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Raman microspectroscopy of algal lipid bodies: β-carotene quantification

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Abstract Advanced optical instruments can serve for analysis and manipulation of individual living cells and their internal structures. We have used Raman microspectroscopic analysis for assessment of β-carotene concentration in algal lipid bodies (LBs) in vivo. Some algae contain β-carotene in high amounts in their LBs, including strains which are considered useful in biotechnology for lipid and pigment production. We have devised a simple method to measure the concentration of β-carotene in a mixture of algal storage lipids from the ratio of their Raman vibrations. This finding may allow fast acquisition of β-carotene concentration valuable, e.g., for Raman microspectroscopy assisted cell sorting for selection of the overproducing strains. Furthermore, we demonstrate that β-carotene concentration can be proportional to LB volume and light intensity during the cultivation. We combine optical manipulation and analysis on a microfluidic platform in order to achieve fast, effective, and non-invasive sorting based on the spectroscopic features of the individual living cells. The resultant apparatus could find its use in demanding biotechnological applications such as selection of rare natural mutants or artificially modified cells resulting from genetic manipulations.

Keywords Raman microspectroscopy · Microalgae · *Trachydiscus minutus* · Biotechnology · Carotenoids

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Introduction

We have been testing the performance of our Raman microspectroscopic setup with biotechnologically interesting samples of algae. Algal cultures are known to provide different valuable chemical compounds such as lipids for biofuel production (Schenk et al. 2008; Gouveia and Oliveira 2009), polyunsaturated fatty acids (PUFA; Carvalho and Malcata 2005; Guiheneuf et al. 2009), various carotenoids (Ben-Amotz et al. 1988; Garcia-Malea et al. 2005), proteins, chlorophyll, glycerol, and saccharides (Yeh et al. 2010; Fuentes et al. 2000; Becker 2007). Some algae contain β-carotene dissolved in high concentrations in the oil in their lipid bodies (LBs), including some strains which are considered useful in biotechnology for lipid and pigment production, e.g. *Dunaliella* sp. and *Nannochloropsis* sp. (Ben-Amotz and Avron 1983; Converti et al. 2009). We have focused on such β-carotene-rich lipid bodies (LBs) of the microalga *Trachydiscus minutus* (Xanthophyceae), which additionally contains high proportions of PUFA in its storage lipids (Rezanka et al. 2010; Samek et al. 2010). Algae were cultivated in a light intensity gradient to induce a gradual increase of β-carotene and storage lipid production. Algal suspension was embedded in agarose gel and the Raman spectra were recorded along with bright-field

(BF) microscopic images. We employed a calibration set of β-carotene solutions in a vegetable oil for absolute reference. From the obtained calibration curve, we have calculated the absolute concentrations of β-carotene in the LBs of the sampled algae. Furthermore, we compared the β-carotene-to-lipid Raman peak ratio with the light intensity during the cultivation and the LB volume calculated from the BF image analysis. The strong signal of β-carotene allows for spectra acquisition with short integration time. Raman-based non-invasive β-carotene concentration sensing may be useful for many advanced biotechnological applications such as the Raman assisted cell sorting.

Materials and methods

Experimental organism and cultivation conditions *Trachydiscus minutus* (Bourrelly) Ettl, CCALA # 838, was obtained from the Culture Collection of Autotrophic Organisms, CCALA (Institute of Botany, Academy of Sciences of the Czech Republic, Trebon). *T. minutus* was cultivated in 50-mL air-bubbled batch cultures at 22°C, 1× BBM medium (Bold 1949; Bischoff and Bold 1963) buffered to pH 7.5 with 15 mM HEPES and modified to contain 1 mg L^{-1} molybdenum (Setlik 1967). The experiment was conducted with 16 cultures arranged along a gradient of photosynthetically active radiation (PAR) flux density Q_p ranging 80–800 μmol photons m^{-2} s^{-1}. The cells were harvested after 10 days of cultivation and stored in dark at 4°C. The cultivation apparatus consisted of 60-mL Pyrex test tubes situated in an aquarium with circulating water. Lighting was supplied with white LED panel (PSI, Drasov, Czech Republic). Aeration was effected with an aquarium air pump with a manifold splitting the air supply to the individual tubes. The light intensity was measured with the Li-Cor photon flux meter LI-250 (Licor Instruments, USA) inside the empty aquarium in front of the test tube posts.

Raman microspectroscopy For in vivo microspectroscopic experiments with spatially immobilized algal cells, 2–4% *w/v* solution of agarose (Sigma, type XI: low gelling temperature) in deionized water was tempered to 40°C and mixed by pipetting with 20% *v/v* of algal suspension directly on a microscope cover slip. The resultant drop was covered with one more cover slip as fast as possible to aid heat dissipation. Raman microspectroscopic experiments with living algal cells were carried out using a homebuilt experimental system based on a custom-made inverted microscope frame. In order to extract quantitative information from the experimentally obtained spectral data, we adopted the rolling circle filter (RCF) technique for background removal (Brandt et al. 2006). Detailed description of the apparatus and the processing of the spectra can be found in literature (Samek et al. 2010). In the Raman microspectroscopic setup, a CCD camera (piA1600, Basler AG, Germany) coupled with an IR-optimized water-immersion ×60, NA 1.20 UPLSAPO objective (Olympus corp., Japan), was used for orientation in the sample and to capture the images of the spectrographed cell. Images were processed in ImageJ (ver. 1.43u, NIH USA public domain sw.), and the diameters of the lipid bodies were measured manually. Volume (V_{LB}) was calculated based on a spherical approximation of the LB shape. The Raman laser beam was positioned in the center of the field of view and focused along with the focal plane of the microscope. Individual agar-embedded cells were placed with their lipid body into the center and then finely steered to reach the maximal intensity of the spectrum. In total, five to seven lipid bodies were spectrographed for each experimental culture. All LB measurements had integration time 10 s at 40-mW laser power in the objective rear aperture, excitation wavelength was 785 nm. Artifacts resulting from imprecise targeting of the excitation laser were prevented by manual elimination of the spectra with excessive fluorescence or Raman signatures atypical for lipid body. In order to calibrate the measurements, pure β-carotene (Sigma–Aldrich Corp., USA) was dissolved in commercial extra virgin olive oil to a concentration 10 g L^{-1}. An aliquot of the solution was sequentially diluted with oil, resulting in six standard solutions containing 0.007–10.0 g L^{-1} of β-carotene, which were spectrographed with integration time 0.1 s at 150-mW laser power, 785 nm. Characteristic Raman peaks of β-carotene and lipids were selected and their magnitudes were used for calculation of various simple and combined ratios in order to obtain the best correlation with the β-carotene concentration in the oil solutions. The most suitable ratio was subsequently used for calculation of a calibration curve, and the β-carotene concentration in the algal LBs. Each calibration point was averaged from two to three measurements, except 2.5 g L^{-1} represented by a single value. The examined Raman features are summarized in Table 1. In order to prevent miscalculations due to the minor drift of the Raman peaks, we evaluated the maximal intensity of the peak from a 20 cm^{-1} interval centered on the nominal Raman shift value.

Table 1 Raman peaks of β-carotene and lipids

Raman peak	Symbol	Raman shift
β-carotene C–C stretching	ν_A	1,157 cm^{-1}
β-carotene C=C stretching	ν_B	1,525 cm^{-1}
Lipid CH_2 scissoring	ν_{CH2}	1,445 cm^{-1}
Lipid C–C stretching	ν_{C-C}	1,656 cm^{-1}

Results

BF microscopy was used to examine the ultrastructural features of the studied algal cells. Cells were spherical to discoidal in shape. Dominant ultrastructural features were one or more brown-red spherical LBs and green oval chromatophores, which were well formed and uniformly distributed in low illumination cultured cells. At increasing PAR intensity, the abundance and volume of LBs increased, while the chromatophores became gradually more misshaped, discolored, reduced in number, and restricted to a smaller area, exposing the translucent vacuole. The color of the culture shifted from green to yellow-brown as the PAR intensity increased, indicating the progressive buildup of β-carotene. In this experiment, PAR flux density over 500 μmol photons m^{-2} s^{-1} induced substantial enlargement of the lipid bodies. Selected cells of *T. minutus* cultivated in low, medium and high illumination and the spatially resolved Raman spectra of their intracellular lipid bodies are depicted in Fig. 1.

Intensive β-carotene Raman spectral signatures were detected, while the lipid signals were comparatively weak. In general, the Raman signal of β-carotene was increasingly dominant in cells with intensifying PAR illumination, and particularly in LBs of large volume. We have employed a set of β-carotene solutions to calibrate the Raman measurements of LBs on the absolute concentration scale, see Fig. 2. The

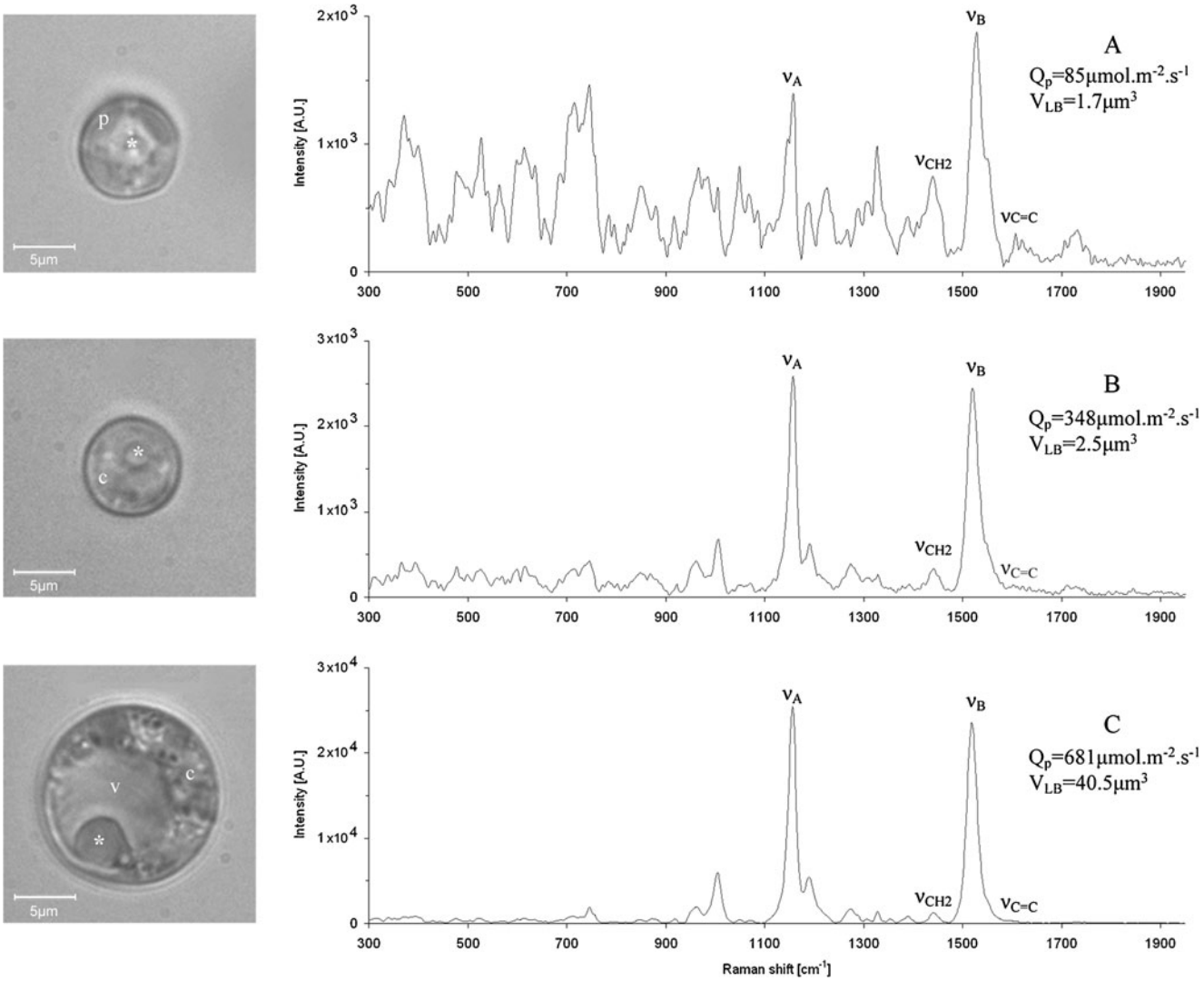

Fig. 1 Raman spectra of *T. minutus* lipid bodies. The images on the *left side* show the spectrographed cells. Lipid bodies targeted by the Raman laser are indicated by the *white asterisks* (*). Lipid body volume V_{LB} increased with PAR intensity Q_p. Oval chromatophores (*p*), well formed and uniformly distributed in low PAR illumination, became gradually more misshaped, reduced in number, and restricted to a smaller area of cytoplasm (*c*), exposing the translucent vacuole (*v*) with increasing PAR intensity. Raman spectra of the respective LBs on the right side show the increasing magnitude of β-carotene peaks at 1,157 cm^{-1} (ν_A) and 1,525 cm^{-1} (ν_B), while the spectral features of other cellular components including lipids at 1,445 cm^{-1} (ν_{CH2}) and 1,656 cm^{-1} ($\nu_{C=C}$) present comparatively small changes in their absolute magnitude. The ν_A/ν_B ratio changes considerably with the LB volume and cultivation conditions

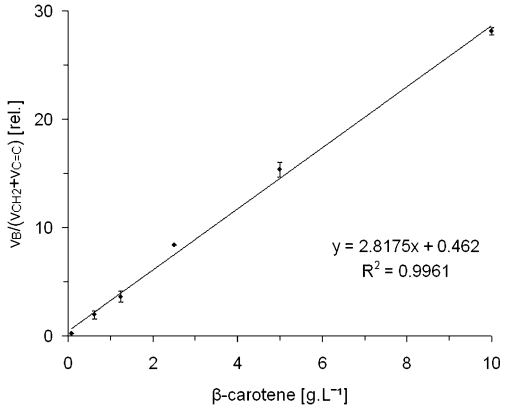

Fig. 2 Calibration of Raman signal ratio with β-carotene solutions. β-carotene solutions were spectrographed and the calculated ratios $\nu_B/(\nu_{CH2}+\nu_{C=C})$ were correlated with the known β-carotene concentrations, yielding the coefficient of determination R^2=0.9961. The absolute concentration of β-carotene was calculated from the Raman signal ratio using linear regression to obtain the equation

ratio of Raman signals used for β-carotene quantification was $\nu_B/(\nu_{CH2}+\nu_{C=C})$. The correlation of this Raman ratio with β-carotene concentration reached coefficient of determination R^2=0.9961. The calibration equation (y=2.8175x+0.462) was derived from a linear fit and it was used to calculate the absolute β-carotene concentrations in the examined lipid bodies. The incorporation of both saturated and unsaturated lipid indicators may improve the measurement reliability in variably saturated lipid mixtures.

The maximal detected β-carotene concentration in the storage lipids of *T. minutus* was around 8 g L^{-1}. We have attempted to correlate the calculated β-carotene concentrations in individual LBs to their respective volumes (V_{LB}), see Fig. 3. In general, the experimental cultures displayed light stimulated LB volume enlargement in direct proportion with the increasing β-carotene concentration. The variability between the individual specimens was high, considerable proportion of the examined cells was showing either relative overproduction or underproduction of β-carotene per LB volume.

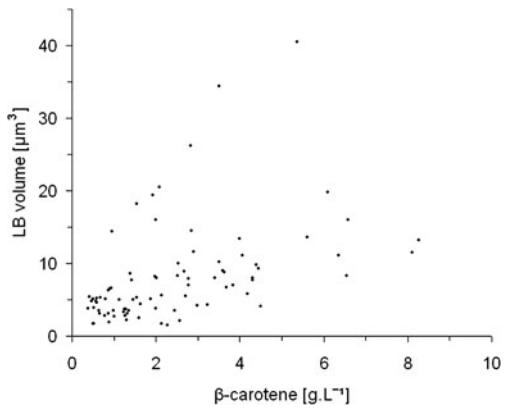

Fig. 3 Proportionality of β-carotene concentration and LB volume. The concentration of β-carotene was derived from the Raman signal ratio and plotted against the volume of the lipid body V_{LB}. Cells displayed PAR stimulated LB volume increase which was loosely proportional with the β-carotene accumulation. The cells with abnormal quantities of β-carotene can be clearly distinguished. In total, 85 measurements were conducted

The β-carotene concentration and V_{LB} were found to be proportional in certain extent to the PAR intensity, see Fig. 4. PAR intensity over 500 µmol photons m^{-2} s^{-1} induced lipid accumulation, on average the LBs reached around 2.6-fold increase in volume compared to the low-light variants. The detected concentration of β-carotene followed a similar trend, reaching in average 2.8-fold increase. The described simple Raman-assisted method of β-carotene concentration estimation could find its use in the non-invasive detection of β-carotene overproducing cells. Moreover, this technique could aid fast semi-quantitative discrimination between small (e.g. V_{LB}<10 µm³) and large (e.g. V_{LB}>10 µm³) lipid bodies. Thanks to high intensity of β-carotene spectral signatures, the detection of very large or β-carotene rich lipid bodies for the purpose of Raman-assisted sorting could be achieved with integration times in order of milliseconds with laser power in 100 mW range at 785-nm wavelength. Preliminary results of β-carotene concentration determination from HPLC of pigments and GC-MS of fatty acids were in agreement with the Raman approach.

Discussion

We demonstrated the potential of Raman microspectroscopy for simple, non-invasive, spatially resolved characterization of the parameters of selected intracellular compartments in individual living algal cells. High spatial selectivity was

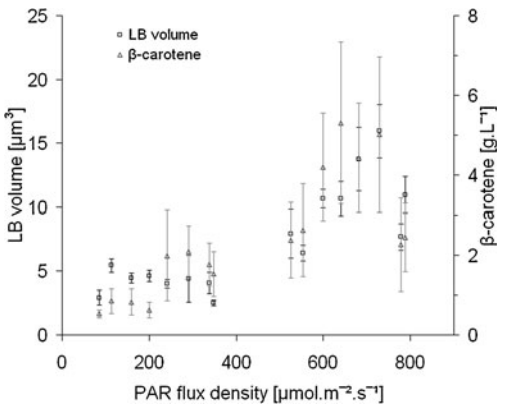

Fig. 4 Proportionality of β-carotene concentration and LB volume to PAR intensity. The lipid body volume V_{LB} and the Raman-derived β-carotene concentration were averaged within the 16 individual experimental variants and plotted against the PAR flux density. Q_p> 500 µmol photons m^{-2} s^{-1} induced intensive lipid accumulation. On average the LBs reached around twofold increase in volume compared to the low-light variants. The mismatch between V_{LB} and β-carotene values can be attributed mainly to the variable PAR influence. *Error bars*: 2 standard deviations

achieved with tightly focused excitation beam from high NA objective. In particular, we have examined the lipid storage bodies. We found a Raman-based method to measure the β-carotene concentration and to estimate the LB volume, which are important parameters for selection of the production strains for biotechnology and industry. The link between β-carotene and lipid deposition was described in literature (Rabbani et al. 1998) and it is species dependent. Induction of β-carotene and storage lipid synthesis by increased PAR intensity was employed in several published experiments (Lers et al. 1990; Lamers et al. 2010). We have calculated the relative concentrations of β-carotene in the storage lipids from the Raman spectral peak ratios. We have used the ν_B peak due to its proximity to ν_{CH2} and $\nu_{C=C}$, reducing the calculation susceptibility to the artifacts from eventual background residues and the dependence on the slope of the Raman setup sensitivity. The absolute concentrations were determined from the calibration based on β-carotene oil solutions. The maximal concentration of β-carotene detected in LBs reached over 8 g L^{-1}, while the oil solution was saturated at 10 g L^{-1}. This suggests that LBs might contain nearly saturated β-carotene solution. The analysis of the image of the spectrographed cell allowed us to calculate the volume of algal lipid bodies in absolute units, to be used as a reference measurement. The overall sensitivity of the Raman analysis could be increased by several orders of magnitude using the resonance effect taking place when the excitation wavelength matches the absorption maximum, around 490 nm for β-carotene (Parker et al. 1999). We have observed severalfold increase in sensitivity using pre-resonant excitation of β-carotene at 532 nm. This modification may allow simple and precise quantification of β-carotene concentration in optically trapped cells for high-throughput Raman-assisted sorting. Additionally, the use of carotenoids as a proxy of lipids has been researched in various spectroscopic settings, in order to achieve label-free, "green" lipid quantification (Su et al. 2008; Solovchenko et al. 2009; Solovchenko et al. 2011), while some research has been focused on artificial fluorescence probes, such as BODIPY® (Molecular Probes, USA), or Nile Red (Elsey et al. 2007; Huang et al. 2009). We believe that Raman microspectroscopy has the potential to become fast and versatile tool for applications in lipid and pigment biotechnology, engineering, and industry. The usefulness of Raman spectroscopic analysis especially when combined with optical trap was already demonstrated in experiments with algae and other microorganisms (Xie et al. 2004; Heraud et al. 2007; Huang et al. 2010; Wu et al. 2011; Samek et al. 2011). With combination of Raman spectroscopy, optical trapping, and microfluidic system, one can realize a high-throughput label-free and non-invasive automated cell sorting based on spectroscopic features of individual living cells, which could find its use in challenging biotechnological applications such as the selection of rare natural mutants or artificially modified cells resulting from genetic manipulations.

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