensors.

**Baseline Subtraction and Data Processing**

For reproducible FSRS data collection, the laser room temperature and humidity need to be maintained (at least ±1°F and ±5% relative humidity). Despite the best effort, the time-resolved FSRS data commonly display a broad, transient baseline which arises from the Raman pump-induced change of the transient absorption profile [14,21,22]. The general way is to draw a smooth spline baseline that cuts through the noise level of the ground-state-subtracted excited-state spectrum, after adding back the ground-state peaks and solvent peaks to fill spectral dips [7-10]. The baseline subtraction yields excited-state Raman peaks across the spectral window typically over 1600 cm\(^{-1}\), which can be analyzed to reveal the mode frequency, intensity, and width dynamics.
Conclusion
The implementation of FSRS requires careful consideration of these procedures to manifest its power in illuminating the microscopic world from proton transfer to biosensor imaging. We expect the research and development labs from academia to industry to use FSRS and gain structural dynamics insights like never before.

Acknowledgement
Special thanks to Dr. Weimin Liu and Liangdong Zhu, and the U.S. NSF CAREER grant (CHE-1455353) to C.F.

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