BROADBAND SRS MICROSCOPY DESIGN CONSIDERATIONS

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1. Summary

The biggest difficulty in implementing broadband SRS with dispersed detection is achieving sensitivities comparable to traditional SRS. It all comes down to how many photons you can measure per second and array detectors generally have to compromise between well depth and clock speed. Even with the best camera I've found so far, our *absolute* sensitivity would be 3 orders of magnitude less than that of traditional SRS. However, there are a few tricks we can play to boost our signal magnitude that might allow broadband SRS to have the same effective limit of detection as traditional SRS. In case the necessary technology to implement broadband SRS is not available I've thought of an alternative way to measure full Raman spectra that uses a fast photodiode as the detector and thus preserves the sensitivity that this type of detector entails. Of course, this second method, analogous to impulsive stimulated Raman spectroscopy, has its own problems and potential solutions.

2. Background

Wei Min (a student of Sunney Xie's, now an assistant professor at Columbia) gave a talk a few weeks ago from which I got a better feel for what's considered "state-of-the-art" for SRS imaging:

- Pixel dwell time = 10 μs (for strongly scattering molecules, more commonly they use 100 μs).
- 100 mW pump, 50 mW stokes (he used twin 80 MHz oscillators meaning he has pulse energies of 1.25 and 0.625 nJ).
- They modulate the pump at 10 MHz and detect the probe.
- They can detect changes in the probe intensity down to 10^{-8} with 1 second integration times. He claims this translates into a limit of detection of 3k molecules in the focal volume, depending on the molecule.
- They've started to do some vibrational tagging with two goals, increasing signal strength and making it easier to differentiate between species. The latter is needed because its hard to record more than a few spectroscopic frequencies.
- They have an x/y resolution of about 300 nm and z of 500 nm (confocal scanning microscopy).

The pixel dwell time means that a 512×512 frame takes about 3 seconds to acquire for a single spectroscopic frequency.* Both the pump and stokes beams in most of these set-ups are picoseconds, whereas I'm proposing a broadband, femtosecond probe (stokes) beam. From theory, the intensity of the signal should only depend on the intensity of the pump so the difference between picosecond and femtosecond probes is not expected to make a significant difference.

3. Signal to Noise

Out biggest issue will be achieving acceptable signal to noise and the biggest source of noise is $1/f$ noise meaning that we need to increase the rep rate. Increasing the rep rate will have the added benefit of reducing the minimum pixel dell time which is twice the inverse of the rep rate; we need to collect one Raman-pump-on probe spectrum and one Raman-pump-off probe spectrum for each complete SRS spectrum. For instance, for a 500 kHz laser our minimum dwell time will be *two* laser shots, or 4 μs.

I should note, that they way my group and most other spectroscopists define the signal and they way most SRS micrcoscopists define it differ; we define the signal as

$$S_1 \propto \ln\left(\frac{I_{Pr}^{Pu}}{I_{Pr}}\right)$$

while they define it as the modulation depth of the probe intensity when the pump is modulated (i.e. the difference instead of the division).

$$S_2 \propto I_{Pr}^{Pu} - I_{Pr}$$

Where I_{Pr} is the intensity of the probe without the pump and I_{Pr}^{Pu} is the intensity of the probe with the pump. In both cases $I_{Pr}^{Pu} = I_{Pr}(1 + \alpha I_{Pu})$ in the limit of small α,† where α is a collection of constants, one of which is the Raman cross section for that particular Stokes frequency. This means that S_1 and S_2 reduce to

$$S_1 \propto \ln(1 + \alpha I_{Pu})$$
$$S_2 \propto \alpha I_{Pu} I_{Pr}$$

You might wonder, as I did, if the difference in signal definition, and the difference in measurement that it implies, might mean a benefit in the signal-to-noise ratio. From my calculations, it doesn't, except for unphysically large values of α.

Assuming we can reach shot-noise limited performance our signal to noise ratio becomes[2]

$$SNR = \alpha I_{pu} \sqrt{I_{pr}}$$

*Sunny Xie has reported a new instrument with better signal to noise that needs only 4 μs dwell times, which translates to 1 fps acquisition times.[1]

†The general equation for large values of α is $I_{Pr}^{Pu} = I_{Pr} \exp(\alpha I_{Pu})$.

Where αI_{pu} is the experimentally observed gain or modulation depth, depending on the signal definition. Sunny Xie and colleagues claim a sensitivity of 10^{-8} with 1 second of integration, if we assume that implies an $SNR = 2$ than that means he is detecting

$$I_{pr} = \left(\frac{2}{10^{-8}}\right)^2 = 4 \times 10^{16} \text{ photons/second}$$

More precisely, he is able to measure 4×10^{16} photo*electrons*/second, this is close to the saturation limit for a standard photodiode.

Reaching this sensitivity with broadband detection will be our *main* challenge. On the one hand, we *must* have a fast detector in order to be able to have high modulate rates thereby obtaining shot-noise limited detection. On the other hand, the overall sensitivity–signal-to-noise ratio–is *completely* determined by the number of probe photons you can detect per second. For array detectors these two properties are usually at odds with one another

4. EQUIPMENT

The big question, as I explained above, is whether we can find a detector that is both fast enough to achieve shot-noise-limited detection *and* has deep enough wells such that enough photons can be collected to reduce said shot-noise as much as possible.

4.1. **Detector.** The best candidate I have found so far, the Hadland DigiStreak™,* has 1280 pixels (spectroscopic frequencies) and can capture 543k full spectra per second. The data acquisition time is only limited by the onboard memory which is about 2 GB. This is by far the fastest camera I have found. I did talk to an engineer at Hamamatsu that claimed they could make a 100-200 kHz camera, but I haven't followed up with him yet. I've also looked into streak cameras, but they're limited by the data readout time.

We could also build our own streak camera in which we would get a fast CCD camera (with a kHz readout rate) and we would use a 1D galvo to sweep the beam across the sensor such that each row would correspond to one pulse. This is exactly how the Hadland DigiStreak works. For a 1024×1024 camera with 1,000 fps read out speeds we could approach MHz detection speeds but at the cost of much more engineering.

One problem, in general, is that as you increase the sensor's readout speed you reduce the well depth, in general. The DigiStreak sensor has a well depth of ~63,000 e^- whereas the camera I'm currently using, the PI PIXIS 100F, has a well depth of over a million e^-. For the DigiStreak, If we approached shot-noise limited detection then we should achieve a per pixel sensitivity of, with 1 s of integration, of

$$2/\sqrt{63000 \times 543000} \approx \times 10^{-5}$$

*www.hadlandimaging.com

Which is *three* orders of magnitude less than our competitors. We can make up some of the difference by increasing the Raman pump power and boosting the signal. I think, with the laser systems described below and an etalon filter, that we could reach 1 μJ/pulse energies. This would be a thousand times more energy per pulse than traditional SRS meaning that we would have the same limit of detection. However, the drawback is that we would be dumping 0.5 W of average power into our samples which could blow them apart. If we could find a camera with a full well depth of 1 Me^- then we would only need 50 mW of average power for the Raman pump. The instantaneous power will still be 100 times that of regular SRS experiments but we'll be operating at 1040 nm so we might be able to get away with it. In any case, the only strict requirement for the camera, other than as large a per pixel capacity as possible, is that it can acquire full spectra at 65 kHz, this number will be made clear below.

The other big question is the light source.

4.2. **Light Source.** There are three options:

(1) An ultra high power, ultra broad band oscillator
(2) A broadband/narrowband oscillator pair
(3) A high rep-rate/high-power amplified system

Each option has a few drawbacks and advantages. With the first option it would be very difficult to get a high power Raman pump, we might be able to use the spectral compression technique pioneered by Cerullo and co-workers[3] but this would give us a ~400 nm Raman pump thereby losing the advantages of working in the infrared. The second option would be the most expensive and ensuring that the pulse trains are phase locked will increase the cost, however, because of the time symmetric nature of the Raman pump in this case a small amount of timing jitter (< 100 fs) will be acceptable.

My personal opinion is that an amplified system based at ~1030 nm (Yb^+) is going to be the best option. There are a few manufacturers (so hopefully we can get a good price); Light Conversion's Pharos or Carbide, Spectra-Physics' Spirit and Clark-MXR's IMPULSE. In all cases the fundamental has about 100 cm^{-1} of bandwidth so we won't lose much power with basic spectral filtering. The main difference between these competitors is the maximum power available, the Carbide is on the low end at 4 W, the Pharos can go up to 15 W, the Spirit to 8 W and the IMPULSE maxes out at 10 W. Both the Spirit and Pharos/Carbide are based on crystal technology while the IMPULSE is a fiber laser, its not clear whether that means it will be more stable or require less maintenance. On the other hand, the Spirit was initially designed as an industrial laser so it *probably* has the lowest maintenance and highest stability, but that is by no means a fact.

The open question is whether we can generate the broadband pulse efficiently. I'm thinking that generating white light continuum in sapphire (as we do now in my current lab) will be enough. I've spoken to both Spectra-Physics and Clark-MXR and they've both have offered to run experiments generating continuum in sapphire at a 500 kHz rep rate.

They also gave me informal quotes of $250k and $230k, respectively. Light Conversion hasn't gotten back to me yet.

As I explained above, the way we will perform this experiment will mean that the probe controls the background noise while the pump controls the signal intensity. This means that we'll want to nearly saturate the detector with every probe pulse. The DigiStreak has a well depth of ~63,000 e^- and a quantum efficiency that averages to about 5% in the 800-1040 nm range* meaning we would want about 0.5 nJ of white light per pulse. Of course, what we really want is a camera with a much deeper well depth, but I think we could easily get 10 nJ of white light if we needed to.

One way in which we could improve our SNR is to boost our signal by doubling our Raman pump to 520 nm. Pre-resonance Raman cross sections generally scale as

$$K\nu\nu_s^3\left[\frac{\nu_e^2+\nu^2}{(\nu_e^2-\nu^2)}\right]^2$$

Where K is a scaling factor, ν is our Raman pump frequency, ν_s is the frequency of the vibrational transition and ν_e is the electronic transition. Assuming that $\nu_e = 30{,}000$ cm^{-1} (an absorption band at 333 nm) the ratio of the Raman cross-sections at 520 nm versus 1040 nm is about 100. This would help a lot in achieving high sensitivity while still performing broadband detection as in this case we'll only need to use a 10 nJ Raman pump pulse (50 mW average power). There are a few caveats, mainly that we'll be working with higher energy photons which might increase the risk of photo damage and we will want to avoid samples that have visible absorbance spectra, i.e. no samples that absorb at wavelengths longer than about 500 nm.

If we can find an acceptable camera and light source then the other equipment is relatively easy to choose.

Spectrograph: I haven't given a whole lot of thought to this, but anything simple and small will do. I don't expect we'll want, or have, any tunability in the Raman pump so it won't even need to be computer controlled. Honestly, we could probably build one ourselves.

Digital micromirror device: Texas Instruments is really the only game in town when it comes to DMDs. The one that we would want is the DLP Discovery™ 4100 which has a maximum pattern rate of 32.552 kHz and has 1024×768 pixels. This will almost certainly be the bottle-neck in imaging speed. For this experiment that means that we will want to collect the Raman pump on and the Raman pump off spectra at this rate, which explains the lower limit for camera speed given above.

Standard microscope optics: I'm assuming you have more than a few of these lying about and that most anything we'd need that you don't have would be readily in stock.

*We would probe on the anti-stokes side of a 1040 nm Raman pump

Raman pump modulator: This is a solved problem, we need an acousto or electro-optic modulator that can be synced to our light source.

Raman pump filter: This will depend on the light source (for instance if we go with the twinned oscillators than it will be unnecessary). I would think an etalon filter would be the best choice, though it might make the timing tricky if we decide to try out the light sheet idea that I've outlined below.

5. Compressed Sensing

One of the main innovations of my proposed approach is leveraging compressive sensing (CS) in order to further reduce the imaging time by removing the need measure every pixel. The main ideas behind this are outlined in a paper co-authored by Candes[4] on applying CS to fluorescence microscopy. I have started looking into the actual technical details and while I haven't gotten a firm grasp on everything, I believe that this is a solved problem and as long as we can collect data we can use nearly black box reconstruction algorithms to "decompress" the data, at least initially. That being said, one of the other great advantages to this technique is that it has the potential to get better, much better, with just a software update.

5.1. **Imaging speed.** Our minimum "effective" pixel dwell time is the ultimate limit on imaging speed and is the best measure with which to compare this technique to traditional SRS. It can be determined as $1/f_{mask}R$ where f_{mask} is the speed with which we can update the pixel mask and R is the compression ratio. Assuming that we can collect acceptable spectra as fast as we can update the pixel mask (33 kHz) and have a compression ratio of 10 we would have an effective dwell time of ~3 μs. This is three times better than standard SRS for a *single* spectroscopic frequency.

If we acquire data, compressively, in three dimensions we may be able to increase our compression ratio by another factor of 10 (the data becomes sparser with increasing dimensionality) and so our effective pixel dwell time should be reduced by another order of magnitude to ~0.3 μs.

5.2. **3D.** For imaging in 3D I've had a new idea.* Because FSRS is self-phase matched the signal is, necessarily, collinear with the probe beam. This means that we could, potentially, turn the pump beam into a light sheet allowing us to greatly improve our axial resolution with the added benefit of being able to sample efficiently in 3D while removing any out-of-focus contributions, which could make image reconstruction easier. Because we could sample in 3D we can design more efficient sampling algorithms which should improve our compression ratio and therefore our overall imaging speed.

*Actually we may have discussed this during my interview and I've just remembered it as my own, if that's true my apologies.

6. Time Domain Stimulated Raman Microscopy

6.1. **Frequency-Combs.** To my mind, the dispersed detection instrument I've described above will not work unless we can find a much better camera even if we double the Raman pump frequency and increase its power. I've been thinking about using an alternative method of recording Raman spectra developed by Hänsch and co-workers.[5]

They've termed the technique "dual-comb CARS" and essentially, it down-converts molecular vibrations into radio-frequencies which are then measured directly in the time domain. The single biggest advantage to this method is that everything is measured on a single photodiode like traditional SRS microscopy meaning that we should be able to achieve the same absolute sensitivities. The experiment itself is quite simple, you overlap the pulse trains (or combs) of two femtosecond oscillators whose frequencies differ by an amount δf. This frequency difference means that for each period the pulse separation between the oscillators increases by a time step of $\Delta t = 1/f - 1/(f + \delta f)$. In this way the first pulse generates a vibrational coherence and the second pulse probes it for every pair of pulses thereby tracing out an "interferogram" of the molecular vibrations. This has the effect of down sampling a molecular vibration by an amount of $\delta f/f$.

While this technique should be able to achieve much higher sensitivities it has two intertwined problems:

(1) The interferogram only refreshes with a rate equal to δf which means that the minimum possible dwell time is equal to $1/\delta f$. Moreover, any possible dwell time *must* be an integer multiple of $1/\delta f$.
(2) The Nyquist frequency for the measurable molecular vibration is $\nu_{max} = 2/\Delta t = f(f + \delta f)/2\delta f$

For instance, if $\delta f = 100$ Hz and $f = 1$ MHz then $\nu_{max} = 1667$ cm^{-1} and the dwell time is 10 ms. We can easily increase both of these by using GHz oscillators, in this case $f = 1$ GHz and $\delta f = 10$ kHz then ν_{max} is the same as before but the dwell time is reduced to 100 μs. This is still far slower than traditional SRS microscopy but there are two tricks you can play. One is to use compressive sensing which would decrease the *effective* dwell time by 10, for 2D imaging, and 100, for 3D imaging.* The other is to dynamically change δf which can, Hänsch claims, increase the duty cycle by up to 50 fold.

Either of these tricks alone would reduce the dwell time to the μs level allowing video rate imaging. Furthermore, these dwell times are for *entire* spectra and because we are performing the measurement in the time domain our resolution is only limited by the intrinsic vibrational dephasing time of the sample (usually on the order of a few cm^{-1}). However, it is important to note that the maximum detectable frequency is not only limited by Nyquist sampling as discussed above but also by the intrinsic bandwidth of the laser pulses. That being said, 15 fs oscillators should have no problems up to 2000 cm^{-1}, though higher

*ISRS is also self phase matched so we can also combine this technique with light sheet microscopy.

frequencies will need better SNRs to detect. In comparison, the broadband instrument I described above will be much more adapt at measuring high frequency signals. I should note that this technique is identical (in theory) to interferometric CARS, which Meng Cui did as a graduate student, but that the acquisition time is, potentially, much faster because the time delay between pulses is controlled by the comb frequency mismatch and not by a physical delay stage.* The other issue is measurement, we would need to be able to record data at near GHz rates, standard lock-in amplifiers won't work but we could use an oscilloscope.

There are a few other parameters that could be adjusted to improve this technique. One very simple one is to have the two oscillators running with different spectra so that the "probe" oscillator could be easily separated from the pump oscillator. In this case we would be able to detect many more probe photons and thus increase our SNR. In addition, if we could find another, faster, way to sweep the time delay–such as that described below–we could really improve the duty cycle. The real issue for this instrument is finding the right laser sources, especially ones with rep rates that can be changed quickly and accurately.

6.2. **Acousto-Optic Programmable Dispersive Filter.** This is a different approach to sweep the time delay between pulses without a mechanical delay stage. The basic principles of operation are covered in depth in ref. 6. Briefly, an oscillator pulse train is passed through an acousto-optic crystal (in this case TeO_2) while at the same time an acoustic wave is launched into it. Because of the huge separation between the propagation time of the acoustic and EM waves, each laser pulse essentially encounters a the acoustic wave, which appears stationary to the laser pulse, at different points along the length of the crystal. Interaction of the light pulse with the acoustic wave rotates a portion of the laser pulse and because the crystal is birefringent the o and e portions of the pulse propagate with different speeds. The time delay between the o and e components will depend on the axial position of the acoustic wave at the time of the acousto-optic interaction. Of course, the change in the axial position of the acoustic-wave between subsequent laser pulses depends on the speed of the wave relative to the repetition rate of the laser. In effect the time delay between the o and e pulses is linearly scanned with a time step of

$$\Delta t = \frac{(n_e - n_o)}{c} \frac{v_{sound}}{f_{rep}}$$

Where Δt is the increase in delay between the o and e pulses for subsequent input laser pulses, v_{sound} is the speed of sound in the crystal, f_{rep} is the repetition frequency of the oscillator and n_o, n_e and c have their usual meanings.

Schubert et al.[6] have demonstrated a system based on an Er^+ fiber laser with a f_{rep} = 40 MHz and a commercial acousto-optic programmable dispersive filter (Dazzler, Fastlite).

*To get the same speed with a physical delay stage you'd need one that could move at 3 km/s!

The oscillator's repetition rate translates into a $\Delta t = 5$ fs (a Nyquist limit of 3336 cm^{-1}). The Dazzler has a maximum repetition rate of 34 kHz consequently the maximum delay of the scan is about 6 ps, long enough to sufficiently sample the vibrational free induction decay. Additionally, the 34 kHz rep rate corresponds to a minimum pixel dwell time of 30 μs, significantly shorter than that for the frequency combs mentioned above.

There are a few advantages to using this method over the dual-comb method described above. Firstly, you only need one oscillator, reducing the cost substantially. Moreover, we can use telecom wavelengths ($\lambda = 1560$ nm) for which there are many readily available components and longer wavelengths should reduce photo-toxicity.* Short pulses (8 fs) are relatively simple to produce pulses using highly nonlinear fibers and various compression strategies (i.e. prism compressors). Second, the implementation is much easier; there is only one beam to align. Finally, the pump and probe beams are polarized orthogonal to one another meaning they can be easily separated after the sample which could improve the signal-to-noise. However, it is important to note that the orthogonal polarizations may reduce the signal, for most bands the signal intensity will be reduced by 25% but for polarized bands it could be greater. Even so, the ability to separate the two beams may mean we can employ better detection strategies, such as balanced detection, which could boost our signal-to-noise significantly.

7. Limits of Detection and Power Considerations

In order to evaluate the utility of a broadband SRS microscope we need to ensure that it will have a limit of detection that is comparable to a traditional single wavelength SRS microscope. Luckily, the theories of time domain (impulsive) stimulated Raman and frequency domain stimulated Raman are nearly identical meaning that they have the same dependence on the same parameters. For both the intensity of the signal is given by

$$\frac{\Delta I_{pr}}{I_{pr}} = \left(\frac{\pi\alpha'}{nc}\right)\frac{\omega_l}{\omega_\nu} N l I_{pu}$$

Here α' is the polarizability derivative of the molecule with respect to the normal mode of interest (this can be directly related to the Raman cross-sections available in the literature), n is the index of refraction of the medium, c is the speed of light, ω_l is the frequency of the laser, ω_ν is the frequency of the normal mode of interest, N is the number density of molecules, l is the path length and I_{pu} is the time integrated intensity of the pump pulse (i.e. the energy flux of the pump). We can use this equation directly for molecules

*Previous authors[7] have indicated that two photon absorption is the cause of most photo-toxicity because the resulting wavelengths are in the near UV. For telecom wavelengths the two photon absorptions will be in the visible.

with reported Raman cross-sections.* Alternatively, we can use data from traditional SRS microscopists.

In the SRS literature Wei Min has the most detailed reports on the SRS detection limits for various molecules. His instrument is a confocal laser scanning SRS microscope and it has the following parameters:

Power: 120 mW for both pump and probe (240 mW total power)
Spot size: 300 nm
Dwell Time: 100 μs

With these parameters he can achieve sensitivities close to 10^{-7} which leads to the following limits of detection (in number of molecules in the laser focus volume):

Retinoic Acid: 3,500[2]
Phenylalanine (Benzene rings): 10,000[8]
Alkynes: 12,000[9]
Choline: 10,000-20,000[10]

One surprising observation is that Wei Min and colleagues are *not* cooking their samples even though the intensity at the focus is in excess of 300 MW/cm^2. However, this fact leads to an important realization: one *needs* that kind of intensity to get a decent signal, which means that wide field imaging will be impossible. Moreover, traditional SRS microscopists use picosecond pulses which are in general less damaging to biological tissue than femtosecond pulses. However, moving to telecom wavelengths should mitigate the risks to some extent. Because we can no longer use wide field imaging my initial ideas for incorporating compressive sensing strategies will not work. Of course, it is still possible to sample the image incoherently with a laser scanning instrument but determining the proper scanning parameters and patterns will require some more thought.

Now to the question of whether or not this microscope would be worth the trouble. I still think it would be. This approach would offer be able to collect an entire spectrum with roughly the same signal to noise in the same amount of time a traditional SRS microscope would take to collect a single spectroscopic frequency. This means we could measure many more species simultaneously while for a traditional SRS instrument the acquisition time would scale linearly with the number of species to measure. For instance, tracking the diffusion of dopamine, serotonin and adenosine simultaneously[11] with traditional SRS would be difficult; all acquisition times would be at minimum three times longer. In fact, the spectra of those three neurotransmitters are quite similar and to accurately quantitate their spatial concentrations would most likely require more than three spectral points in order to deconvolute the signals. Moreover, it would be easier to study small molecule production, transport and interactions within cells with broadband SRS.

*Raman cross-sections are directly related to polarizability and transforming from one to the other is simple.

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