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Non-invasive investigation of the morphology and optical properties of the upside-down jellyfish *Cassiopea* with optical coherence tomography

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The jellyfish *Cassiopea* largely cover their carbon demand via photosynthates produced by microalgal endosymbionts, but how holobiont morphology and tissue optical properties affect the light microclimate and symbiont photosynthesis in *Cassiopea* remain unexplored. Here, we use optical coherence tomography (OCT) to study the morphology of *Cassiopea* medusae at high spatial resolution. We include detailed 3D reconstructions of external micromorphology, and show the spatial distribution of endosymbionts and white granules in the bell tissue. Furthermore, we use OCT data to extract inherent optical properties from light-scattering white granules in *Cassiopea*, and show that granules enhance local light-availability for symbionts in close proximity. Individual granules had a scattering coefficient of $\mu_s = 200–300$ cm$^{-1}$, and scattering anisotropy factor of $g = 0.7$, while large tissue-regions filled with white granules had a lower $\mu_s = 40–100$ cm$^{-1}$, and $g = 0.8–0.9$. We combined OCT information with isotopic labelling experiments to investigate the effect of enhanced light-availability in whitish tissue regions. Endosymbionts located in whitish tissue exhibited significantly higher carbon fixation compared to symbionts in anastomosing tissue (i.e. tissue without light-scattering white granules). Our findings support previous suggestions that white granules in *Cassiopea* play an important role in the host modulation of the light-microenvironment.

1. Introduction

Characterization of anatomical structures and morphology in photosymbiotic animals is important to understand the interplay between host light-modulation and the spatial organization of photosymbionts in complex holobionts, such as corals and other photosymbiotic cnidarians. External structures can be relatively easily identified using macro- and microscopic imaging in the visible wavelength range on intact individuals with various levels of preparation [1,2]. However, imaging deeper into tissue is challenging with conventional or laser scanning microscopes, where the observational depth is typically limited to a few hundred micrometres before absorption and scattering of light reduces image quality substantially, and fluorescent labelling of structures is often necessary to achieve sufficient contrast [3]. Invasive tissue clearing of fixed specimens can enhance light penetration for three-dimensional reconstruction of intact whole organisms [4]. However, serial sectioning in
combination with light or electron microscopy is often required to obtain a combination of macrostructural observations and detailed information of internal organization [5]. This is both destructive and slow, and involves tissue sample preparation steps that can create artefacts in tissue organization [6,7].

In this context, optical coherence tomography (OCT) presents an attractive alternative imaging modality [8–10], as it enables non-invasive in vivo imaging of live specimens using near infrared (NIR) wavelengths, allows for high-resolution tomographic scanning of tissue in real time, and enables three-dimensional reconstruction of large samples with a potential depth of penetration of several millimetres depending on the scattering properties of the sample [8,11]. Previous studies have used OCT for visualizing and quantifying structural characteristics, such as surface area, volume, and porosity in biofilms [12,13], terrestrial plants [14] and marine invertebrates [15], including scleractinian corals [11,16,17].

The OCT measuring principle is analogous to ultrasound imaging and is based on the detection of directly backscattered light from refractive index mismatches in a sample, measured as the echo time delay and intensity of directly backscattered light from internal microstructures in materials or tissues [18]. OCT imaging uses low coherent NIR light to generate tomographic data composed of axial point measurements (A-scan) that are used to generate two-dimensional cross-sectional images (B-scan) or three-dimensional data cubes (C-scan) of backscattered photons using interferometry. Besides providing structural information from tomographic measurements of relative backscatter intensity, OCT systems can also be calibrated for absolute measurements of reflectivity. In combination with a theoretical bio-optical model based on an inverse Monte Carlo method [19], calibrated OCT datasets can be used to extract the scattering coefficient, μs (in cm⁻¹), with lower μs = less scattering and more photons passing into the medium without deflection, and higher μs = more scattering of photons, increasing the average path length of photon travelling in the medium per vertical distance traversed), as well as the anisotropy of scattering, g (where g = 1 indicates completely forward scattered, and g = 0 indicates isotropic scattering). More details on the basic principle of OCT can be found elsewhere [8–10]. Furthermore, we note that OCT can also be combined with spectroscopic measurements to obtain a wider range of optical parameters and higher spatial resolution [20].

The optical properties in corals have received increased attention over the last decade, and both coral tissue and skeleton have been found to exhibit strong light scattering properties [20–25]. Host-produced fluorescent pigments (FPs) in particular enable cnidarians to enhance scattering of light incident in the tissue [26], and the backscatter of light from the coral calcium carbonate skeleton further enhances light capture and photosynthesis by the endosymbiotic dinoflagellate algae [21,22,24,27–30]. Wangpraseurt et al. [11] used OCT imaging for real-time quantification of tissue movement in coral polyps, providing insights into symbiont and FP movement and density. They demonstrated how optical properties not only differ between tissue regions, but that tissue movement can lead to dynamic modifications of local optical properties of the coral tissue, potentially enhancing or reducing photon absorption [16]. However, we are not aware of such use of OCT for investigating other cnidarians.

The so-called upside-down jellyfish Cassiopea is currently promoted as an important model system for cnidian photosymbiosis [31,32]. Cassiopea hosts photosynthetic microalgae belonging to the same dinoflagellate family (Symbiodiniales) as symbionts found in scleractinian corals [33]. Adult Cassiopea medusae live mostly in shallow benthic environments, where they position themselves up-side down on the sediment basking in the sun to expose their photosynthesizing algae that supplement the carbon demand of the heterotrophic host with autotrophically acquired carbon [34–36]. The symbiont algae are found concentrated in the host tissue on the oral side of the bell and in oral arms of the medusa [5,37]; see electronic supplementary material, figure S1 for an overview of Cassiopea morphology. Cassiopea exhibit a range of colours from purple-blue to reddish [33,38] but most commonly have a blue pigmentation associated with the bell disc and oral vesicles [39], which was recently identified as a new group of pigments (i.e. rhizostomins [40]). Another common trait of adult Cassiopea is the whitish appearance of certain tissue regions containing granules, which is most pronounced in the oral arms and stretches radially from manubrium to the bell margin and rhopalia [41]. The function of the white granules in Cassiopea is still unknown, but it has been suggested to play a role in the mitigation of photodamage and enhancement of photosynthesis [39,42], where the white granules and coloured pigments in Cassiopea might serve similar functions to that of the skeleton and host pigments in corals.

Here, we used non-invasive OCT imaging to visualize the structure of live Cassiopea sp. medusae and provide details of specific anatomical features in intact, living specimens as well as cut-out tissue samples from living specimens. We identified two highly heterogeneous tissue regions in the bell, namely the rhopalia canals containing a high density of white granules and the anastomosing tissue without white granules visibly present. Optical properties were extracted from these regions using the OCT data, and a labelling experiment using stable isotopes of 13C-bicarbonate and 15N-ammonium with nanoscale secondary ion mass spectrometry imaging (NanoSIMS) was conducted to test if the different optical properties altered photosynthetic performance of algal symbionts found in the two tissue regions.

2. Methods

(a) Cassiopea husbandry and preparation

Cassiopea sp. medusae (acquired from DeJong Marinelife, Spijk, Netherlands) were cultivated at the Marine Biology Section (MBS), University of Copenhagen (Helsingør, Denmark) in a 50 l aquarium. Medusae were fed 5 times per week with recently hatched Artemia nauplii and supplemented with AF Amino Mix (Aquaforest) 2–3 times per week. Medusae were kept at 25°C in artificial seawater (ASW) with a salinity of 35 and a pH of 8.2. Water was recycled inside the tank using a small internal filter, as well as UV light filtration. Illumination was maintained with a programmable LED aquarium lamp (Triton R2, Pacific Sun) running a 12/12 h day night cycle with a downwelling photon irradiance (400–700 nm) of 350 µmol photons m⁻² s⁻¹, as measured just above the water surface using a calibrated spectroradiometer (MSC-15, GigaHertz-Optik). We used Cassiopea medusae in a size range from 7 mm up to several cm in diameter to scan the internal and external anatomy of the Cassiopea medusa life stage.
(b) Optical coherence tomography
We used a spectral domain OCT imaging system (Ganymede II, Thorlabs GmbH) comprised of a base unit with a non-coherent NIR light source (930 nm; GAN611) and a scanning system (OCTG9) fitted with either the OCT-LK3-BB lens kit (field of view 10 × 10 mm, lateral resolution 8 μm, effective focal length 36 mm) for complete medusa scans, or an OCT-LK2-BB kit (field of view 6 × 6 mm, lateral resolution 4 μm, effective focal length 18 mm) for close-up scans with a higher resolution. The axial resolution was 5.8 μm. For imaging, animals and cut-out tissue samples were kept in a small Petri dish submerged in either filtered ASW or 100% glycerol, typically with 4 mm liquid on top of the tissue surface. Prior to the OCT imaging, animals were anaesthetized with MgCl2 (50% w/v in ASW) that was added dropwise to the Petri dish until the individual stopped pulsating. Cut-out tissue, if not stable in a favourable orientation relative to the OCT system, was fixed with hypodermic needles onto a cork surface covered with dark-cloth to reduce light reflection.

(i) Extraction of optical parameters using OCT
Inherent optical properties like the scattering coefficient, μs (cm−1), and the anisotropy of scattering, g, can be estimated from OCT scans of biological tissues using theoretical models of light propagation based on the inverse Monte Carlo method [43,44]. More detailed description of optical parameter extraction from OCT scans of tissue can be found elsewhere [10,16].

Briefly, OCT B-scans were acquired with a resolution of 581 × 1024 pixels, over a fixed depth interval of 2.8 mm, and variable distance in the x-plane. The setup was optimized to yield the highest signal at a fixed distance of 0.4 mm from the top of the scan. The OCT reflectivity (R) was calibrated before measurements using homemade reflectance standards with an immersion oil-glass, a water-glass, and an air-glass interface. R values from the standards were determined using Fresnel’s equation:

\[ R = \left( \frac{n_1 - n_2}{n_1 + n_2} \right)^2, \]  

(2.1)

using the refractive index (n) for air (1), water (1.33), immersion oil (1.46) and quartz glass (1.52). The OCT signal (in decibel, dB) from the samples, was then converted to the depth-dependent R via a linear fit of log10(R) versus OCT intensity values (see Wangprasert et al. [16] for details).

Calibration of the focus function of the objective lens was performed by measuring the OCT signal fall off, in steps of 0.1 mm, from either side of the focal plane (z = 0.4 mm) to z = 0 and 0.8, respectively. The signal loss from the focal plane follows an exponential decay function. The determined R values from the sample scans were corrected by dividing with the exponential fit. Then the corrected R values were plotted against sample depth (z, distance from focal volume) and fitted to the exponential decay function:

\[ R(z) = \rho \times e^{-\mu s z}, \]  

(2.2)

where ρ (dimensionless) is the light intensity and μs is the signal attenuation (cm−1) from the focal volume. The fit was considered satisfactory if R2 > 0.5. The first few pixels from each scan were excluded due to high reflectivity arising from the refractive index mismatch at the water-epidermis interface.

Using the grid method [19], values of ρ and μs were matched to g and μs, based on the theory described in previous studies [16,45]. It was assumed that the Cassiopea tissue absorption at 930 nm was negligible and dominated by water (μs ≈ 0.43 cm−1). The effective numerical aperture (NA) was 0.11. The refractive index of the investigated tissue was estimated to be 1.473 (see results and discussion).

The targeted areas for extraction of optical properties in Cassiopea tissue were (i) bell tissue with white granules (along rhopalia canals), and (ii) symbiont clusters in anastomosing tissue. Scans in ASW were done on intact tissue, and on cut-out tissue where the epidermis was removed by gently peeling the epidermis from the underlying bell using tweezers.

Raw OCT files and three-dimensional animations of figures 1–5 can be found in the electronic supplementary material.

(c) Isotopic pulse labelling experiment

(i) Experimental setup
A tank with approximately 10 l of deionized water was placed on top of 3 magnet stirrers (RCT basic, iKA GmbH). A heater and a small water pump were fitted in the bath to keep water homogeneously at 25±0.5°C. A white LED lamp (KL2500 LED; Schott AG) equipped with a 3-furcated fibre guide was used for homogeneous illumination over each magnet stirrer. Each fibre guide was fitted with a collimating lens, and light was adjusted to an incident photon irradiance of 350 μmol photons m−2 s−1 (400–700 nm), as measured with a calibrated spectroradiometer (MSC-15, GigaHertz Optik). The photon irradiance was chosen based on variable chlorophyll fluorescence imaging of the maximum PSII quantum yield, and measurements of the non-steady-state effective PSII quantum yield as a function of photon irradiance, so-called rapid light curves (RLC) in ROI’s representative of different Cassiopea tissue regions (electronic supplementary material, figure S2c) [46], where all individuals showed light saturation above 500 μmol photons m−2 s−1 (see more details in the electronic supplementary material). Isotopic labelling was done in 100 ml plastic beakers placed in a thermal bath and fitted with a magnet bar, as well as a mesh at the bottom to separate medusae from the rotating magnet (200 rpm).

(ii) Water preparation and pulse labelling
Prior to the labelling experiment, 1 l of isotopically enriched artificial seawater (ASW) was prepared as follows: Filtered (0.2 μm; Millipore) ASW (salinity of 35, pH 8.2) was stripped of dissolved inorganic carbon by lowering pH to <3 with 1 M HCl, and was then flushed with atmospheric air over night. The ASW was then enriched with 3 mM NaH13CO3 (99 atom%, Sigma Aldrich), back up to experimental pH (8.2) with 1 M NaOH, and 3 μM 15NH4Cl (99 atom%, Sigma Aldrich) was added. The mix was left to equilibrate with the experimental temperature of the thermal bath (25°C) before pulse labelling began.

Three Cassiopea medusae of approximately 3.5–4 cm in diameter were chosen for the labelling experiment. For each medusa; 3–4 oral arms were removed using a tweezer and razor blade in order to expose one half of the medusa bell surface area. Medusae were subjected to 6 h of pulse labelling in 80 ml of isotopically enriched ASW, with a change of isotopically enriched ASW every 2 h. At the end of pulse labelling, one quarter of the bell from each medusa was chemically fixed in 10 ml of 2.5% [v/v] glutaraldehyde (Electron Microscopy Sciences), 4% [v/v] paraformaldehyde (Electron Microscopy Sciences), and 0.6 M sucrose (Sigma-Aldrich) mixed in 0.1 M Sörensen phosphate buffer. Chemically fixed Cassiopea samples were kept at room temperature for 2 h and then stored at 4°C in fixative until further processing.

(iii) Histological sectioning for NanoSIMS analyses
A detailed description of NanoSIMS analyses including sample preparations can be found in Lyndby et al. [36]. Briefly, isotopically labelled animals had a piece of the bell cut out including both anastomosing tissue and the rhopalia canal with visible white granules. The cut-out tissue samples were divided into the two respective tissue regions, and a smaller piece roughly...
halfway between manubrium and margin was extracted and embedded in Spurr’s resin. Semi-thin histological sections with a thickness of 200 nm were then cut with an Ultracut S microtome (Leica Microsystems), placed on 10 mm diameter round glass slips, and gold coated with a layer of 12.5 nm gold (Leica EM SCD050, Leica Camera AG) for NanoSIMS imaging.

(iv) NanoSIMS image acquisition
Isotopic imaging of semi-thin histological sections was performed with a NanoSIMS 50L instrument [47]. Images (40 × 40 μm, 256 × 256 pixels, 5000 μs pixel⁻¹, 5 layers) were obtained with a 16 KeV Cs⁺ primary ion beam focused to a spot-size of about 120 nm (with a beam current of 2.2 pA). Secondary ions (12C₂⁻, 13C12C⁻, 12C14N⁻ and 12C15N⁻) were counted in individual electron-multiplier detectors at a mass resolution of about 9000 (Cameca definition), sufficient to resolve all potential interferences in the mass spectrum [48–52].

Isotopic images were analysed using the software L’IMAGE developed by Prof. Larry Nittler at the Arizona State University. Contours were drawn in each image around the epidermis as well as around individual amoebocytes and dinoflagellate cells. The epidermis in each NanoSIMS image was treated as a single region of interest (ROI; n = 1). Similarly, amoebocytes were treated as one ROI per image unless their cell clusters were clearly separated. Due to low numbers of symbionts in the exumbrella tissue, NanoSIMS analyses only include subumbrella tissue. Drift-corrected maps of ¹³C- and ¹⁵N-enrichment were obtained from the count ratios ¹³C¹²C⁻/¹²C₂⁻ and ¹⁵N¹²C⁻/¹⁴N¹²C⁻, respectively. Measured enrichments were expressed in δ notation:

\[ \delta ^{13}C \, (\%e) = \left( \frac{R_{\text{enriched}}}{R_{\text{control}}} - 1 \right) \times 1000 \]

and

\[ \delta ^{15}N \, (\%e) = \left( \frac{R_{\text{enriched}}}{R_{\text{control}}} - 1 \right) \times 1000, \]

where \( R_{\text{enriched}} \) and \( R_{\text{control}} \) are the count ratios of an enriched sample and a control (i.e. unlabelled) sample, respectively. Total numbers of technical replicates (ROIs) are provided in electronic supplementary material, table S1.

(d) Symbiont density estimation
The density of symbionts was estimated from bell tissue corresponding roughly to AOIs drawn in ImagingWin (Walz GmbH). For each cut-out section, the subumbrella epidermis was carefully peeled off using a pair of fine tweezers, leaving the thick mesoglea (bulk mass of the bell) with the ex-umbrella epidermis still attached; simply referred to as ‘mesoglea’ from this point. The subumbrella epidermis and the mesoglea were analysed separately by homogenizing the tissue in 1.5 ml of filtered (0.45 μm) ASW using a T10 Standard Ultra Turrax handmixer (IKA) until

Figure 1. (a) Three-dimensional scan (C-scan) of an entire juvenile Cassiopea sp. medusa, using a FOV of 7 × 7 × 2.8 mm (xyz), a resolution of 350 × 350 × 1024 pixels, corresponding to a voxel size of 20 × 20 × 2.74 μm. Rhopalia can be seen along the bell rim (purple arrows), and first oral arm bifurcation (black arrow) and an oral groove (white dashed circle) are clearly visible on the oral arms. (b) Tomographic cross section (B-scan) in the xz-plane through the bell, revealing the in vivo distribution of dense symbiont clusters underneath the subumbrella epidermis and diffusely spread deeper in the bell (white arrows), and large patches of white granules (purple dashed circles) underneath epidermis. A faint line of the exumbrella epidermis is visible at the bottom (green arrow). (c) Overview photo of the animal under OCT observation, the dashed white line indicates the B-scan presented in (b). White scale bar represents 1 mm. Coloured scale bar indicates relative OCT signal strength. An animated three-dimensional representation (electronic supplementary material, movie S1) and the raw OCT file can be found in the electronic supplementary material.
samples were completely homogenized. Then 1 ml of the tissue slurry was transferred to a Sedgewick–Rafter cell S52 (Pyser-5C0), and dinoflagellate cells were counted in 10 random squares on a microscope (AxioStar Plus, Zeiss). A total of three biological individuals were used, each of which 6 tissue regions were extracted (3 rhopalia canals and 3 anastomosing tissue regions).

(e) Statistical analyses

We used R (version 4.0.5) with the package nlme (version 3.1–157) to perform statistical analyses. Linear mixed model (LMM) analyses were used to test the relationship between isotopic enrichment in Cassiopea holobiont compartments in 2 bell tissue regions (near rhopalia canals and in anastomosing tissue) taking into account the biological replicate as a random factor. $^{13}$C enrichment data for amoebocytes, and $^{15}$N enrichment data for symbionts and host epidermis were square root transformed to achieve normality.

3. Results and discussion

(a) Imaging Cassiopea morphology

Due to the near-neutral buoyancy of Cassiopea jellyfish, even small vibrations during OCT imaging can cause the sample to move, creating distortion in the scans. Scanning speed and reference light intensity was thus adjusted to achieve a relative short scan-duration without sacrificing signal intensity. The field of view (FOV $7 \times 7 \times 2.8$ mm; $xyz$) was adjusted to contain the entire animal, and the number of pixels in each direction was kept to a minimum ($350 \times 350 \times 1024$ pixel; $xyz$) (figure 1a). The scan pattern resulted in a voxel size of $20 \times 20 \times 2.74$ µm ($xyz$). Given that the average diameter of dinoflagellate symbionts found in Cassiopea are typically around 10 µm [53,54], this voxel size provided sufficient resolution to identify symbiont clusters in form of amoebocytes (that typically host more than 10 cells per unit, e.g. [36]), and symbiont clusters were identifiable even from complete specimen scans (figure 1b).

Externally, rhopalia were visible along the bell margin, and the first oral arm bifurcation and groves were clearly visible. Individual arms showed signs of vesicles forming apically, while digitata were not yet present (e.g. [55,56]) (figure 1a). The bell-disc surface is not perfectly flat, but appears ridged in a radial pattern, possibly due to the musculature lining the subumbrella epidermis [57]. Some shading is apparent from oral arms and the marginal lobe reaching in over the bell disc, and the OCT generally did not manage to penetrate through the entire animal system from sub- to exumbrella epidermis due to multiple scattering effects degrading the contrast with depth. Internally, symbiont clusters and white granules were found in high densities near the subumbrella epidermis (e.g. [36,37]), and white granules can be seen extending further down into the exumbrella mesoglea along rhopalia canals (figure 1b).

Changing to a higher magnification objective, it was possible to zoom in on the above-mentioned structures to reveal more details, while also gaining more non-invasive information on the internal tissue organization of Cassiopea. We used both intact animals and cut-out tissue regions from multiple specimens of various sizes to scan a broad range of anatomical structures at different growth stages.

Rhopalia are specialized eyespots in Medusozoa that allow medusa to detect light and orientate themselves in the water column [58,59]. Cassiopea have on average 16 rhopalia distributed along the bell margin [41]. Close-up scans with OCT showed that these eyespots are well nestled between two marginal lappets along the rim (figure 2a–c). Rhopalia canals are major canals of the gastric network extending from the center of the bell, out towards the rhopalia [41] (figure 2d,e). Cross-sectional scans near one of the rhopalia showed that these major canals fork into two canals, each reaching out into a marginal lappet around the rhopaliun (figure 2d,e).

Oral arms are highly branched at the center of the bell (figure 3a). They typically occur in four pairs in Cassiopea, and each arm harbours secondary mouths and vesicular appendages. Vesicular appendages could be seen containing clusters of cassiosomes ready for deployment to catch prey [60] (figure 3b). Cassiosomes are released from the vesicular epidermis in amorphous ‘popcorn’ shaped tissue balls, with newly developing cassiosomes being formed underneath fully developed ones [60]. However, it was not possible to confidently discern individual cassiosomes underneath the surface of the cluster, possibly due to the accumulated layers of dermis from each individual cassiosome that substantially lowered OCT scan penetration depth into these clusters (figure 3b).

(b) Improving depth penetration

During this OCT imaging work, several factors that decreased the signal penetration were identified. Host epidermal tissue, symbionts, and white granules all greatly scattered the NIR light from the OCT light source decreasing the contrast with depth in the jellyfish tissue. To reduce the OCT signal loss at the upper tissue interface, the refractive index of the surrounding medium was matched to the epidermis in Cassiopea. Glycerol has a refractive index of 1.473, and submerging a piece of tissue in pure glycerol demonstrated that the Cassiopea epidermis has a very similar refractive index, thus permitting OCT imaging of deeper tissue layers (electronic supplementary material, figure S3). Sectional scans of a vesicular lappet clearly showed the entire structure, and revealed a cavity in the center of the lappet (electronic supplementary material, figure S3b). Contrary to tissue scanned in water, where the endodermis/gastrodermis appeared thin or transparent, the endodermis of the lappet cavity appeared much clearer due to less backscattering in the epidermis. Individual symbiont clusters also became more distinctly visible throughout the lappet system when imaged in pure glycercol (electronic supplementary material, figure S3).

While pure glycerol matches the refractive index of the epidermis well, the high viscosity of glycerol makes it difficult to image larger samples, and submerging intact medusae in glycerol did not improve depth penetration due to water and mucus sticking to the surface of the animal, which resulted in lower signal penetration and contrast.

To explore the deeper layers of the bell, we cut a piece of bell tissue from a medusa and then carefully removed the subumbrella epidermis and mesoglea using a pair of fine tweezers. Removing the subumbrella epidermis greatly improved the OCT depth penetration, because the symbiont cell clusters and the epidermis were the main sources of light scattering in these anatomical structures (figure 4a). When the tissue was cut, grabbing and ripping off the sub-umbrella epidermis proved easy, and even kept parts of the
exumbrella gastrodermis from radial canals still visibly attached to the exumbrella mesoglea (figure 4b).

Removing the subumbrella epidermis greatly improved visibility of symbiont clusters and white granules present in the exumbrella mesoglea. A side-by-side comparison of the exumbrella mesoglea, with half the scan covering the rhopalia canal full of white granules, and the other half covering anastomosing tissue only with symbiont clusters, revealed a major heterogeneity in the structure and content of the two tissue areas (figure 5).

**Figure 2.** Rhopalia of *Cassiopea* medusae of approximately 4 cm (a) and approximately 1 cm (c) in diameter. (b) The rhopalium is highly scattering of NIR light and appears almost solid (white arrow). (d) The rhopalia canal can be observed running out to the base of the rhopalia (purple dashed circles), before splitting into two canals that reach out into the marginal lappets on each side of the rhopalia (e). Throughout the scans, clusters of symbionts and white granules were diffusely spread out in the bell margin and marginal lappets along the rhopalia canal and into the bell margin (purple arrows). The two-dimensional scan in panel (b) is derived from the three-dimensional scan in panel (a), and panel (d,e) is derived from panel (c) (white dashed lines). Scale bars represent 200 µm in panel (b), and 100 µm in (d,e). Coloured scale bar indicates relative OCT signal strength.

**Figure 3.** (a) Oral arm with oral groove (purple dashed circle; approximately 1 mm in length). Sectional scans of the arm showed that the oral groove extended into the arm and further into the manubrium (not shown here). Oral vesicles (white arrow) were found apically on the arm, along with secondary mouths covered in frigid digitata (blue arrow). (b) Close-up of vesicle with a developing cluster of cassiosomes (red dashed circle). White scale bars in mm. Coloured scale bar indicates relative OCT signal strength.
Figure 4. (a) OCT scan of bell tissue with part of the subumbrella epidermis and mesoglea removed (right side of dashed line), improving scan penetration depth and clarity in this area. (b) Overview of bell tissue with subumbrella layer removed. The remaining gastrodermis from the radial canals was still attached to the exumbrella mesoglea showing how these canals branch out over the entire bell. A dense carpet of white granules was found in the exumbrella mesoglea underneath radial canals. White scale bars in mm. Coloured scale bar indicates relative OCT signal strength.

Figure 5. (a) Three-dimensional scan of a rhopalia canal with the subumbrella layer removed, revealing the density of symbiont clusters in anastomosing tissue (left side) versus white granules (right side). (b) A sectional scan showing that white granules appear much denser than symbiont clusters. White granules were mainly spherical in shape (up to 80 µm in diameter), and appeared to be solid particles as judged from the OCT signal. (c) Close-up scan of symbiont clusters. (d) Close-up scan of white granules. Due to the density of granules, it was not possible to confidently discern symbiont clusters in this tissue region. White scale bars in panel (a,c,d) in mm. White scale bar in panel (b) represents 200 µm. Coloured scale bar indicates relative OCT signal strength. An animated three-dimensional representation of panel a can be found in the electronic supplementary material, movie S2.
brella layer (electronic supplementary material, figure S4). We also found that the symbiont populations were more than 3-fold higher in the subumbrella layer relative to the exumbrella epidermis, as compared to the intact tissue, which showed that symbionts were more abundant near the subumbrella epidermis, as compared to the rest of the Cassiopea medusa (figure 1b).

(c) High-resolution scans

High-resolution scans with a voxel size of 0.5 × 0.5 (figure 5c) or 1.0 × 1.0 µm area (figure 5d), and 2.7 µm in height, were used for detailed scans of the spatial distribution of symbiotic clusters and white granules. White granules were found in high density along the rhopalia canal, and appeared as ‘solid’ spheres of roughly 80 µm in diameter (figure 5b). Symbiont clusters were also found in surprisingly high density in the exumbrella epidermis, although more diffusely spread out compared to the white granules. Symbionts appeared to be positioned in elongated clusters, stretching up to 80 µm across. Given that the average size of Symbiodinium cells in hospite with Cassiopea are normally around 10 µm in diameter [53,54] this indicates that amoebocytes can harbour more than 10 symbionts depending on clustering formation, consistent with a previous estimate [36]. Due to the high density of white granules near rhopalia canals, clusters of symbionts were hard to detect or distinguish from the granules in this region (figure 5d). However, symbiont densities quantified from the two regions by cell counting were found to be equally distributed between the anastomosing and rhopalia canal tissue (electronic supplementary material, figure S4b). We also found that the symbiont populations were more than 3-fold higher in the subumbrella layer relative to the exumbrella layer (electronic supplementary material, figure S4b). The data are in agreement with the larger OCT scans of intact tissue, which showed that symbionts were more abundant near the subumbrella epidermis, as compared to the rest of the Cassiopea medusa (figure 1b).

(d) Optical properties of Cassiopea bell tissue

Studies of the optical properties of cnidarian tissue is an expanding field, yielding important information about how host tissue and symbionts modulate and interact with their light environment. Such information is also important for the development of models that simulate radiative transfer in corals, for example [61]). While the function of the white granules in Cassiopea remains unknown, other pigments in Cassiopea have been speculated to be either photoprotective or photo-enhancing [39,42], similar to host pigments found in corals (e.g. [22,62]). The high density of the heterogeneously distributed, light scattering white granules in the bell and oral arms suggests that the light microenvironment could be greatly altered in these tissue regions relative to tissue with no white granules. We estimated the optical properties of Cassiopea bell tissue with light scattering white granules using optical parameter extraction from OCT scans [16].

The optical properties of tissue with white granules near the rhopalia canals were radically different from the anastomosing tissue (figure 6). The scattering coefficient of tissue with white granules were estimated to be $\mu_s = 200–300 \, \text{cm}^{-1}$, and with an anisotropy factor of $g = 0.7$ for individual white granules (i.e. analyses targeting narrow regions with individual granules; electronic supplementary material, figure S5b). Estimates from larger areas of tissue (i.e. analyses averaged over large areas, comprising multiple granules with a width of 10–20 granules; electronic supplementary material, figure S5b) indicated that $g$ increased to 0.8–0.9 and the scattering coefficient dropped to $\mu_s = 40–100 \, \text{cm}^{-1}$. This suggests that the arrangement of light scattering white granules in the tissue increases both the photon residence time and depth penetration, thereby enhancing the chance of absorption by symbionts near white granules, similar to reflective (and fluorescent) host pigments in corals [16,22].

It was not possible to determine the optical properties of symbiont clusters in anastomosing tissue. Symbiont densities in the exumbrella mesoglea were on average 3-fold lower than in the subumbrella, with few clusters that were diffusely spread in the mesoglea, as judged from B-scans of this tissue region (figure 5b,c). This prevented the extraction of meaningful optical properties, but indicates that light travels through the exumbrella mesoglea in anastomosing tissue relatively unhindered. These differences suggest that symbionts in mesoglea with white granules might be more light-exposed based on an increased light scattering in this region, as compared to other tissue regions.

(e) Photosynthetic performance in different Cassiopea tissue compartments

Given that the optical properties of light scattering white granules can drastically alter the local light microenvironment, a 6 h isotopic labelling experiment was conducted in combination with NanoSIMS isotopic analyses [36,63–65]. We used $^{13}$C-bicarbonate and $^{15}$N-ammonium incubations to investigate...
whether carbon and nitrogen assimilation rates differed between symbiont algae in the subumbrella anastomosing tissue and rhopalia canal regions. We combined such measurements with variable chlorophyll fluorescence imaging to investigate the photosynthetic performance and carbon assimilation of symbionts in the two tissue regions.

Variable chlorophyll fluorescence imaging showed no differences in maximal PSII quantum yield \( F_{v}/F_{m} \) between algal symbionts found along rhopalia canals versus anastomosing tissue in intact bell tissue (electronic supplementary material, figure S2a), and the effective PSII quantum yield \( \Phi(\text{II}) \) was also similar for the two regions, as measured with RLCs (electronic supplementary material, figure S2b), indicating no difference in light acclimation or health in symbionts between the two tissue regions across the three medusae. However, removing the subumbrella layer, and running RLCs on the sub- and exumbrella layers side-by-side showed that symbionts deeper in the bell had a drastically lower threshold for the sub- and exumbrella layers side-by-side showed that symbionts deeper in the bell had a drastically lower threshold for light saturation, and rETR saturated already around 100–200 \( \mu \text{mol photons m}^{-2}\text{s}^{-1} \) (electronic supplementary material, figure S2c,d), relative to saturation at 500 \( \mu \text{mol photons m}^{-2}\text{s}^{-1} \) for symbiont photosynthesis in the subumbrella layer (electronic supplementary material, figure S2c,d). This indicates that symbionts in the exumbrella epidermis are acclimated to lower light levels, suggesting that most light is absorbed at the subumbrella epidermis directly, or is diffused/scattered by the white granules. A detailed description of variable chlorophyll fluorescence imaging and parameters can be found in the electronic supplementary materials.

Stable isotope labelling of three sub-adult specimens of Cassiopea showed a significant difference in the \( ^{13} \text{C} \) assimilation by symbiont algae near white granules, relative to symbionts found in anastomosing tissue (LMM, \( F_{1,4} = 8.8, p = 0.041 \); figure 7a), suggesting that photosynthesis is locally enhanced by the light scattering from white granules. No differences in \( ^{13} \text{C} \) enrichment were observed in amoebocyte cells (LMM, \( F_{1,4} = 1.2, p = 0.342 \)) or host epidermis (LMM, \( F_{1,4} = 0.5, p = 0.508 \)). Finally, no differences in \( ^{15} \text{N} \) uptake were found for symbionts (LMM, \( F_{1,4} = 0.04, p = 0.854 \)), amoebocytes (LMM, \( F_{1,4} = 0.5, p = 0.508 \)), or host epidermis (LMM, \( F_{1,4} = 0.2, p = 0.649 \)), comparing between rhopalia canals and anastomosing tissue (figure 7b), indicating that each region had equal access to nutrients during the labelling experiment.

The presence and role of optical microniches in Cassiopea for symbiont photosynthesis has hitherto been unexplored. Here, we provide evidence that the Cassiopea host directly alters the light microenvironment, benefitting nearby symbionts. White light scattering granules in Cassiopea provide a means for the host to not only enhance photosynthetic performance of symbionts, but also to propagate light further down into the host tissue, potentially supporting symbiont populations present in shaded parts of the animal. While it remains unknown what triggers production of white light scattering granules in Cassiopea, it is possible that individuals living in overall more shaded environments (e.g. mangroves) would be more inclined to produce white light scattering granules to enhance photosynthesis of symbionts.

We found highest symbiont densities near the subumbrella epidermis due to higher light availability. However, enhanced propagation and homogenization of light through the Cassiopea tissue via scattering might explain why roughly one quarter of the symbiont population is found deeper into the bell and exumbrella layers. Furthermore, light penetrating all the way through the animal to the substrate could potentially be backscattered up into the animal, additionally enhancing photon availability in the umbrella.

4. Conclusion

Optical coherence tomography can provide detailed scans of the in vivo tissue organization of intact Cassiopea, showing the distribution of symbiont clusters, white granules, and other structures at high spatial resolution. The methods presented here enable detailed mapping of the distribution of entire symbiont populations or similar distinguishable objects in intact, living cnidarian systems, providing new means for non-invasive monitoring of the internal dynamics of symbiotic cnidarians, under both homeostasis and during stressful events. Additionally, this study provided the first insight into how light scattering granules in Cassiopea medusae can enhance the photosynthetic performance of their symbionts near these granules. These observations support previous suggestions that white tissue regions with granules in Cassiopea play several roles in modulating the light microenvironment of Cassiopea, by enhancing light availability via scattering, thus exhibiting both photosynthetic and photoprotective properties. The presented
approach to study structure and function in Cassiopea is also applicable to other photosymbiotic cnidarians (e.g. cnidarian model systems such as Exaiptasia sp. and Hydra sp.), but we propose that OCT could also find broad application for non-invasive monitoring of structure and morphology of particular tissues or whole specimens in many types of invertebrates.

Ethis. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. Raw OCT data files are publicly available via the Dryad Digital Repository: https://doi.org/10.5061/dryad.jwstjgci [66].

Supplementary material is available online [67].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors’ contributions. N.H.L.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, writing—original draft, writing—review and editing; S.B.: formal analysis, investigation, methodology, software, validation, writing—original draft, writing—review and editing; S.B.: formal analysis, investigation, validation; S.L.J.: formal analysis, investigation, resources; A.M.: conceptualization, funding acquisition, project administration, supervision, validation, writing—original draft, writing—review and editing; M.K.: conceptualization, funding acquisition, methodology, project administration, resources, software, supervision, validation, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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