Plant oleosomes are uniquely emulsified lipid reservoirs that serve as the primary energy source during seed germination. These oil bodies undergo significant changes regarding their size, composition and structure during normal seedling development; however, a detailed characterization of these oil body dynamics, which critically affect oil body extractability and nutritional value, has remained challenging because of a limited ability to monitor oil body location and composition during germination in situ. Here, we demonstrate via in situ, label-free imaging that oil bodies are highly dynamic intracellular organelles that are morphologically and biochemically remodelled extensively during germination. Label-free, coherent Raman microscopy (CRM) combined with bulk biochemical measurements revealed the temporal and spatial regulation of oil bodies in native soya bean cotyledons during the first eight days of germination. Oil bodies undergo a cycle of growth and shrinkage that is paralleled by lipid and protein compositional changes. Specifically, the total protein concentration associated with oil bodies increases in the first phase of germination and subsequently decreases. Lipids contained within the oil bodies change in saturation and chain length during germination. Our results show that CRM is a well-suited platform to monitor in situ lipid dynamics and local chemistry and that oil bodies are actively remodelled during germination. This underscores the dynamic role of lipid reservoirs in plant development.
Owing to the initial absence of photosynthesis in germination, nearly all energy for initial development in plants comes from lipids via lipolysis of TAGs by surface-bound lipases, β-oxidation in glyoxysomes and catabolism in mitochondria [3]. As a result, the density of oleosomes, and correspondingly their concentration, nearly all energy for initial development in plants comes (from approx. 100 Da to approx. 300 kDa) [24,25]. Indeed, such probes have been shown to perturb native lipid physiology. MALDI-IMS allows detection with high sensitivities and signal strength by resonant enhancement by up to six orders of magnitude and circumvents challenges with auto-fluorescence [31–33]. Our results demonstrate that oil body lipid composition is insufficient to interrogate individual oil bodies (0.05–3 μm diameter) at this time [24,27].

An alternative approach for local lipid analysis is chemical imaging via nuclear magnetic resonance (NMR) or vibrational microscopy, which requires little to no sample preparation (fixation at most). Lipids are readily identifiable with both techniques via their intrinsic molecular spectroscopic signatures and thus require no labelling. NMR-based imaging has the ability to image specific signals from lipids, water or other metabolites as well as acquire hyperspectral data—an NMR spectrum at each voxel—in macroscopic specimens with voxel sizes as small as approximately 10 × 10 × 100 μm³ [28]. This gives a unique overview of lipid organization in an entire specimen and has been used to show a gradient in lipids within soya bean cotyledons, with higher TAG content in the central and adaxial (inner) region than in the abaxial (outer) region [29,30]. NMR imaging is non-invasive, label-free, chemically specific and offers spatial resolution on the micro-scale—comparable to MALDI-IMS; however, it is similarly not well suited to image individual oil bodies or body clusters in cells because of the limited spatial resolution. Furthermore, acquisition times can be relatively long (approx. 10 h) for high-quality hyperspectral datasets [28].

Raman scattering also derives contrast from intrinsic molecular vibrations in the sample, e.g. from the abundant CH₂ groups that are strongly present in lipid acyl chains. Furthermore, because of the chemical specificity of this approach to functional groups, similar to NMR, it is possible to image entire macromolecular species, e.g. all lipids, all proteins, all nucleic acids, etc., within a sample. Since the excitation for Raman scattering is visible light, the diffraction-limited optical resolution, approximately 250–300 nm in plane, offers the ability to localize individual (or small clusters of) oil bodies within single cells as an imaging modality. Therefore, chemical imaging based on Raman scattering potentially provides a non-invasive molecular fingerprint of lipids within oil bodies in situ, which is, to the best of our knowledge, not possible with any other method.

In this work, we use coherent Raman microscopy (CRM)—specifically coherent anti-Stokes Raman scattering (CARS)—to image lipids in situ and in vivo without labels and quantify biochemical changes of these oil bodies during germination. CARS effectively boosts the Raman signal strength by resonant enhancement by up to six orders of magnitude and circumvents challenges with auto-fluorescence [31–33]. Our results demonstrate that oil body size and spatial distribution are dynamic and are paralleled by changes in lipid composition as germination proceeds. CRM, together with additional biochemical analyses, shows that lipid-associated proteins, and specifically oleosins, disappear as larger oil bodies appear in the cotyledon. Interestingly, we also observe that oil bodies again decrease in size after initially growing. These results show that oil bodies are not static depots during germination but rather are actively remodelled to transform from oleosin-coated oil bodies to...
non-oleosin-coated oil bodies [34] with dynamic lipid species contained within them.

2. Material and methods

2.1. Soya bean germination

Ten grams of soya beans (Glycine max; Davert GmbH, Aschberg, Germany) were mixed in one line on a sheet of aluminium foil in between four layers of wet Kimtech science precision wipes 21 × 11 cm (Kimberly Clark, Roswell, GA, USA). They were covered with a second piece of aluminium foil (30 × 140 cm in total) and dipped in deionized water (18 cm distance from bean to water) to imbibe the beans and continuously moisten them during growth [35]. Soya beans were incubated at 25 °C in the closed aluminium foil up to day 5, after which their cotyledons were released by removing the upper part of the foil. The exposed seedlings were returned to the water and cultivated under the same conditions up to day 9. For each day, representative soya beans were selected and documented by total plant size and weight (electronic supplementary material, figure S1) based on a set of criteria: root size ± 25% (for days 2–6), colour of cotyledons, secondary roots and primary leaves (for days 6–8).

2.2. Oil body purification

In total, 11–30 soya beans matching the above-defined germination stage criteria were selected at the specified day after imbibition and ground to extract oil bodies as described previously [36, 37]. Briefly, the selected beans were placed into 100 ml water and ground in a Thermomix TM31 (Vorwerk, Wuppertal, Germany) at the highest speed (10 200 r.p.m.) for 60 s. This slurry was then filtered through two layers of 21 × 11 cm Kimtech science precision wipes to obtain raw soya milk. Twenty-five per cent sucrose (w/w) was added to the raw soya milk, and the pH was adjusted to 11.0 with 1 mol L−1 sodium hydroxide to remove weakly bound proteins, such as conglycinin, glycmin, allergenic proteins and oleosin proteases. This made subsequent Bradford measurements more specific for only strongly bound proteins such as oleosins, calloseins and steroleosins [16, 36, 38]. We note that using high alkaline pH reduces the total amount of oleosins compared with neutral extraction [39], which could cause a systematic offset for subsequent oil body size measurements from laser diffraction and Bradford measurements of protein quantity. The solution was poured into 50 ml centrifuge tubes and centrifuged in a Multi- 
Fuge X1R (Thermo Heraeus, Hanau, Germany) at 15 000 g for 10 min to remove any remaining storage proteins in the oil bodies [37] and to denature the proteases Bd 30 K and P34 [16]. Finally, the washed oil bodies were collected with a spoon, dispersed in 10 ml phosphate-buffered saline (PBS), and stored at 4 °C for protein and lipid analysis. Prior to any lipid or protein analysis, the dry weight of the purified oil bodies was determined using a precision moisture balance HR83 (Mettler Toledo, Geissen, Germany).

Protein quantification of the oil bodies was determined using a standard Bradford assay with the Bradford reagent B6916 (Sigma, Steinheim, Germany) by using the same dry weight of oil bodies. For detailed protein analysis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the NuPAGE-System (Life Technologies, Darmstadt, Germany). A 10% NuPAGE resolving gel was used with the manufacturer’s instructions but without heating the samples (10 mg ml−1 purified oil bodies) to avoid lipid smear. Instead, they were solubilized overnight at room temperature with the NuPage LDS Sample Buffer and the NuPage LDS Reducing Agent. Protein bands were visualized with Coomassie staining, G-250 SimplyBlue SafeStain (Life Technologies, Darmstadt, Germany).

2.4. Fixation and slicing of tissue sections

In fixed samples, cotyledons of representative germinated soya beans were cut into quarters and fixed with 4% paraformaldehyde with 150 mM sucrose at 4 °C overnight. These samples were subsequently flash frozen in liquid nitrogen and sliced with a cryotome into 15 μm thick slices at −20 °C with an MTC cryostat (SLEE Medical, Mainz, Germany). Care was taken to obtain slices from a location 3–5 mm from the embryo. These slices were mounted on glass slides, sandwiched in 40 μl PBS by attachment of a no. 1 coverslip, and sealed with nail polish prior to imaging. Representative Z-stacks at the specified Raman shift were acquired at each day from the adaxial (flat part), centre and abaxial (curved) regions of the cotyledon cross-section (see the electronic supplementary material, figure S2) [30].

2.5. Coherent anti-Stokes Raman scattering microscopy

CARS was used to image oil bodies in situ soya bean coty-ledon. CARS is a nonlinear Raman microscopy method involving three-photon interaction: pump (v_pump), Stokes (v_stokes) and probe (v_probe) photons to generate a fourth anti-Stokes photon (v_anti-Stokes = v_pump − v_stokes + v_probe), where v_pump, v_stokes, v_probe and v_anti-Stokes are the energies of the pump, Stokes, probe and anti-Stokes photons, respectively. The Raman shift Δ_Raman = v_pump − v_stokes was set to 2845 cm−1 for all images in this study. Laser excitation was provided by picosecond pulses at 1064 nm and 817 nm (APE, Berlin, Germany). CARS microscopy was performed on an SP 5 II TCS CARS microscope equipped with transmitted and reflected light photomultiplier tubes (Leica, Mannheim, Germany) and a 1.49 numerical aperture (NA), 60× oil-immersion objective (Nikon, Tokyo, Japan). The laser power varied by 4% over the entire imaging period (three weeks), while all other imaging and scanning parameters were held constant. Oil bodies were imaged in the transmitted photomultiplier tube with an additional 660 nm bandpass filter (full-width half-maximum of 8 nm) (Photomet GmbH, Seefeld, Germany) in addition to the standard Leica CARS 2000 filter set. Samples were imaged either as fixed tissues or as live beans.

2.7. Quantification of oil bodies in CARS images

Z-stacks were acquired at a line rate of 200 Hz, with 1024 × 1024 lateral pixels in each field of view, and 28–33 axial slices. The pixel sizes were 0.252 μm (lateral), 1.01 μm (axial).
(lateral) and 0.545 μm (axial). Three beans were imaged for each cotyledon region for each day. Sub-stacks from the centre of each Z-stack, consisting of six images, were selected with ImageJ. These sub-stacks were maximum intensity projected and analysed for particles with a diameter larger than 1 μm and a circularity of 0.5. The threshold criteria were set via ImageJ ‘Robust Automatic Threshold Selection’ (RATS; noise = 11, λ = 3, min = 205) [40].

2.8. Hyperspectral CARS microscopy
A dual-output laser source (Leukos-CARS; Leukos) was used for the pump and Stokes beams. The source was a passively Q-switched 1064 nm microchip laser, delivering sub-nanosecond pulses at a 32 kHz repetition rate and an average power of approximately 300 mW. For pump (and probe), the fundamental beam at 1064 nm was used, and the Stokes was a fibre-generated super-continuum with a spectral density of more than 100 μW nm⁻¹ from 1050 to 1600 nm. The Stokes and pump/probe beams were matched in time and space at the focus of our microscope, as described previously [41]. Briefly, we used a reflective collimator (ROD4APC-P01; Thorlabs) to collimate the Stokes beam and filtered it through a longpass 700 nm filter (FEL0700; Thorlabs) and longpass 830 nm filter (LP02-830RS-25; Semrock). The Stokes and pump beams were combined at a dichroic mirror (LP02-1064RU-25; Semrock) and introduced into a modified inverted microscope (Eclipse Ti; Nikon). The pump and Stokes pulses were tightly focused onto the sample with a near-infrared objective (PE IR Plan Apo 100×, NA 0.85; Olymups), resulting in approximately 32.5 mW of total laser power at the sample. The sample was mounted on a stepper-motor stage for coarse positioning (Microstage; Mad City Labs) with a nested piezo stage (Nano-PDQ 375 HS; Mad City Labs) for sample scanning. Together, this provided a 100 μm travel range with sub-nanometre resolution. The CARS signal generated by the sample was collected in the forward direction by another objective (M-10×, NA 0.25; Newport) and sent through notch (NF03-532/1064E-25; Semrock) and short-pass filters (FES1000; Thorlabs) to remove the pump and Stokes radiation. The filtered CARS signal was dispersed by a spectrometer ( Shamrock 303i, 300 lines mm⁻¹, 1000 nm blaze; Andor) and detected on a deep-depletion CCD (Newton DU920P-BR-DD; Andor). The sample was raster scanned across the laser focus with steps of 0.3 μm in plane. For each position in the sample, a CARS spectrum in the range between 600 and 3400 cm⁻¹ was acquired. CARS data were acquired with a pixel dwell time of 100–200 ms, depending on the signal level. The spatial resolution of the instrument was independently measured to be approximately 0.5 × 0.5 × 3.5 μm³, and the spectral resolution was limited by the camera pixel pitch to approximately 4 cm⁻¹ per pixel. The entire CARS microscope is controlled with custom software written in LabVIEW (National Instruments).

2.9. Hyperspectral data analysis
Raw CARS spectra were analysed with custom routines in IgorPro v. 6.37 (Wavemetrics). Initially, data were normalized by exposure time. Treatment of the data with a modified Kramers–Kronig algorithm and error phase correction removes the non-resonant contribution to the signal and retrieves the imaginary component of the third-order Raman susceptibility. For error phase correction, we use an iterative, noise-maintaining approach based on [43] that is modified to be model-free by employing a second-order Savitzky–Golay smoothing filter, having a window size of 101 spectral points (404 cm⁻¹ width), for repetitive baseline estimation. The window size and order were determined empirically but are the same for all data in this work. These resulting spectra are linear in concentration and can be quantitatively analysed similar to Raman spectra, and we refer to them as Raman-like (RL) spectra. Images of particular Raman frequencies were generated in IgorPro for subsequent analysis. Intensity plots of the CH₂ symmetric vibrations were constructed by integrating the intensity from RL CARS spectra from 2845 to 2852 cm⁻¹, and these images were used for identifying oil bodies in all samples imaged with hyperspectral CARS microscopy. Average RL spectra were collected from regions of interest (ROIs) of identified oil bodies and curves were fitted to a sum of Lorentzian peaks for quantitative analysis (electronic supplementary material, figures S5 and S6, tables S1 and S2).

3. Results
3.1. Spatio-temporal oil body changes during germination
It has been reported that oil bodies undergo morphological changes after imbibition of soya beans, but the dynamics of these changes regarding lipid distribution within the native, germinating cotyledon is not known [45–47]. Therefore, we monitored oil bodies in different parts of the soya bean cotyledon using CARS microscopy, which is a type of CRM. In CARS, multiple excitation lasers are employed: pump, Stokes and probe, to excite Raman vibrational modes in a sample. When the energy difference between the pump and Stokes lasers, ΔvRaman = vStokes − vPump, is resonant with a vibrational transition of bonds in the focal volume of the lasers, the CARS signal is strongly enhanced, providing a chemically specific, label-free signal suitable for in situ imaging. By tuning ΔvRaman to 2845 cm⁻¹, the resonance frequency for CH₂ (methylene) groups in lipids, we imaged the oil bodies in living and fixed cotyledons with three-dimensional spatial resolution similar to multi-photon fluorescence microscopy without the need for exogenous labels [48].

Because sample perturbation by the excitation light is minimal (as explained below), CARS microscopy allows for three-dimensional time lapse imaging in living, germinating soya beans. In figure 1, we show still images from a 24 h time-lapse video starting at day 4 (post imbibition) from the adaxial (inner) region of the cotyledon. The orthogonal views as well as the three-dimensional reconstruction illustrate that the penetration depth for imaging is sufficient to span at least one cellular layer into a living cotyledon. From day 3 to day 5, we observed a quadruplication of root length (electronic supplementary material, figure S1), and the live imaging movie shows highly mobile lipids in living cells (electronic supplementary material, movie S1) over the same period. The live CARS imaging of lipid distribution in soya bean cotyledons shows that oil body size and distribution are highly dynamic during germination. Importantly, we note that the imaged cotyledon developed into a full plant within 8 days (electronic supplementary material, figure S3), which proves that extended CARS imaging was not cytotoxic.

Upon examination of three spatial regions within fixed and sectioned cotyledons: the (flat) adaxial, centre and (curved) abaxial (outer) regions over days 1–8 of germination, we clearly see a depletion of lipids from abaxial cell layers and an increase in lipid signal in the adaxial and central regions (figure 2n). Large round features appear in the centre and adaxial regions by day 6. CARS images reveal
that oil bodies in different regions of the cotyledon undergo unique morphological changes throughout germination. Considering that different regions of the cotyledon become different parts of a plant, this is not unexpected. The adaxial region differentiates into the typical morphology of a leaf-like mesophyll by day 8 [49]. All cells in the abaxial cotyledon will eventually become a (woody) epidermis, which should be void of organelles, leaving behind virtually no lipid-containing structures. This is consistent with the CARS images in figure 2, which show that the abaxial region is devoid of lipid signal by day 6. Interestingly, the images of the adaxial and centre regions of the cotyledon show an increase in oil body size at day 6, followed by a decrease at day 8.

Quantification of the number of CH$_2$-rich oil bodies larger than 3 $\mu$m in diameter (defined as ‘large’ oil bodies) from the three different cotyledon regions shows the spatio-temporal behaviour of oil body morphology during germination (figure 2c). Growth and subsequent shrinkage of oil bodies was most apparent within the centre region, and to a lesser extent in the adaxial region, with the centre region having a slightly delayed oil body size increase compared with the adaxial region. The abaxial region of the cotyledon showed a marginal oil body size increase but significantly less than either the adaxial and centre regions. In addition to oil body size quantification from CARS, oil body size was measured using laser diffraction measurements on extracted oil bodies from day 1 to day 8 germinating plants. These results, which are binned over the entire germinating soya bean cotyledon, independently confirm that oil body size increases, then decreases and is consistent with the quantification of large oil bodies from CARS (electronic supplementary material, figure S4).

3.2 Oil body lipid composition undergoes subtle changes while protein emulsifier disappears during germination

With multiple experimental methods confirming oil body morphological changes during germination, we next investigated whether the biochemistry of oil bodies changed during germination. We used hyperspectral CARS imaging—where a full vibrational spectrum at each spatial location is acquired—to measure oil body composition in situ in fixed cotyledon sections. After appropriate processing, these spectra provide a directly quantifiable vibrational RL spectrum, depicting local chemistry within each spatial voxel (0.5 x 0.5 x 3.5 $\mu$m$^3$) [50]. Hyperspectral datasets were obtained from days 1, 5 and 9 of germinating soya bean cotyledons from the central region.

Oil bodies in hyperspectral CARS images were identified from images rendered by plotting the CH$_2$ (2845–2852 cm$^{-1}$) intensities that are characteristic of aliphatic carbons (figure 3a–c). This is a similar contrast metric to that used to produce figures 1 and 2. We note that, in contrast to the semi-quantitative data in figures 1 and 2, the data in figure 3a–c are amenable to quantitative band analysis [50]. To quantitatively examine oil body-specific chemistry during germination, spectra from at least four CH$_2$-rich ROIs for each time point were averaged; these are shown in figure 3d. In order to better compare differences in RL spectra from...
each day, we plot the two regions of the spectra as normalized spectra in figure 3d. For reference, an RL spectrum of refined, analytical soya bean oil (analytical standard from Sigma) is also shown in figure 3d (black curve). The ‘fingerprint’ region (900–1800 cm$^{-1}$) and ‘CH’ region (2700–3100 cm$^{-1}$) were normalized by the amide I/C=C vibration (1646 cm$^{-1}$). Changes in local lipid composition were further quantified by calculating the ratios of specific vibrational resonances in the spectra, which correspond to different functional groups. This was done by multi-peak fitting the fingerprint and CH spectra from at least four different ROIs from each day with sums of Lorentzian functions corresponding to specific vibrations with centre and width parameters found in the established literature [51–53]. Results from the fitting are shown in the electronic supplementary material, figures S5 and S6, and were in good agreement with the RL spectra from each day, and parameters of each Lorentzian as well as the specific vibration are given in the electronic supplementary material, tables S1 and S2.

The peak at approximately 3005 cm$^{-1}$ originates from =CH vibrations from unsaturated carbons in acyl chains. Therefore, the ratio of peak areas $A_{R}/A_{C=C, sym}$, where $A$ is the peak area obtained from multi-peak fitting, is a metric for the relative degree of lipid unsaturation [44,54]. The observed level of unsaturation was markedly increased at day 5 after imbibition ($0.80 \pm 0.09$) (mean $\pm$ s.d.) compared with day 1 ($0.49 \pm 0.08$) and dropped below the starting value by day 9 ($0.33 \pm 0.11$) (figure 3e).

Beyond relative unsaturation, our data permitted calculation of the molar average lipid chain length and number of unsaturated (C=C) bonds. This was done by measuring the RL spectrum of a series of TAGs as well as a protein solution and using these spectra to construct a series of component spectra via least-squares decomposition that represent the TAG background, TAG number of CH$_2$ groups, and protein (see the electronic supplementary material, Methods). These component spectra are shown in the electronic supplementary material, figure S7. To validate this method, we calculated the average chain length and number of C=C bonds of analytical standard olive, sunflower, canola, soya bean and peanut oils as well two TAG mixtures by using non-negative least-squares fitting of measured spectra with derived component spectra (see the electronic supplementary material, Methods, equation S1). This process is related to partial least-squares decomposition, which has been extensively used for characterization of fatty acids and proteins in a variety of plant and food sources with near-infrared spectroscopy [55–57].

The fits with the derived component spectra model match well with the measured spectra (electronic supplementary material, figure S7), and produced average chain lengths and numbers of C=C bonds that were accurate to within 0.5 C and 0.1 C=C bonds, respectively, when compared with molar averages of analytical standard oils from manufacturers’ provided mass spectrometry data (electronic supplementary material, table S3). We found that the average lipid chain was 17.9 C with 1.1 C=C per chain at day 1; 18.0 C and 1.4 C=C per chain at day 5; and 14.4 C and 0.5 C=C per chain at day 9 when fitting the spectra shown in figure 3d. These numbers are summarized in table 1.

The increase in unsaturation at day 5 observed from our increased lipid unsaturation calculated from the spectral ratios (figure 3e). In addition to chain length and unsaturation, we quantified changes in lipid order in the oil bodies. The ratio of peak intensity of the CH$_2$ Fermi resonance (approx. 2885 cm$^{-1}$) to CH$_2$ symmetric (approx. 2845 cm$^{-1}$) is related to the ‘order’ or packing of aliphatic side chains [58,59]. During germination, the order parameter, $R = I_{CH_2 \, Fermi}/I_{CH_2 \, sym}$, where $I$ is the intensity of the RL
spectra at 2885 cm$^{-1}$ ($I_{\text{CH2 Fermi}}$) and 2845 cm$^{-1}$ ($I_{\text{CH2 Sym}}$), increased from $0.94 \pm 0.07$ at day 1 to $1.13 \pm 0.13$ at day 5 and decreased to $1.02 \pm 0.16$ at day 9 (figure 3f).

In situ spectroscopy data also allowed for determination of the relative amount of protein associated with oil bodies and how it changed during germination. The amide III vibration (1252 cm$^{-1}$) is a proxy for protein concentration, and a decrease in the amide III band is clear, especially at day 9. The ratio of peak areas, $A_{\text{amide III}}/A_{\text{CH2 deformation}}$, was then used as a metric of protein-to-lipid ratio associated with oil
Table 1. Chain length, number of C–C bonds, and protein : lipid ratio for spectra in figure 3d determined using the method described in the text and electronic supplementary material. Methods. Error was determined by RMS deviation from gas chromatography molar averages of analytical standard oils (electronic supplementary material, table S3). The protein : lipid ratio is a relative metric for comparison among samples but is not an absolute, stand-alone metric.

<table>
<thead>
<tr>
<th>day</th>
<th>molar average chain length</th>
<th>molar average number of C–C bonds</th>
<th>protein : TAG ratio (unscaled)</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 1</td>
<td>17.9 ± 0.5</td>
<td>1.1 ± 0.1</td>
<td>0.17 : 1</td>
</tr>
<tr>
<td>day 5</td>
<td>18.0 ± 0.5</td>
<td>1.4 ± 0.1</td>
<td>0.38 : 1</td>
</tr>
<tr>
<td>day 9</td>
<td>14.4 ± 0.5</td>
<td>0.5 ± 0.1</td>
<td>0.27 : 1</td>
</tr>
</tbody>
</table>

bodies. Amide II/ACH2 deform showed a decrease to 0.5 ± 0.15 at day 9 (figure 3g) after a slight increase from day 1 to day 5 (0.65 ± 0.01 to 0.8 ± 0.09). This is consistent with the trend in protein : TAG ratio obtained from spectral fitting for our chain length calculations (table 1). We found that the protein : TAG ratio is 0.17 at day 1, 0.38 at day 5, and 0.27 at day 9. In support of these quantitative metrics, we generated images of protein distribution via the ring breathing mode (approx. 1003 cm⁻¹) and compared them with images of the CH2 symmetric vibration (approx. 2845 cm⁻¹) at days 1, 5, and 9 of germination (electronic supplementary material, figure S9). While it was not clear from these images if a quantitative increase or decrease in oil body–associated proteins occurred during germination, it was certainly clear that oil bodies in cotyledons were always surrounded by a substantial pool of proteins and that less lipid–protein colocalization (yellow in the electronic supplementary material, figure S9) appears at day 9 than at day 1 or day 5. Finally, from both hyperspectral CARS quantification and imaging, we note that oil bodies became more heterogeneous with increasing germination time. This is reflected by the increased spread of the quantities reported in figure 3e–g (i.e. the error bars increase with time) as well as the less structured images in the electronic supplementary material, figure S9.

Since hyperspectral CARS analysis of the oil bodies at days 1, 5 and 9 showed that protein concentration associated with oil bodies changes throughout germination, we further analysed protein content, and specifically oleosin emulsifiers, associated with extracted oil bodies. At day 1, we observed that 3.8 weight % of the extracted oil weight was protein, and this decreased steadily to 0% by day 8 as measured by a Bradford assay (figure 4b). From laser diffraction experiments, where we observed the average diameter of oil bodies at each day of germination (electronic supplementary material, figure S4), and the Bradford measurements, we determined the average protein content per oil body at each day. This was done by calculating the number of oil bodies analysed in the Bradford measurement, assuming (i) a spherical shape for oil bodies with diameter as determined for the electronic supplementary material, figure S4, and (ii) a constant oil density (0.917 gm ml⁻¹ at 25°C) throughout germination. We found that protein content per oil body increases sharply at day 4 and rapidly decreases again before reaching zero at day 8 (figure 4b, black). When we compare the protein per oil body from the Bradford measurements with the protein per oil body expected from oil body size changes alone—i.e. a constant Bradford signal at each day—(figure 4b, red), it is clear that changes in oil body size alone cannot explain the experimentally determined protein density. The measured protein per oil body is consistently lower from day 2 onward. Interestingly, the increase (from day 1 to day 4) and decrease (from day 4 to day 8) in measured protein density in figure 4b is similar to the trend seen in figure 3g (and table 1), where the protein per lipid increases at day 5 relative to day 1 and then decreases at day 9.

Lastly, we independently verified that oleosins associated with oil bodies decreased over the course of germination using SDS–PAGE of purified oil bodies. High (23–24 kDa) and low (15–16 and 17–18 kDa) molecular weight soya bean oleosins were visible starting from day 1 but decreased until they were undetectable at day 5 (figure 4c). Similar degradation of oleosins was previously reported [16,60] in soya beans. This verifies that oil bodies start as oleosin-coated oil bodies to become non-oleosin-coated oil bodies during the early days of germination [34].

4. Discussion

In this work, we monitored oil body dynamics, quantified oil body morphology and quantified changes in oil body chemistry during germination with label-free CARS microscopy in situ via direct visualization using the vibrational modes of methylene groups (lipids) in living, intact tissues. Monitoring lipid morphology with CARS was similar to what was previously done by Cavonious et al. [61]. In that study, changes in oil body size in algae were measured with CARS and correlated with mass spectrometry in control and nitrogen-starved conditions, whereas the current study focused on oil body changes in unperturbed germination. Our results show that oil bodies first grow in size, followed by shrinkage, and these processes occur at different rates, depending on the location within the cotyledon. The abaxial region of the cotyledon empties of all lipids by day 5, while the centre and adaxial regions of the cotyledon show a time-dependent size of oil bodies. The spatially specific oil body dynamics are analogous to the unique spatio-chemical composition of oil bodies in cotton shown by MALDI-IMS imaging [25,26]. In that work, the different locations within the seed showed distinct lipid compositions, and these regions were known to develop into different plant organs/tissues. This underscores the simultaneous heterogeneous metabolic processes during early germination at different locations within a cotyledon. The observed morphological changes of oil bodies were paralleled by changes in their local lipid composition and associated protein composition. We note that our measurements of oil body size and chemistry changes do not distinguish between de novo synthesis of oil bodies and alteration of existing oil bodies. While it is likely that new oil body synthesis is occurring—diacylglycerol acyltransferase is surprisingly active in Arabidopsis germinating seeds and seedlings and has also been found in germinating soya beans—it is typically assumed that germinating soya beans primarily consume existing oil bodies imparted during seed maturation [3,62–64]. This is especially true for etiolated soya bean germination.
Furthermore, TAG content has been shown to clearly decrease throughout soya bean germination [64,65]. Nevertheless, we are unable to rule out the possibility that changes in oil body synthesis during germination, in addition to consumption and metabolism, influence the results presented here.

A pattern of growth and shrinkage of oil bodies in early plant growth was very recently identified in native Arabidopsis embryos using confocal fluorescence microscopy [6]. Our results are generally consistent with that study, even though the plant orders (Brassicales versus Fabales), spatial location in the plant (embryo versus cotyledon) and developmental stage (post germination versus imbibition) of the samples are different. Moreover, our results showing more lipid content in the inner regions of the cotyledon are also generally consistent with those from NMR imaging by Borisjuk et al. [29] in whole soya bean (Glycine max) cotyledons; however, the exact timing (particular day) within germination of the NMR images was not stated.

Quantitative hyperspectral CARS imaging showed that oil bodies exhibit a higher degree of lipid unsaturation at day 5 compared with just after imbibition (day 1) or towards the end of germination (day 9). This is in line with previous studies of germinating seeds, where stearic acid and palmitic acid were reported to be replaced by linolenic acid in germinating sunflower seeds [66]. Such a transformation indicates that saturated lipids are metabolized first following imbibition. The increase in unsaturation we observe was accompanied by a slight increase in aliphatic chain order, which correlates with the degree of packing of lipid side chains, in oil bodies. This counterintuitive result is possibly related to the increase in protein content associated with droplets at day 5; however, further work is warranted to clarify this issue.

Our data show that the average chain length of lipids in oil bodies approximated that of refined soya bean oil (17.5 C from our measurements) at day 1 (17.9 C) and day 5 (18 C). The number of C=C bonds per chain increased at day 5 (to 1.4 C=C bonds), and both the calculated average lipid chain length and unsaturation very closely matched that of refined soya bean oil (1.6 C=C bonds). At day 9, both the chain length and unsaturation of the lipids in oil bodies decreased, perhaps indicative of consumption of deposited oil body lipids during seed maturation, and more representative of de novo synthesized oil bodies. From the perspective that oil consumption (as opposed to biogenesis)
dominates lipid turnover during early germination (day 1 to day 5), this suggests that longer chain lipids are metabolized first, perhaps because more metabolites (acetyl-CoA) in glyoxysomal β-oxidation are derived from longer chain lipids than from lipids with shorter chains. In addition, the use of 16 or 18 C lipid chains in membranes for organelle development could also explain why shorter chains remain at day 9 [67]. The increased unsaturation seen at day 5 is consistent with the work from Yoshida [65], who showed that TAGs containing saturated fatty acids were hydrolysed earlier in germination than unsaturated fatty acids. Finally, we note that germinating soya beans were exposed to light on day 5, so photosynthesis was in strong effect by day 9, as evidenced by the strong green colour. This may have caused changes in lipids stored in oil bodies, e.g. for production of lipids (thylakoid membranes) that support photosynthesis. Clarifying the complete lipid biochemical changes in soya bean oil bodies throughout germination requires further investigation, potentially via MALDI-ISM, to elucidate the dynamics of different lipid subtypes within oil bodies in soya bean cotyledons.

We further explored the protein biochemistry of the oil bodies throughout germination and found a decrease in proteins associated with oil bodies in hyperspectral images (figure 3g and electronic supplementary material, figure S9) and bulk biochemical analysis (figure 4b). It is clear from the electronic supplementary material, figure S9, that protein-rich regions and oil bodies appeared to colocalize (to within 350 nm) at days 1 and 5, but this colocalization was less apparent by day 9, which qualitatively agrees with the quantitative data from spectral analysis. Oil body-bound oleosins completely disappeared by day 6, as seen from the SDS–PAGE analysis. Decreased presence of proteins, and specifically oleosins (which are very strong emulsifiers because of their strong negative charge and large steric hindrance), consigns only the phospholipid monolayer to stabilize the oil bodies. This will weaken the emulsification system and promote oil body coalescence, consistent with the oil body size increase observed at day 5 (figure 2 and electronic supplementary material, figure S4). Interestingly, we observed that oil bodies decreased in size (days 6–8) after initially increasing. This is correlated with maximal lipolytic activity in glyoxysomes [68,69] and β-oxidation of fatty acids [70] as well as oxygenation of polyunsaturated activity by lipoxygenases [69,71], suggesting a possible connection between enzymatic hydrolysis of TAGs, oleosin bound to the oil body and lipid metabolism. This conjecture is further supported by the close proximity of glyoxysomes to oil bodies in soya beans that has been observed with electron microscopy [72,73].

Finally, it is interesting to note that soya beans are known to have especially small oil bodies immediately after imbibition. Comparing oil body remodelling in different plants that have larger initial oil bodies using in situ CARS microscopy might help elucidate the underlying physiological reasons for the morphological changes in oil body size and composition during plant development. Furthermore, combining the CARS label-free approach for lipid (and protein) imaging with organelle-specific stains for mitochondria or glyoxysomes will be a useful platform to explore how oil body storage and metabolism are related in different parts of cotyledons during germination.

5. Conclusion

In this work, we used label-free CARS microscopy to observe intracellular morphological and chemical changes in oil bodies in germinating soya bean cotyledons in situ. We showed that CARS imaging over a 24 h period did not arrest cotyledon development and that CARS is a convenient, label-free method to analyse spatio-temporal oil body chemistry as it does not rely on dye permeability or intricate sample preparation. Based on in situ chemical imaging with hyperspectral CARS, we found marked variations in the lipid composition within, and protein associated with, oil bodies throughout the germination process. Bulk biochemical analyses of parallel samples used for CARS imaging showed that proteins associated with oil bodies, and especially oleosin emulsifier proteins, were reduced over the first 6 days of germination, coincident with oil body size increase. The spatio-chemical changes in oil bodies accessible with label-free CARS microscopy opens new possibilities for studying lipid dynamics and flux of lipids from oil bodies into other organelles during plant development.

Data accessibility. For numerical data in the main text of this manuscript, see [74]. The image data and supplementary numerical data are available from the corresponding author.

Authors’ contributions. G.W. and S.H.P. designed the research. G.W. and N.B. acquired the CARS images and performed the image processing. G.W., S.S. and H.J. grew samples and optimized fixation and CARS imaging protocols. G.W. and S.S. did the laser diffraction and biochemical analyses. M.B. and T.A.V. contributed ideas on the emulsion principles and interpretation. All authors contributed to discussion of the results and revision of the paper.

Competing interests. We declare we have no competing interests.

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