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Resolving carotenoid distribution in living cells of *Haematococcus pluvialis* with hyperspectral confocal Raman microscopy

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Carotenoids can be classified broadly in photosynthetic organisms as primary, or essential to photosynthetic processes and located in the chloroplast, or secondary, being unnecessary for photosynthesis and located in the cytoplasmic space, usually in lipid-rich globules. *Haematococcus pluvialis* is a freshwater alga, known to accumulate massive amount of the red secondary ketocarotenoid astaxanthin under stress conditions. Here, we use hyperspectral confocal Raman microscopy and multivariate analysis to extract multiple pure spectral components from a complex dataset containing resonance-enhanced Raman and fluorescence features from different cellular morphotypes *of H. pluvialis*. Carotenogenesis and the accumulation of astaxanthin in *H. pluvialis* is poorly understood. The high specificity and spatial resolution of Raman microscopy allows the opportunity to investigate these processes *in vivo.* Multivariate curve resolution (MCR) algorithms resolve spectral features representing pure spectral components and have a distinct advantage over simple band integration in that highly over-lapping features can be separately resolved, assuming their intensities vary across different pixels. Multiple spectral images of *H. pluvialis* cells were combined into a single dataset (225,739 spectra) and multivariate curve resolution algorithms are applied to minimize the sum of residuals *de novo*. The analysis yields a least-squares fit of > 99% for all spectral variance and 4 spectral components; Raman signatures for astaxanthin and beta-carotene, as well as cellular autofluorescence and chlorophyll emission. From the MCR analysis, we find astaxanthin in punctate and globular regions outside of the chloroplast and in association with some minor amounts of beta-carotene.

Chlorophyll was associated more dominantly with beta-carotene and was located in the chloroplast. The results demonstrate the broad potential of hyperspectral confocal Raman microscopy paired with multivariate analysis to resolve spectral signatures of highly similar chromophores in living cells.

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