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Raman and fluorescence microscopy sensing energy-transducing and energy-storing structures in microalgae

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ABSTRACT

Chlorophylls and carotenoids are core components of photosynthetic energy transduction in algal cells, whereas starch, lipids, and polyphosphates represent energy reserves. All these biomolecules exhibit characteristic molecular vibrations that were sensed and localized in individual cells of *Desmodesmus quadricauda* and *Chlorella vulgaris* by confocal Raman microscopy. In the same cells, fluorescence of chlorophylls and of lipid bodies stained with Nile Red was mapped using the same instrument. In the first of the three consecutive scans, a low-power and short-exposure excitation by a green 532 nm laser was used to map chlorophyll fluorescence. In the second scan, the full power of a 785 nm laser was used for Raman mapping of carotenoids. The third scan was performed again with the 532 nm laser to map non-fluorescent biomolecules such as lipids, proteins, starch, and polyphosphates. Before the third scan, chlorophyll fluorescence was suppressed by photobleaching a wide area using a strongly defocused 532 nm laser beam. Raman microscopy was used for the first time to localize polyphosphate granules within a single algal cell in the context of other relevant bio-energetic structures. The new information represents an opportunity for algal cell phenotyping.

1. Introduction

Phytoplankton are responsible for net photosynthesis of the same magnitude as terrestrial plants [1]. It is on this enormous scale that microalgae and other photosynthetic microorganisms have used sunlight since early in the evolution to produce energy-rich biochemical compounds from CO_2, H_2O and inorganic nutrients [2]. This process remains a vital energetic driver of the entire biosphere, including humankind, for which it serves as an origin of food, feed, and multiple biomaterials and ecological services [3]. Microalgae also significantly contributed to the petroleum reserves that fuel modern transportation and provide essential raw materials for industry [4].

Eukaryotic microalgae perform photosynthetic energy transduction in chloroplasts, specialized organelles with thylakoid membranes [5]. Thylakoids contain chlorophyll and carotenoid pigment molecules that serve to capture photosynthetically active radiation, providing energy to oxidize H_2O to O_2 and to reduce CO_2. Reserves of free energy that are accumulated in this way are stored in the form of starch, lipids, and polyphosphates.

Analytical methods such as mass spectroscopy [6], gas, and high-pressure liquid chromatography or nuclear magnetic resonance [7] are used to determine molecular structure and abundance of pigments, starch, lipids, and polyphosphates in algal biomass, but are limited in localizing these biomolecules in individual cells. Mapping of single cells is currently mostly done by the optical microimaging of autofluorescence and the emission of compound-specific fluorescent labels or by element-sensitive scanning electron microscopy [8]. These methods are typically used selectively to identify only one cellular structure.

Significantly, more demanding studies of energy transduction and energy storage in microalgae require rapid and simultaneous imaging of pigments and energy-storing molecules in a single cell. Confocal Raman microscopy is a contactless, non-invasive and often non-destructive method suitable for discrimination between cellular structures of contrasting chemical compositions [9]. The method combines advantages of Raman spectroscopy [10], a scattering variant of vibrational spectroscopy, with confocal optical microscopy. Photons that are inelastically scattered on molecular vibrations provide valuable information about molecular structures, conformations, interactions, and the local environment of the molecules in focus, with a spatial resolution of a few μm^3. Raman microanalysis can be performed *in situ*

Abbreviations: CCD, charge-coupled device; SVD, singular value decomposition.

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and does not require staining of the specimen. When an appropriate experimental setup is used, Raman microspectroscopy can be used inside living cells. This was shown for the first time on the yeast *Schizosaccharomyces pombe* [11]. Although the Raman spectrum of each chemical species bears a wealth of structural information, it can also be taken simply as a spectral signature, a specific fingerprint of the given chemical compound, and used for its identification and spatial localization. Due to the linear relationship between Raman intensity and molecular concentration, Raman spectroscopy is particularly suited for quantitative analysis. If confocal Raman spectra are scanned point-by-point over the specimen, 2D or 3D Raman images can be constructed to visualize the chemical composition of the scanned object.

Due to recent revolutionary advances in instrument technology and signal/image-processing [9,10], confocal Raman microscopy and imaging are increasingly being applied to biological samples including single cells [12]. The application of Raman microscopy and imaging to photosynthetic organisms lags behind research of other biological systems in which detection of Raman scattering is not hampered by the strong autofluorescence of pigments [9]. Nevertheless, the technique was used soon after construction of the first Raman microscope [13] to investigate hydrocarbons in *Botryococcus braunii* [14] and for an *in situ* study of carotenoids in a single cell of *Pyrocystis lunula* [15]. The limitation of the technique caused by strong autofluorescence of photosynthetic pigments remains a major hurdle because the spontaneous Raman scattering of the Stokes type can be much smaller than the fluorescence background. New opportunities for reducing this problem [16–19] are offered by non-linear coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS) that detect signals outside of the fluorescence emission range and increase Raman scattering signals, respectively. In spite of the progress in these emerging techniques, conventional Raman microscopy remains an attractive option because of its robustness, affordable cost, and because it requires only simple operator training. In particular, conventional Raman microscopy remains widely used for the robust and relatively inexpensive localization of pigments and lipids (for reviews see [20,21]). Raman bands of carotenoids are relatively easy to detect because they are resonantly enhanced in the visible part of the spectrum, typically by 488, 514.5, and 532 nm laser excitations, as well as in the near infrared range, such as the frequently used 785 nm laser line [22]. Similarly, a pre-resonance enhancement allows detection of chlorophylls by Raman scattering of the near infrared 785 nm excitation [22]. Consequently, Raman microscopy of pigments was successfully exploited, for example, to investigate β-carotene and lipid accumulation in nitrogen-starved *Dunaliella tertiolecta* [23]. Co-registration of the resonance Raman signal from lipid-solubilized carotenoids, along with endogenous autofluorescence of chlorophylls that visualizes chloroplasts, was recently suggested as a label-free, diffraction-limited method to localize lipid bodies and to quantify the lipid yield on a single-cell basis in various microalgal strains instead of conventional staining by Nile Red [24]. The current state of Raman spectroscopy of microbial pigments with more examples can be found in a recent review [25].

Another opportunity is offered by specific molecular vibrations that are sensitive to the number of double bonds in fatty acid chains of lipids [26]. This opportunity was exploited to determine the saturation degree (iodine number) of neutral lipids in living algal cells [27]. Wider perspectives of the conventional Raman approach in algal lipidomics were further explored in [28]. Recently, time-gated CARS microscopy was used for the quantitative monitoring of lipid content and fluidity at the subcellular level in the living microalgae *Phaeodactylum tricornutum*, probing the inherent molecular vibrations of lipids [16]. Both spontaneous Raman and CARS spectra of triacylglycerols have been used for imaging lipid droplets in the model green alga *Coccomyxa* under nitrogen depletion [18]. Accumulation of triacylglycerol lipids in nitrogen-starved algal cells was studied by conventional Raman microscopy of whole cells in Huang et al. [29] and Wang et al. [30]. Another type of lipid, triterpenes of *B. braunii*, were analyzed in Weiss et al. [31]. Raman spectroscopy was also used to quantify macroscopic samples of algal biomass [32].

Starch serves in most microalgae as the primary, easy-to-mobilize energy storage. The capacity of Raman microspectroscopy to quantify cellular starch was recently demonstrated in *Chlamydomonas reinhardtii* and in *Chlorella pyrenoidosa* [33] and was suggested as an attractive alternative to conventional analytical methods used in research and industry. In an interesting application, the algal organelle, the pyrenoid, which is surrounded by starch granules, was identified by Raman microscopy as a carbonaceous kerogen in petrified Precambrian microorganisms [34].

Polyphosphates are still largely enigmatic polymers that played a significant role in biological evolution [35]. Quantification and localization of polyphosphates within a single living cell remain a significant challenge that limits a better understanding of these vital molecules [36]. Confocal Raman microscopy was used for an *in vivo* study of polyphosphate accumulation in vacuoles of the yeast *Candida albicans* [37]. The potential of Raman microscopy in polyphosphate monitoring was demonstrated with polyphosphate-accumulating bacteria that are used in enhanced phosphorus removal from waste water [38]. Microalgae are also considered to be of enormous practical potential in this process [61]. However, to the best of our knowledge, polyphosphates in microalgae have not yet been studied by Raman microscopy.

Here, we use conventional confocal Raman and fluorescence microscopy for simultaneous detection and visualization of pigments, lipids, starch, and polyphosphates in a single algal cell. For the first time, we demonstrate that polyphosphate bodies can be localized microscopically in an algal cell by Raman spectroscopy. The sharp Raman peak of polyphosphate at around 1158 cm^{-1} allows quantitation of the polymer content in the structural and functional contexts of starch, lipid, and pigment distributions.

2. Experimental section

2.1. Microalgae cultivation and sample preparation

The chlorococcal alga *Desmodesmus quadricauda*[1] was obtained from the Culture Collection of Autotrophic Organisms, Institute of Botany (CCALA, Czech Acad. Sci., Třeboň). This species is a well-studied model organism with an interesting cell cycle during which a significant accumulation of starch reserves occurs [41–43]. Its cells are also large enough to allow good resolution of different cellular compartments. *D. quadricauda* was cultivated in a glass tube with air bubbling as the sole source of carbon and in a nutrient medium based on the chemical composition of microalgal biomass as in Doucha and Lívanský [44], except for nitrate as a source of nitrogen. The glass tubes with the algal suspension were immersed in a thermostated waterbath (30 °C) and exposed constantly to 200 μmol(photons) m^{-2} s^{-1} from a panel of warm-white, light-emitting diodes.

Chlorella vulgaris, strain CCALA 256, was also obtained from the culture collection in Třeboň and cultivated in a plastic V-bag photobioreactor (NOVAgreen, Vechta-Langförden, Germany) in the medium prepared according to Doucha and Lívanský [44] with urea as a source of nitrogen. This species was selected for the Raman experiments because it is used in the Forschungszentrum Jülich for mass production of algal neutral lipids.

[1] The culture collection also describes the earlier taxonomic classification *Scenedesmus quadricauda* (Turpin) Brébisson, (strain Greifswald) under which the organism was identified in numerous publications. The genus *Desmodesmus* (Chlorophyta, Chlorococcales, Scenedesmaceae) was separated from *Scenedesmus* by An et al. in 1999 [39]. A later phylogenetic analysis based on plastid genomics has further distinguished *D. quadricauda* from the closely related *Desmodesmus communis* [40].