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Screening of Protein Crystallization Trials by Second Order Nonlinear Optical Imaging of Chiral Crystals (SONICC)

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Abstract

Second order nonlinear optical imaging of chiral crystals (SONICC) is a promising new method for the sensitive and selective detection of protein crystals. Relevant general principles of second harmonic generation, which underpins SONICC, are reviewed. Instrumentation and methods for SONICC measurements are described and critically assessed in terms of performance trade-offs. Potential origins of false-positives and false-negatives are also discussed.

Keywords

SHG; microscopy; lipidic mesophase

1. Introduction

Interest in the structure and function of integral membrane proteins is driven largely by their role in human disease. High-resolution protein structures can provide information on ligand binding locations and protein function. The most common strategy for obtaining such structures is currently X-ray diffraction, often performed at synchrotron sources. Complications commonly plaguing soluble protein crystal detection in high-throughput systems are exacerbated in studies of membrane proteins for several reasons. Consequently, crystallizing membrane proteins is notoriously difficult, and often requires screening a large number of trials.

Several factors complicate high-through crystallizations of integral membrane proteins, most of which arise from the combined requirements of providing both hydrophilic and hydrophobic environments around the proteins. Many of the most promising matrices for membrane protein crystal formation routinely result in defects and scattering centers, contributing to the turbidity of the mother liquor and complicating small crystal detection. These effects are rampant in lipidic mesophase crystallizations, including those performed in lipidic cubic phase (LCP). [1–4] LCP crystallizations and related methods represent some of the most promising new matrices for the routine production of diffraction-quality crystals of integral membrane proteins. However, slow diffusion of membrane proteins within LCP and related phases and high nucleation rate can routinely result in the formation of numerous smaller crystals rather than a few larger, well-formed crystals, further complicating unambiguous protein crystal detection.

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A host of imaging approaches has evolved to characterize protein crystallization trials. Bright field searching provides good contrast for large crystals, but cannot distinguish between salt and protein crystals and has difficulty finding smaller crystals in scattering media. Birefringence studies have many of the same strengths and weaknesses as bright field approaches, [5, 6] but provides higher contrast for crystals through suppression of the background. Background birefringence and scattering of the crystallization matrix or platform ultimately limit the detection capabilities. UV-autofluorescence can also provide image contrast for crystals and discriminate between salt and protein. However, the intrinsic brightness for UV fluorescence can vary significantly across proteins. In addition, UV-fluorescence cannot easily discriminate between protein aggregates versus protein nano and microcrystals. Finally, contrast is reduced by auto quenching and can damage proteins if long or repeated exposures are required. Trace fluorescence labeling can also discriminate between salt and protein, but at the expense of additional complexity and the possibility for impacting crystal structure quality. Although many additional techniques are continually emerging, this set represents a reasonable compromise between complementary information content while retaining the imaging speeds required for high-throughput analysis.

Recently, second order nonlinear optical imaging of chiral crystals (SONICC), based on second harmonic generation (SHG) microscopy, [7] has been developed as an alternative approach for detection and characterization of protein crystals. SONICC has the potential to provide sensitive and complementary information when used in combination with the existing suite of methods. The primary advantages of SONICC are arguably the selectivity for crystalline forms and the sensitivity to allow reliable detection of nano and microcrystals. These capabilities are particularly well suited for studies of membrane proteins, given the growing use of lipidic mesophase crystallizations. An example is shown in Figure 1, [8] in which SONICC micrographs are compared with a suite of conventional imaging approaches, including bright field imaging, birefringence imaging [5, 6], ultraviolet autofluorescence microscopy, and fluorescence imaging based on labeling. [9, 10] In the limited data set used, SONICC proved particularly advantageous in detecting small crystals in highly scattering media.

The primary purpose of the present work is to describe the instrumentation, approaches, and methods underpinning practical implementation of SONICC. Specific aspects include ultrafast optical sources, rationale for rapid beam-scanning, critical discussion of numerical aperture, field of view, and depth of field, specifics of the SHG detection configuration, sources of false-positives and false-negatives and their mitigation, and the compatibility of SONICC with different crystallization platforms. Finally, a discussion of the current challenges is provided along with untapped future possibilities.

2. Overview of Nonlinear Optical Interactions

2.1. Classical anharmonic oscillator model

Many of the critical qualitative features of SHG can be understood by considering a classical anharmonic oscillator for a hypothetical one-dimensional molecule (Figure 2). [11–13] An external electric field induces a dipole on a molecule by perturbation (sloshing) of the electron cloud. Larger fields produce larger induced dipoles, with the magnitude of the dipole described by a potential energy surface (PES). In general, the efficiency of generating an induced dipole is not necessarily identical for fields polarized in opposite directions, which contributes to the anharmonicity in the PES. For moderate driving fields, only the bottom-most harmonic portion of the PES is sampled, which describes the linear polarizability of the molecule.

However, if the driving fields are sufficiently strong, the induced dipole can be large enough to sample the anharmonic regions in the PES. In this case, the polarization is distorted in time by the anharmonicity. The shallower region of the PES will allow longer residence times of the electron cloud in that configuration relative to the opposite polarization. Consequently, the net distorted time-dependent polarization is illustrated by the solid black line in Figure 2.

From the presence of this time-domain distortion, Fourier transformation recovers the frequency content required to generate it. In addition to the major component at the fundamental frequency, a small but nonzero contribution at the doubled frequency emerges as well as a direct current (DC) offset. Once generated, the different frequencies can be easily separated by wavelength and the SHG light selectively detected.

The net coherent polarization of a material arises from the collective interactions of all the individual oscillators. In linear optics, the coherent polarizability describes reflection and refraction at the materials level. If there were to two molecules of identical structure but opposite orientation immediately adjacent to the one another, the polarization of the system at the fundamental would be identical, but the SHG arising from the anharmonic distortion would be inverted in sign between the two molecules. The net polarization would therefore result in perfect cancellation of the SHG fields.

The simple, one-dimensional model described in the previous section illustrates some key elements of SHG in general, and SONICC in particular. First, the molecular response requires asymmetric anharmonicity in the polarizability. Second, the effects only become significant at high driving fields that can sample the anharmonic portions of the molecular polarizability. Finally, the total field is described by the net collective response of all the molecules within the focal volume. Unordered systems result in no net coherent response, whereas ordered assemblies have the potential to generate SHG. However, the net effect depends sensitively on both the molecular response and the packing arrangement of the crystal.

2.2. SHG Activity of Crystals

In three-dimensional space, the induced molecular nonlinear polarization describing SHG is dependent on two driving fields (usually from the same beam). Mathematically, this dependence is expressed as a $3 \times 3 \times 3$ $\chi^{(2)}$ tensor, each χ_{ijk} element of which describes the efficiency and sign/phase of generating i -polarized SHG when driven by j - and k -polarized fundamental fields. [11, 14] Similarly, the nonlinear optical properties of crystals will also be described by a $3 \times 3 \times 3$ $\chi^{(2)}$ tensor. However, the symmetry operations associated with the crystal will play as much or more important a role as the protein tensor itself in ultimately dictating the net SHG properties of the crystal.

Protein crystals, due to their chirality, can only form 11 of the 32 possible crystal classes. [11, 14] Of these classes, SHG is symmetry allowed in all but the relatively rare, high-symmetry O (432, cubic) class. However, generation of strong SHG is not guaranteed simply because a particular unit cell lacks inversion symmetry; other symmetry considerations are also important as they can lead to internal cancellation of the SHG signal. As a general trend, lower symmetry classes are likely to produce stronger SHG than higher symmetry classes. For example, the C_1 (1) point group (triclinic crystals) is most likely to exhibit strong SHG response based on symmetry. The C_2 (2) point group (monoclinic crystals) reduces the number of nonzero hyperpolarizability tensor element from 27 to only 13, but is still generally quite bright. Similarly, the C_3 (3) point group (trigonal), the C_3 (4) point group (tetragonal), and the C_6 (6) point group (hexagonal) all exhibit polar order and are likely to be relatively bright for SHG. Although lacking polar order, the D_2 (222) point

group (orthorhombic) is also relatively bright for SHG. However, there is a precipitous reduction in SHG efficiency for symmetry classes that contain four-fold or six-fold rotation or skew axes AND an orthogonal 2-fold rotation or skew axis [i.e., D_4 (422, tetragonal) and D_6 (622, hexagonal) crystals]. [15]

Within the context of these general trends, there is also substantial intrinsic variability within each point group. While the large majority of crystals within a low symmetry class are likely to produce detectable SHG, there will always remain a subset that produces very bright SHG and another weak-producing subset.

3. Instrumental Considerations for SONICC

3.1. Why ultrafast laser sources?

Nonlinear optical interactions scale nonlinearly with the input intensities. The reason for this requirement is apparent by inspection of Figure 2. For modest driving amplitudes, the corresponding amplitude of the induced polarization is weak, and the harmonic portion of the potential energy surface dominates to ensure a radiated field at precisely the same frequency as the driving field. It is only for sufficiently high driving forces that the anharmonic portions of the potential energy surface are sampled and frequency-doubled output can occur. As a consequence of this nonlinear scaling, high optical peak powers are required to achieve sufficient intensity for efficient nonlinear optical conversion. In contrast, low average powers are preferred for laser scanning microscopy in order to minimize perturbations to the sample.

Obviously, the simplest way to achieve high peak power with low average power is by using a pulsed laser source. Shortening the pulses by a factor of 10 while maintaining the same average power yields a 100-fold increase in SHG signal. An ultrafast laser source producing 100 fs pulses at 100 mW of average power at an 80 MHz repetition rate produces peak powers of 12,000 W.

In principle, reducing the pulse duration from 100 fs to 10 fs can again improve the signal to noise by an additional 100-fold. However, in practice, the use of sub 100 fs pulses is nontrivial in microscopy due to the large optical bandwidth requirements. Dispersion in the beam path, the objective, and the sample often introduces chirp that can be challenging to compensate without additional efforts as the pulse shortens below ~30 fs.

Several ultrafast laser sources are commercially available for SHG microscopy. Historically, Ti:sapphire lasers are an established, proven technology as ultrafast oscillators. However, the most common center wavelength at ~800 nm can produce interfering two-photon excited fluorescence for some proteins. Two-photon absorption can also be problematic if the sample or other elements within the field of view absorbs around 400 nm. For example, attempts to imaging crystals contained within Mitogen loops with an incident fundamental wavelength of 800 nm can result in some spectacular effects from rapid local heating of the loop at high laser fluence. The yellow color of the loops is consistent with this trend, suggesting relatively strong two-photon absorption corresponding to transitions in the blue. Fortunately, high-powered Ti:sapphire oscillators often have considerable tunability, achieving fundamental wavelengths >1 μm , but with additional expense.

The relatively recent development of ultrafast fiber lasers has made substantial end-roads in providing viable alternatives to solid-state ultrafast lasers. In fiber lasers, the gain medium is doped directly within the fiber, with the fiber itself serving as the optical cavity of the laser. Doping of the fibers with different rare earth element gain media changes the lasing wavelength range, with wavelengths of 1064 nm, 1030 nm, and 1550 nm routinely available

(Nd, Yt, and Er, respectively). Pulse durations of ~100–200 fs are commonplace, with average powers from ~100 mW to 1 W or above are available, consistent with the requirements for measurements by SONICC. In principle, ultrafast fiber lasers [16] have the potential to be highly stable, low cost, and capable of hands-free operation for extended periods of time. In practice, the technology is still maturing, with considerable variability across systems in the degree of laser tinkering required for stable operation.

3.2. Why perform beam-scanning measurements?

The most common method used for SHG microscopy is to focus a high-repetition rate (>10 MHz) oscillator beam and perform imaging one pixel at a time by scanning the focus of the beam through the field of view (Figure 3). [17–21] In this case, single-channel detectors (e.g., photomultiplier tubes or avalanche photodiodes) are used for detection, with the intensity in each pixel effectively encoded in time through control of the beam-scanning mirrors. This approach takes full advantage of the nonlinear dependence of the signal with incident intensity. Only the focal volume experiences sufficient intensity to enable efficient SHG, such that little or no significant background signal is produced out-of-plane.

The highly localized excitation in SHG is particularly advantageous in studies of turbid crystallization matrices, which are increasingly encountered in membrane protein crystallization protocols. Incident fundamental light that is scattered never makes it to the focal volume. Consequently, only the “ballistic” unscattered component of the light results in a high enough intensity within the focal volume to contribute to the detected signal. [22, 23] Scattering can impact the efficiency of SHG, but will not generally directly affect image resolution. This approach is distinctly different from conventional confocal microscopy, in which the detected light is descanned back through the scan head before focusing through a pinhole or onto a small active area detector (e.g., avalanche photodiode). The pinhole serves to reject out-of-plane fluorescence generated during scanning. The detected beam must be descanned first in order for the light from every location within the field of view to be directed through a fixed-position pinhole. However, no such requirement exists for nonlinear optical processes, since the nonlinear dependence on intensity alone is sufficient to provide localization of the signal to the focal volume. Furthermore, even if the relatively few photons of SHG light are scattered multiple times prior to detection, all SHG can be assumed to have originated from that same focal volume. Consequently, collection efficiency is generally optimized by placing the photodetector close to the back of the objective to minimize losses and by using a large-area photodetector (e.g., PMT) and collecting light from a large field of view.

Beam scanning is typically performed using either a galvanometer mirror pair or by combining a slow-axis galvanometer mirror with a fast-axis resonant scanner. The primary advantage of the resonant scanner is to minimize possible sample damage or perturbation from local heating. [24–26] Alternatively, imaging can also be performed by illuminating the entire field at once with each pulse and recording the image on an array detector (e.g., charge-coupled device, CCD). This latter approach has the benefit of capturing dynamics in pump-probe measurements, but requires much higher peak powers of excitation, since all pixels are illuminated in parallel. In practice, the laser requirements necessary to achieve full field of view illumination with high signal to noise are prohibitively complicated for bench-top measurements. Furthermore, this approach is susceptible to out-of-plane interferences and losses in image quality due to scattering, limiting its use to transparent media.

3.3. Field of View, Depth of Field, Working Distance, and Frame Rate Considerations

Several trade-offs specific to nonlinear optical imaging arise depending on the specific nature of the crystallization platform investigated. First and foremost, the quadratic

dependence of SHG on the peak intensity within the focal volume results in a steep dependence on numerical aperture and tightness of focus.

For nanocrystals much smaller than the focal volume, the SHG intensity scales approximately with the square of the peak intensity, or equivalently with the fourth power of the numerical aperture (NA), suggesting a nearly 500-fold difference in the detection limits between a 0.3 NA 10x objective and a 1.4 NA 100x immersion objective. For this reason, tight focusing is highly advantageous for nanocrystal detection. Alternatively, recovery of signal can also be achieved by increasing the laser power to compensate for the loss in signal. A ~22-fold increase in the laser power can also produce a 500-fold increase in signal, such that images acquired with 20 mW through a 1.4 NA immersion objective and with ~450 mW through a 0.3 NA objective will generally produce comparable signals for nanocrystals. Practical factors affecting the use of higher laser power are the corresponding costs of such laser systems and the increased potential for sample perturbation by local heating, as discussed in greater detail in Section 3.4.

In comparison, the SHG intensity can be more forgiving with respect to the NA if the crystals under investigation approach or exceed the focal volume in size. The depth of field is approximately equal to the wavelength divided by the numerical aperture squared for an air-coupled objective. In addition, the beam radius in the focal plane is approximately equal to the wavelength λ divided by the product of π and the NA when imaging with a low NA, such that the confocal volume is approximately $[(\pi\lambda^3)/NA^4]$. As such, tight focusing reduces the number of unit cells contributing to the SHG signal, which largely offsets the gains expected by tight focusing. Additional complicating factors related to phase matching also come into play in nonlinear optical imaging of larger crystals. However, as a general rule of thumb, the SHG intensity measured from crystals much larger than the focal volume does not change substantially with changes in the NA. As in conventional bright-field microscopy, increasing the depth of field by reducing the NA also results in fewer Z-slices required to probe the entire volume of a crystallization droplet.

3.4. Susceptibility of Membrane Protein Crystals to Sample Perturbation from Laser Exposure

The unique nonlinear nature of SHG also impacts the mechanism and degree of possible sample damage in ways distinct from X-ray and UV exposure. In both of the latter two cases, cumulated protein damage arises from reactions initiated by high-energy photon absorption and scales linearly with dose. However, SONICC intentionally is most commonly performed with near infrared incident wavelengths (0.8 – 1 μm) that are low energy and are not significantly absorbed by the proteins. From numerous damage studies performed using similar instrumentation in multi-photon excited fluorescence microscopy in living tissues, two fundamentally distinct damage mechanisms routinely dominate in nonlinear optical imaging of biological tissues and samples: 1) plasma formation from high-order multi-photon absorption and ionization, and 2) effects from rapid local heating.

Plasma formation can be minimized by decreasing the NA and/or increasing the pulse duration and by rapid beam-scanning. Plasma formation can occur through direct absorption of a high multi-photon absorption leading to ionization. If a large enough fraction of the molecules present within the focal volume undergo ionization, plasma formation and bright white-light generation can result. Since this mechanism scales significantly higher than quadratically with peak power, reducing the tightness of focus minimizes plasma formation. By providing a more uniform intensity profile across the focal volume, reducing the NA maintains comparable signal to noise and minimizes the possibility of plasma-related damage, provided that the protein crystals are still comparable to or larger than the focal volume defined by the lower NA.

For the low NA measurements typically performed for protein crystal imaging, damage from local heating is significantly more of a concern than plasma formation. Whereas plasma formation generally tends to depend on the peak power of single pulses, local heating effects are generally cumulative over many pulses. In brief, even near-infrared light can produce some weak absorption from vibrational overtones and combination bands of water. Local heating can also arise from chromophores exhibiting strong two-photon absorption cross-sections at the doubled frequency. If the rate of heat deposition exceeds the rate of heat dissipation, rapid local heating can arise. Since heat dissipation scales with the temperature gradient, the local temperature will increase until the two rates are again matched.

One of the most effective strategies to minimize the impact of local heating is to employ rapid beam-scanning. Resonant scanners operating at 4 kHz, 8 kHz, and even higher frequencies are readily available to translate quickly across the field of view. Rotating polygonal mirrors achieve similar effects at comparable scan rates. For an 8 kHz sinusoidal scanner imaging a $500\text{ }\mu\text{m} \times 500\text{ }\mu\text{m}$ central field of view with a $1\text{ }\mu\text{m}$ beam waist at the focus, the beam spends only $\sim 60\text{ ns}$ in a single focal volume before moving to the next. As a result, heat is deposited into a single focal volume from just 5 pulses of the laser for an 80 MHz ultrafast laser in a single pass. Retracing the same line with the focused beam again allows an average of $60\text{ }\mu\text{s}$ of dead time for heat dissipation prior to the deposition of heat from another set of 5 pulses under these conditions. Numerous passes of the mirror can be performed to increase the integrated exposure of each pixel. For comparison, imaging with a more conventional galvanometer for the fast-scan axis results in typical sustainable scan rates of $\sim 200\text{ Hz}$ (although galvanometers up to 1 kHz are available), corresponding to about 5000 consecutive laser pulses per focal volume per pass and a roughly 1000-fold greater sensitivity to potential complications from local heating. By using a combination of rapid beam-scanning and low-NA focusing, SHG microscopy can routinely be performed with laser powers approaching or even exceeding 1 W without introducing obvious sample damage. For comparison, high-NA measurements commonly employed in biological multi-photon excited fluorescence and SHG microscopy using water or oil immersion objectives are typically limited to a few mW of power before significant sample damage arises.

Selection of an appropriate NA is therefore typically highly dependent upon the anticipated sizes of the crystals to be studied. In studies of nanocrystals that are small relative to the wavelength of light, the highest signal to noise will be achieved with tight focusing and a high NA. If instead the crystals approach or greatly exceed the wavelength of light as is typically the case, reducing the NA increases the field of view and the depth of field without substantially impacting the S/N measured in transmission.

However, this general guideline has an important caveat. This simple analysis neglects the influence of the linear optics and coherence lengths in affecting the detected SHG. In brief, the fundamental and the second harmonic light walk off each other in phase as they propagate through the crystal, such that the next incremental contribution to the signal from each infinitesimal slab can transition from constructive to destructive interference. Because the SHG and the fundamental move in opposite directions in the epi-detected direction, the backwards coherence length is typically on the order of $\sim 100\text{ nm}$, such that crystals with thicknesses along the beam greater than $\sim 100\text{ nm}$ no longer produce increases in epi-detected SHG. In transmission, phase-walk occurs much more slowly from dispersion in refractive index between the fundamental and the second harmonic, such that the forward coherence length is typically on the order of $\sim 5\text{--}10\text{ }\mu\text{m}$ (but highly dependent on the crystal). Birefringence can complicate this scenario, most commonly producing additional increases in the forwards coherence length, but preferentially favoring a smaller subset of possible polarization combinations. Consequently, the SHG efficiency in transmission can begin to decrease substantially with decreasing NA once the Rayleigh length significantly

exceeds the forwards coherence length. For a 10 μm forward coherent length and $\sim 1 \mu\text{m}$ light, this transition corresponds to an NA of ~ 0.33 . Fortunately, losses arising from imaging and lower NA can often be compensated to some extent by increasing laser power.

3.5. Image Acquisition Times

In modern high-throughput screening applications, the read time for a complete 96 well plate should be less than ~ 10 – 15 minutes for reasonable throughput, which corresponds to just 5–6 s per well. When using an 8 kHz resonant scanner, frame rates for SONICC as high as 15 frames per second can be achieved, corresponding to only a few laser pulses per pixel for an 80 MHz laser source. Given the low probability of observing more than one or two dark counts from the detector across the entire image with modern photomultiplier tube detectors, even a few counts within a small number of pixels can be sufficient to suggest the presence of crystals (Figure 4). However, images of quality sufficient for definitive crystal identification or for compatibility with image analysis algorithms for crystal identification will generally benefit from longer integration times, particularly if the crystals are relatively weak SHG-generators.

3.6. Reflection vs. Transmission Detection

Because SHG is a coherent process, the total intensity ultimately results from interference between all the crystalline regions contained within the focal volume of the laser. This interference effect introduces several unique aspects to SHG imaging compared to more conventional microscopy techniques for protein crystal screening. First, the coherence lengths for SHG in the forward (transmission) and epi (reflection) directions can be quite different in a crystal. In practice, the coherence length in transmission is much longer (typically several μm) than the coherence length in epi (typically $< 100 \text{ nm}$). As a result, SHG intensity continues to increase in transmission with increasing crystal thickness up to values approaching the forward coherence length or the depth of field, whichever is smaller. In contrast, the SHG measured in epi typically plateaus at around 100–200 nm in thickness. This trend results in a greater dynamic range of signals in transmission for larger crystals.

Clearly, detection in transmission has a significant advantage in terms of S/N. However, epi-detection still retains some practical benefits. First and foremost, only a single objective lens is required to perform the imaging when measuring in the epi-direction. In instances where collection in transmission is prohibitively challenging from a practical perspective, epi-detection still remains a viable alternative. Even if SHG is detected in transmission, epi-detection can still be done in addition through fairly straightforward modification of the beam path. Because the ratio of the epi versus transmitted SHG depends sensitively on particle size, the combined measurement carries significant additional information on crystal size that is lost in the independent measurements alone. Finally, once the core elements for epi-detection of SHG are integrated into the microscope, the additional overhead to integrate two-photon excited fluorescence into the same beam path is typically low. The main practical challenges are acquiring and storing the additional channels of detection, and maintaining calibration between the detectors.

4. False Positives and False Negatives

Low false-positive and false-negative rates are obviously highly desirable in protein crystal screening. False positives correspond to the apparent detection of a protein crystal where none is present, and false negatives correspond to the inability to reliably identify a protein crystal when one is present.

False positives can arise in SONICC primarily from other crystals that are of appropriate symmetry to generate SHG, but are not protein crystals. One of the key advantages of

SONICC is the selectivity to noncentrosymmetric crystals. The large majority of achiral salts adopt centrosymmetric crystals, while the intrinsic chirality of proteins requires that they assemble into noncentrosymmetric lattices. However, salts or precipitants of chiral molecules other than proteins and certain achiral salts also form SHG-active structures that can produce SONICC signals.

Identification of salts can often be determined by complementary methods. The large majority of inorganic and organic salt crystals produce little or no UVF, such that relatively large ($>10\text{ }\mu\text{m}$) salt crystals can often be identified by conventional ultraviolet fluorescence (UVF) or by two-photon-excited ultraviolet fluorescence (TPE-UVF) as an orthogonal analysis method. [27] Furthermore, SONICC signals from the SHG-active salts tend to be relatively strong. Consequently, it is reasonable to suspect salt crystals from crystals exhibiting high SONICC brightness that are negative for UVF and are formed using one of the above salts as a precipitant. Additional complementary and independent techniques can also be employed to discriminate between salt and protein crystals, including focal Raman microscopy, crush tests, etc.

False negatives can arise when protein crystals assemble into structures that are macroscopically weak for SHG and fall below the detection limits of the instrument. The primary purpose of simulations performed in the next section was to provide an estimate of anticipated false negative rates in SONICC imaging of protein crystals. Relatively weak SHG-efficiency can potentially arise for numerous reasons. In brief, SHG is generally uniformly weak in systems with D_4 (422) and D_6 (622) symmetry (subsets of the tetragonal and hexagonal crystal systems, respectively) and overall significantly stronger in systems of lower symmetry. However, a small population of weak SHG generators can be expected even in low symmetry classes simply by random chance resulting in internal cancellation from the protein itself coupled with inopportune arrangement within the lattice.

5. Crystallization Platform Requirements

When assessing the compatibility of SONICC with a particular crystallization platform, practical considerations include optical transparency, the working distances of the objective and transmission collection optics, and the influence of optical scatter must be considered. Plates designed to enable conventional visible-light microscopy are generally also transparent for the near-infrared fundamental light typically used in SONICC. The frequency doubled light is typically in the visible, also falling within the transparency windows of most plates.

Working distance for the illumination objective can impact ease of imaging, particularly for sitting drop crystallizations by nature of the spatial offset from the cover slip and the crystallization droplet. If that displacement is greater than the working distance of the objective, the objective cannot be brought close enough to the sample to enable imaging. The desire for a long working distance is balanced by the corresponding adjustment in numerical aperture. As discussed in Section 3.4, a target NA of ~ 0.3 yields a typical working distance of $\sim 15\text{ mm}$ for a conventional 10x microscope objective. A comparable NA is usually desired for collection in the transmission direction. Depending on the nature of the sample platform, longer working distances can be achieved for collection while maintaining a comparable NA by increasing the overall diameter of the collection lens, making 10x objectives with NAs of ~ 0.3 and working distances of $\sim 33\text{ mm}$ available. Aberration-correction is not as critical for the collection optics.

5.1. Well Plate Screening

In general, no special requirements are needed for performing SONICC measurements in conventional 96-well plates. However, some polystyrene plates have been observed to produce localized false-positives from impurities, inclusions, or imperfections within the plastic. Because signals from crystals greater than a few hundred nm are generally stronger in transmission, well plates that enable visible light collection in transmission will typically result in lower detection limits and enable faster imaging times. Fortunately, such interferences do not arise when the beam is focused inside the crystallization droplet. To our knowledge, a systematic study has not yet been undertaken to assess backgrounds from the numerous different vendors and plates.

5.2. Lipidic Mesophase Crystallizations

Lipidic mesophase crystallizations are typically performed using a sandwich approach, in which the lipid mesophase droplet is first deposited, then pressed against a coverslip to provide a flat face for optical analysis. The use of lipidic mesophases has been highly successful at producing high quality crystals of integral membrane proteins. Although quite different from conventional solution-phase crystallization approaches, this sandwich approach has successfully overcome many complications of lipidic mesophases arising from turbidity. The refractive index mismatch at the interface between the aqueous mother liquor and the lipidic mesophase would otherwise generate refraction and scattering effects frustrating conventional imaging approaches. Despite these successes, the relatively high residual turbidity arising from occlusions and other inhomogeneities still complicates detection of crystals smaller than ~5–10 μm in size by conventional methods.

The relative insensitivity of SONICC to optical scattering makes it well-suited for measurements of crystallizations in lipidic mesophases. [8] Preliminary results are shown in Figure 1 for integral membrane proteins (G-protein coupled receptors, or GPCRs) prepared in lipidic mesophase. [4, 28, 29] These experiments were all performed using the sandwich-based crystallization platforms to enable comparison with conventional imaging methods. In brief, simple, automated analysis of SONICC images reliably identified the presence of protein crystals within the lipidic mesophase droplets, even in cases where manual inspection of images obtained by the conventional suite suggested negatives for crystal formation. In principle, SONICC should not require the use of the sandwich-style approach for lipidic mesophase crystallizations, providing additional flexibility in crystallization architecture.

As a caveat, the presence of SHG-active domains within lipidic mesophase is sometimes observed, even in the absence of protein. The origin of these domains is not entirely clear, but one possible explanation is the formation of small lipid crystals with relatively weak but nonzero SHG activity.

6. Current Limitations and Future Prospects

Although preliminary studies by SONICC are quite encouraging, the technique is still in its infancy. Additional testing and time will be required to better understand the strengths and limitations of SONICC for protein crystallography. Lingering questions yet to be quantitatively addressed include: 1) Under what conditions does the laser result in damage to crystals, and what are the possible damage mechanisms? 2) Approximately what fraction of protein crystals are sufficiently weakly active for SHG to produce false negatives, and what additional approaches (if any) can be brought to bear to complement SONICC in such instances? 3) How does the SHG-activity depend quantitatively on space group, protein structure, packing within the lattice, etc.?

Most of these uncertainties are a consequence of the relative infancy of nonlinear optical imaging in protein crystallography, and will likely be addressed over the coming years as ultrafast optics play an increasing role in protein structure determination.

In addition to these uncertainties, SONICC also holds unique yet untapped opportunities exploiting the intrinsic coherent nature of SHG. For example, the nonlinear beam generated by SHG emerges in a well-defined direction and polarization state that depends both on the polarization state of the incident light and on the symmetry and orientation of the crystal. This polarization-dependence may potentially provide insights into crystal quality for diffraction analysis, crystal polymorphism, the ability to distinguish twinning or multiple crystallographic domains, as well as other yet unforeseen capabilities.

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Highlights

- Improved detection of small crystals.
- Compatibility with high throughput screening
- Discrimination between salt and protein crystals

Figure 1.

Comparison of SONICC and conventional optical methods for protein crystal detection of GPCRs, from six representative outcomes of crystallization trials (a–f). Bright field and birefringence (cross-polarized) images were obtained using white-light illumination, with UV and Cy3 fluorescence excited with ~280 nm and ~543 nm light, respectively. All SONICC images were acquired with 800 nm incident light with detection at 400 nm. Adapted from reference 8.

Figure 2.

Diagram of an anharmonic oscillator potential energy well of a 1 dimensional molecule (left, solid) with the harmonic oscillator overlaid for comparison (left, dashed) and the time dependent trace of an oscillation within the well (right, solid). The oscillation can be largely recovered from a weighted sum of several harmonics (right, dashed and dash-dotted).

Figure 3.
General instrument design for SONICC. PMT = photomultiplier tube.

Figure 4.

Demonstration of rapid plate-reading by SONICC. Top: SONICC data for a 96-well plate. Bottom: comparison of visible, conventional uv-excited fluorescence, two-photon excited UV fluorescence, and SHG for two representative wells. All images were generously provided by Formulatrix, Inc. The identity of the sample is confidential information. Acquisition times were 0.5 s per well for visible, UV, and TPE-UVF and 2.0 s per well for SHG. The field of view of each well is approximately 2 mm on each side. Crystals favorably form on the right edges of the drops. This anomaly is likely due to the dispensing robot not placing the protein and precipitate solution directly on top of one another.