Second Harmonic Imaging of Plant Polysaccharides

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ABSTRACT

The application of second harmonic generation (SHG) microscopy to plant materials has been neglected hitherto even though it would seem to have promise for identification and characterisation of biologically and commercially important plant polysaccharides. We have found that imaging of cellulose requires rather high laser powers which are above optimal values for live cell imaging. Starch, however is easily imaged by the technique at laser fluences compatible with extended cell viability. This also has useful applications in imaging plant-derived starchy food products. Lignin in plant cell walls shows a strong 3-photon excited fluorescence which may be enhanced by resonance effects.

Keywords: second harmonic generation, polysaccharides, cellulose, starch 3-photon fluorescence, food microscopy.

1. INTRODUCTION

Plants depend on polysaccharides for structural functions that in animal cells are carried out by proteins. The plant cell wall consists of a matrix of more or less amorphous polysaccharides reinforced by microfibrils of highly crystalline cellulose, a β 1:4 linked glucan. In some tissues (typically in cells which are no longer living) the structure is given further resistance and rigidity by impregnation with lignin, a phenolic compound. These cell walls are critical for plant structure and function, and also provide the base of the food chain for the animal kingdom. They are also the raw material for many human industries such as timber, fabric and paper. Plants also store polysaccharides - most typically in the form of starch - as energy and carbon reserves and these are of fundamental importance to the human diet, and hence of major economic importance. Starch consists of two forms - amylose, long chains of α 1:4 linked glucose residues, and amylopectin, shorter α 1:4 glucans with branches introduced by α 1:6 linkages. The proportions and properties of these are important in food preparation.

Only two papers to date have looked at plant cells using second harmonic generation (SHG). Mizutani et al[1] imaged starch grains in living algal cells by SHG, using a pulsed neodymium laser in a non-scanning microscope with a CCD camera. Using wide-field imaging in this way should give good lateral resolution, since the wavelength of the emitted light will determine the resolution, but it is not depth-selective and hence discards one of the chief benefits of SHG microscopy, the ability to carry out 3D imaging [2]. Chu et al.[3] used a stage-scanning microscope and generated 3D harmonic images of plant cells using a chromium forsterite laser at 1230nm. They showed images of cell walls, suggesting that they could also obtain an SHG signal from cellulose, as well as images of starch grains in the chloroplasts. They further suggested that chloroplast thylakoid membranes (the stacks of membranes which perform photosynthesis) also gave an SHG signal.

Because of the commercial importance of both cellulose fibres and starch, novel imaging methods which can give information about structure, crystallinity and orientation could have wide applicability. Here we investigate SHG imaging of both living plant cells and starch-containing plant products. Rather than the 1 - 1.2 μm wavelengths used

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previously[1,3], we have used a shorter wavelength titanium sapphire laser which is also suitable for two-photon fluorescent (TPF) imaging, at wavelengths between 800 and 850 nm.

2. MATERIALS AND METHODS

2.1 Plant materials

Stems of *Lantana camara* were collected from the garden of the Instituto Gulbenkian de Ciência, sectioned by hand and mounted in water. Petioles of celery (*Apium graveolens*) were purchased commercially and also sectioned freehand and mounted in water. Some collenchyma ribs from the celery petioles were stripped and mounted on large coverslips on the stage of the inverted microscope in surplus water, so that the cellulose fibre orientation would be transverse to the optic axis. Moss (unidentified species) was collected from a wall in Sydney and fronds with two or three leaflets were examined entire in water.

Rice grains (*Oryza sativa*), were crudely hand sectioned and mounted in immersion oil so that the starch did not become hydrated. Three varieties were used: Thai Jasmine (long grain); Thai glutinous (short grain) and Italian arborio. Starch and starch/lipid pastes of the type used in food production were supplied by Professor Les Copeland and Ms Chiming Tang at the University of Sydney.

2.2 Microscopy

At the IGC, the microscope used was a Bio-Rad MRC1024ES MP confocal microscope, equipped for multiphoton microscopy with a Coherent Verdi-Mira 900f titanium sapphire femtosecond pulsed laser and dual-channel non-descanned detectors, mounted on a Nikon Eclipse TE300 inverted microscope. Plan-Apo 20x NA 0.75 dry and 40x NA 1.3 oil-immersion lenses were used. A 0.85 NA condenser was fitted. For the fluorescence signal we used the external detectors with a D525/50nm band pass filter and a 550nm 50 DCLP dichroic together with a band pass filter HQ575/150nm. A transmission detector was not available and the SH images were collected in the backward direction using 840nm excitation with a band-pass filter D390/70.

In Sydney a Leica DMIRBE inverted microscope, fitted with a Leica TCS-SP2 spectrometric confocal head (Leica Microsystems, Heidelberg, Deutschland), was used. The laser is a Coherent Mira titanium sapphire system, tunable between 700nm and 1000nm, operating in the femtosecond regime and pumped by a 5W Verdi solid-state laser (Coherent Scientific, Santa Clara, Ca.). All additional detectors and optical equipment were supplied by Leica Microsystems, with the exception of the additional filters and dichroics, supplied by Chroma Inc (Brattleboro, Vt.) The setup was as described previously[2,4]. 830nm illumination was used with a 415/10nm detection barrier filter in one of the two transmission channels to collect the second harmonic.

3. RESULTS

3.1 Cellulose

Celery collenchyma walls were chosen as a useful test sample since they are very rich in cellulose and are non-fluorescent, being entirely unlignified. The only substantial fluorescence signal in these cells comes from the chloroplasts, which are not numerous. Both in transmission and backscattered mode we saw only a very weak SHG signal using maximum laser power. Figure 1 shows a transverse section of a celery petiole imaged in both SHG and fluorescent mode. The collenchyma cells of the bundle cap are visible only in the SHG image, while the lignified tracheid walls show strong two-photon excited fluorescence and very little second harmonic. The orientation dependent nature of the SHG imaging process is revealed by the fact that only walls in one orientation are strongly imaged. This is transmission image, taken on the Leica, and it was necessary to average 16 frames for an adequate result.

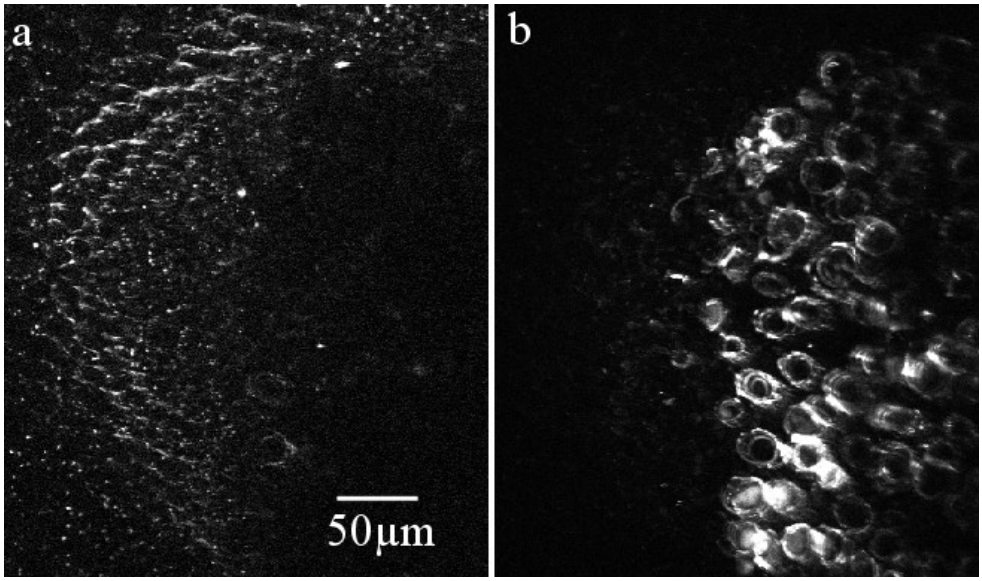

Figure 1. Collenchyma cells in the bundle cap of celery petiole, hand section imaged in the transmission direction. (a) SHG image, in which only the very cellulose rich collenchyma cells are visible. (b) Broad-band fluorescence image (500-600nm) showing the autofluorescence of the lignin in the spirally-thickened tracheids, but no signal in the bundle cap. x20 NA 0.5 dry objective

To investigate the orientation dependence of the signal longitudinal arrays of cells were needed, since the orientation of the cellulose microfibrils in these cells is predominantly along the long axis of the cells[5,6,7]. For this purpose entire collenchyma bundles (ribs) were stripped from the petiole and placed in water on a large coverslip on the inverted microscope (Nikon / Bio-Rad) and imaged in backscattered mode.

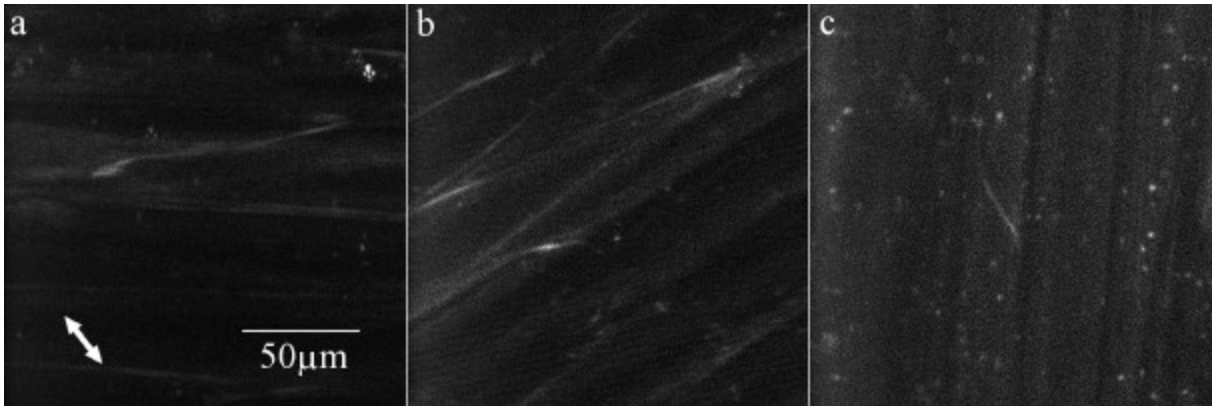

Figure 2. Collenchyma rib from celery petiole, placed at three different orientations. The double arrow shows the direction of polarization of the 840nm laser beam. (a) Left - right (cellulose fibril orientation 0° (b) 45° (c) up-down (90°). x20 objective

Figure 2 shows the result. The strongest signal is seen when the longitudinal walls are at 90° to the direction of polarization (Figure 2b); with the cells at 0° and 90° the strongest signal comes from transverse walls, though in Figure 2c it is a wall parallel to the polarization direction. However it cannot be assumed that the cellulose fibres in transverse walls necessarily have a microfibril orientation following the wall direction; it is only the long walls that show a strong alignment.

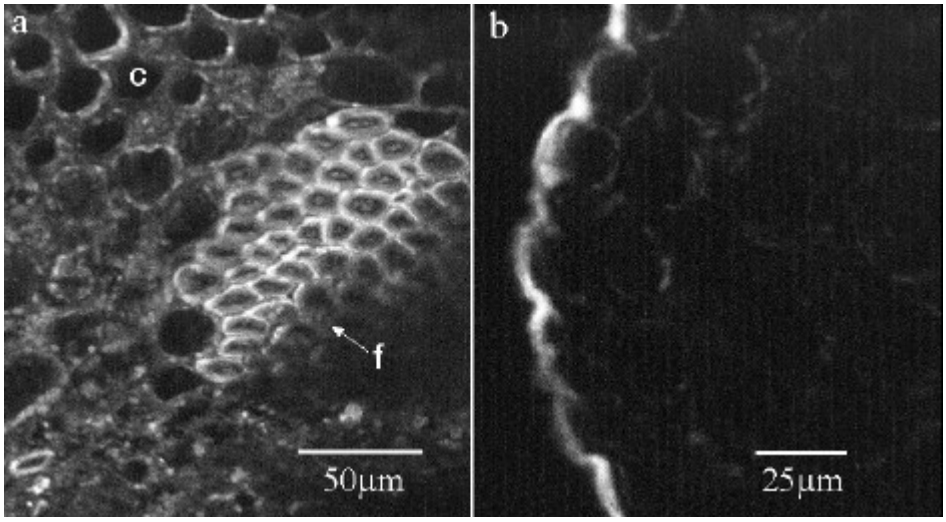

Figure 3. Transverse section of *Lantana* stem. (a) 840nm excitation, 355-425nm detection, showing a weak signal from the collenchyma (c) and a stronger one from the lignified fibres (f); the signal from the fibres seems to be a combination of SHG and 3-photon fluorescence. (b) Epidermis, 870nm excitation showing strong 3-P fluorescence between 355-425nm. x20 NA 0.5 objective

Lantana stems are woody and therefore have lignified fibres but also have collenchyma. As Figure 3 shows, the lignified fibres (f) give a brighter SHG image. The excitation here is at 840nm so the second harmonic is at 420nm and the detected range is 355-425nm. Therefore two-photon excited fluorescence should not be detected. However the middle lamella region is very bright, and this is where the most lignin is present (and is seen in the 2-photon fluorescence channel, not illustrated). This led us to suspect that we might be seeing some 3-photon excited fluorescence in the violet region. However the image was orientation-dependent. Tuning the laser to 870nm (2nd harmonic therefore 435nm) should exclude the second harmonic; in fact the image became much dimmer but did not disappear. It seems likely that there is some degree of resonance-enhanced fluorescence occurring. The cuticle of the epidermis showed very strong violet or ultra-violet three-photon fluorescence under 870nm excitation (Figure 3b); in this case there was little or no change in signal between 840nm and 870nm so it must be a pure fluorescence image.

3.2 Starch

Chloroplasts in celery showed a signal in the SHG image which did not colocalise with the autofluorescence of the chlorophyll, and we attributed this to starch. As a test sample we used a prepared slide of starch grains (Bio-Rad Microscience, Hemel Hempstead, UK). Figure 4 shows that these gave a strong SHG signal. Crystalline starch in starch grains is typically organised with the crystallites in a radial fashion, which gives a characteristic cross image in polarised light[8]. This in turn means that the SHG image will be orientation dependent, and Figure 4 a&b shows that this is so - opposite quadrants are bright when the sample is rotated through 90° (the images have been rotated to bring then back to equivalent positions). The signal is strong, and a very low excitation level is adequate for a high-quality image. Figure 4c shows a maximum brightness projection from a 50-section stack through this sample, and illustrates the quality of imaging obtainable from a higher NA lens (the limitations of our stage mean that rotations are only feasible with a low-power objective).

These results encouraged us to believe that we could image starch in living cells at fluences which would not have adverse effects on normal cell physiology. Starch is formed during photosynthesis and therefore it is particularly useful to be able to image starch is leaves which remain photosynthetically competent. However the leaves of higher plants contain extensive airspaces which hinder effective imaging. Bryophyte (moss) leaves are more suitable since they have only a single layer of cells over most of the lamina, and therefore no sir spaces. Moss leaves do not contain lignin but they do contain substantial amounts of other phenolic compounds[9] which fluoresce in the blue and green, as well as chlorophyll fluorescing in the red.

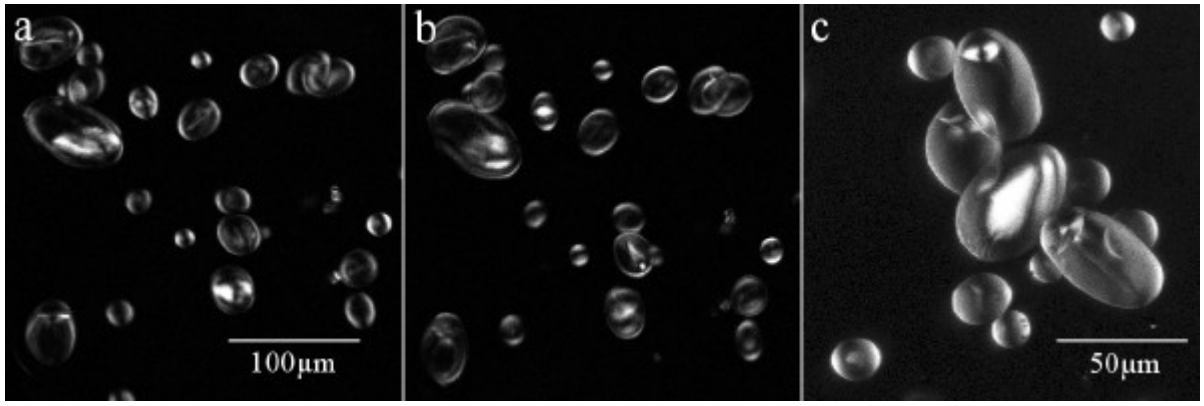

Figure 4. Prepared slide of starch grains, SHG image excited at 830nm, detected with a 415/10 narrow-band filter (Leica TCS-SP2) a & b - single plane images of the same area, showing the orientation dependence of the signal. The slide was rotated through 90° between the images, which have then been rotated to bring them back to the same position. x10 objective NA 0.3. (c) Maximum brightness projection from 50 optical sections taken with a higher power objective (x40 NA 0.75).

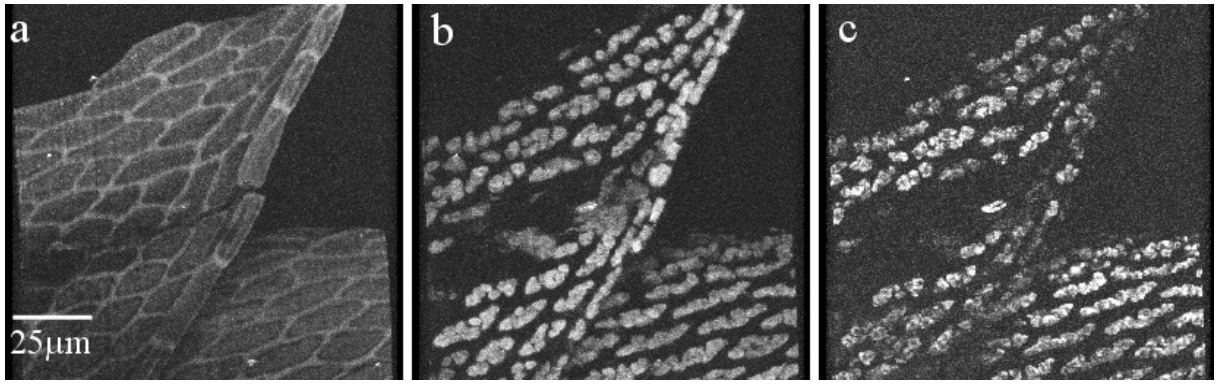

Figure 5. Living moss leaflets in water; maximum brightness projection from 97 optical sections covering a depth of 44 µm. (a) green fluorescence from phenolics, (b) red fluorescence from chlorophyll (c) SHG at 415nm. Leica TCS-SP2, x63 NA 1.2 water immersion objective.

Minimising dose as much as possible it proved simple to collect three-dimensional images as shown in Figure 5, a 256x256x97 voxel stack. The green channel showed the cell walls clearly, presumably from autofluorescence of tannins or other phenolics, while the chlorophyll was detected in the second (red) channel of the back-scattered detector and the second harmonic in the transmission detector. There was no visible deterioration in the cell after the collection of this dataset; in particular the autofluorescence of the chlorophyll, normally a sensitive indicator of damage, was unchanged.

There are many aspects in which the structure of starch is useful to the food industry and we therefore investigated the applicability of SHG imaging to these problems. Conventional microscope preparation of starch seeds such as rice grains inevitably leads to the partial hydration of the starch with concomitant swelling and structural changes. We therefore cut hand sections of grains and mounted them in immersion oil. Figure 6a shows that a strong signal and a detailed image was obtained but it is not easy to interpret; a more sophisticated preparation technique may be needed to take this approach further.

Starch pastes and doughs of one sort or another are fundamental in the food-processing industry and Figure 6b shows a thin layer of rice-flour paste mounted between slide and coverslip with no additional treatment (and without adding additional water). The signal was quite low from this but since it is not living material there is no objection to using moderately high levels of laser power. The images showed good contrast and it was possible to see a lot of detail in the

SHG image which we interpret as showing regions of starch recrystallization. In three dimensional stacks these were seen to be sheet or ribbon-like. These samples also showed considerable autofluorescence which was not uniform but also showed structure, probably reflecting variations in water and lipid content.

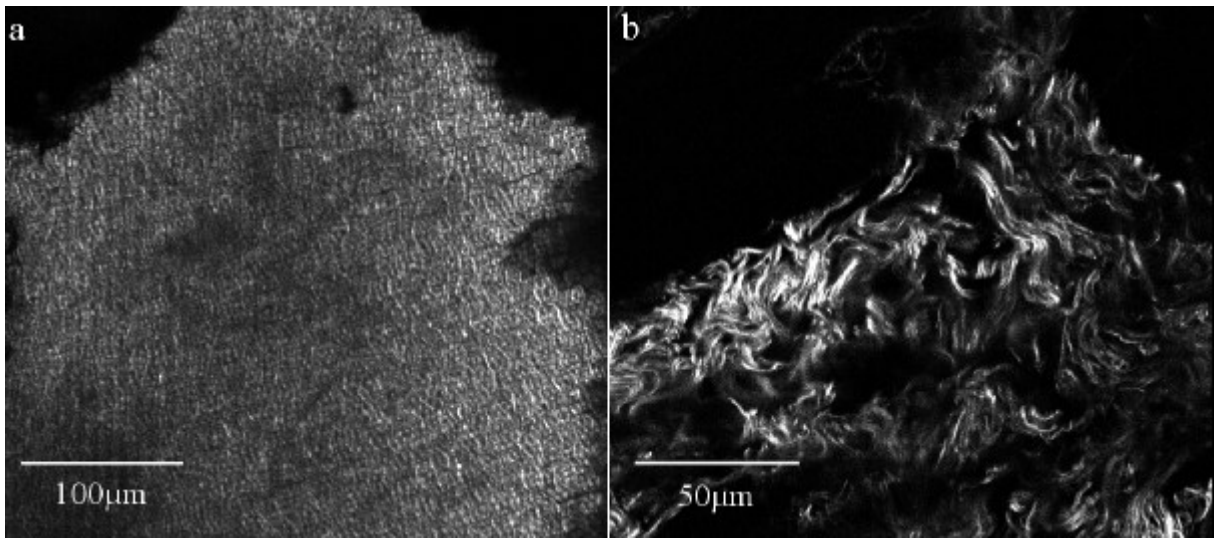

Figure 6. (a) Fragment of a hand-cut section through a grain of Thai jasmine rice. SHG image at 415 nm; x40 NA 0.75 dry objective. (b) Rice-flour paste - SHG image at 415nm; x63 NA 1.2 water immersion objective.

4. DISCUSSION

Cellulose is a β 1:4 linked glucan[8]; alternate glucose residues are in reversed orientations which gives a certain amount of symmetry to the molecule, though the chirality of the glucose means that the symmetry is not complete. (Only the dextro form (D-glucose) is found in plants). Starch, on the other hand, is α 1:4 linked[8], so each glucose molecule in the chain is in the same orientation, making the chain much less symmetrical. One would therefore expect, *a priori*, that starch would be a better generator of the second harmonic than cellulose, and this proves to be the case in practice. Useable images of cellulose in cell walls can only be obtained using high laser power and extended integration times.

Cheng et al.[10] showed that plant chloroplasts are very susceptible to damage from femtosecond titanium sapphire laser irradiation. They used 760nm excitation, substantially shorter than the 830 or 840nm used here, and found that even a single 3.3 second scan or a small area, under their imaging conditions, could be sufficient to reduce the red fluorescence of chlorophyll, leaving residual green fluorescence. Three such scans were sufficient to cause cytochemically demonstrable cell damage. Similar damage is often observed using blue excitation, so it seems the difference is in degree rather than kind of damage Successful imaging of living plant cells in a physiologically normal state will therefore depend on minimising dose, though one could also expect that longer excitation wavelengths than 760nm would reduce the extent of the damage.

Our results presented here suggest that this will not be achieved when imaging cell walls by SHG from cellulose. High laser powers and long accumulation times were needed to obtain even marginally useful images. Possibly, using the longer wavelengths given by a chromium forsterite laser, SH yield from cellulose would be increased and chlorophyll photo-damage decreased. The simple fact remains that the β 1:4 structure of the cellulose chain makes it less than optimal for SHG imaging.

Starch, on the other hand, is also crystalline and structurally a much better harmonic generator. Useful signals are obtained at low excitation doses, and as Figure 5 shows images of starch can be obtained simultaneously with two-

photon fluorescence images of chloroplasts under conditions which show no sign of impacting on cell viability. No loss of red chlorophyll signal was observed in the course of collecting a 97-slice stack; the chlorophyll fluorescence showed no sign of shifting to the green and therefore appearing in the 'cell wall' channel in this series. The use of a longer excitation wavelength probably helped in this regard, but the optimised collection geometry of our non-descanned backscatter detectors was probably also a significant factor. Cheng at al.[10] did not mention any non-descanned detection system so by implication were probably using the confocal detector system of their Olympus microscope; this will always lead to a severe reduction in collection efficiency compared to wide-field detection. Also, the air spaces in the *Arabidopsis* leaves used had been flooded with water[10], possibly compromising normal physiological functioning.

Processing of starchy foods for consumption is fundamentally dedicated to breaking down crystalline starch into an amorphous form which is more hydrophilic, more digestible and more palatable. This process is referred to (confusingly) as gelatinisation in the food processing industry. Subsequent recrystallization (retrogradation) causes defects in products such as lumpiness in sauces and staling of bread[11]. Preventing this to extend shelf-life and improve product texture is a key goal of food technology. Since the strength of the SH signal is strongly dependent on crystallinity SHG microscopy promises to be a very effective tool for imaging this process. Polarised-light widefield microscopy will also reveal crystalline regions, but does not offer spatial resolution in the axial direction. With SHG microscopy the structure and extent or retrograded regions can be investigated in all 3 spatial dimensions.

5. CONCLUSIONS

In the past two or three years there has been substantial interest in using harmonic microscopy to study structural proteins in animal tissues[2,4], but its potential in the plant world has been underappreciated. To the best of our knowledge this is only the third paper published in this field. In principle several polysaccharides could act as sources of SH signals, but only starch and cellulose typically occur in highly crystalline states. Cellulose is a rather weak SH generator and the incident energy needed to give useable signals is likely to be damaging to living cells in many cases. Starch, on the other hand, is easily detected and imaged in three dimensions under conditions which seem not to damage live tissues. Once the crystalline structure has been degraded in food preparation somewhat higher laser fluences are needed but the material is no longer so susceptible to damage and extended imaging seemed to make no difference to the sample. This provides agricultural scientists and food technologists with a useful tool for assessing crystal structure and orientation in starchy foodstuffs.

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