SAND2014-15670PE

Identifying and Localizing Pigments in Living Cells

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Presented at the PARC All Hands Meeting
St. Louis, MO
June 17-18, 2014

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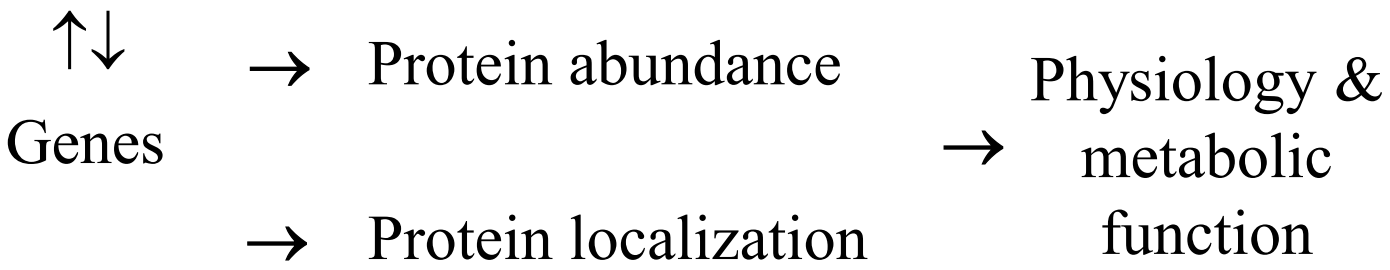
Pigment Localization is Dynamic

- Photosynthetic and metabolic activity is regulated in response to changing environmental parameters



- Traditional biochemical assays determine average parameters, but
  - Generally made on model species
  - No insight into stochastic response

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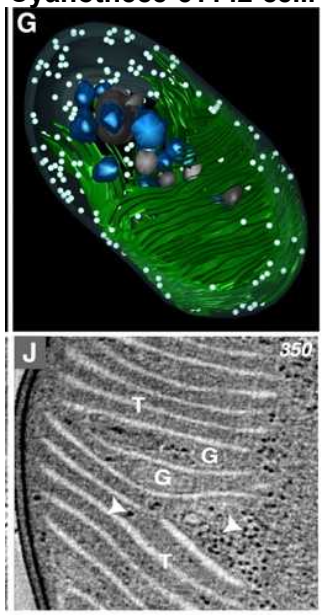
Single Cell Measurements

- Key information on populations
  - Screening for unique phenotypes
  - Population dynamics
- Subcellular resolution possible
- Exquisite spatial resolution offered by electron microscopy
  - Recent extensions to tomography

Tomographic reconstruction of a Cyanothece 51142 cell.



Liberton M et al. Plantphysiol 2011;155:1656-1666

But the need remains to probe pigment dynamics and the cellular level

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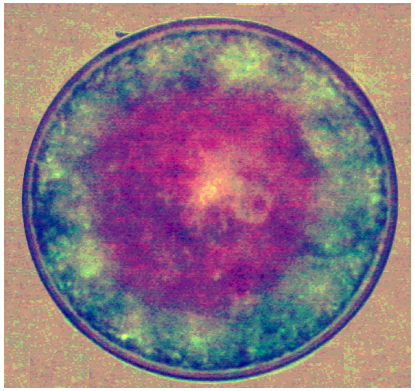
Motivation

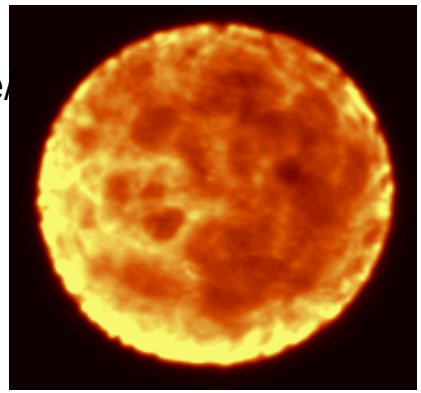
Spatially and Temporally Resolved Biochemical Information at the Cellular Level

Light Micrograph



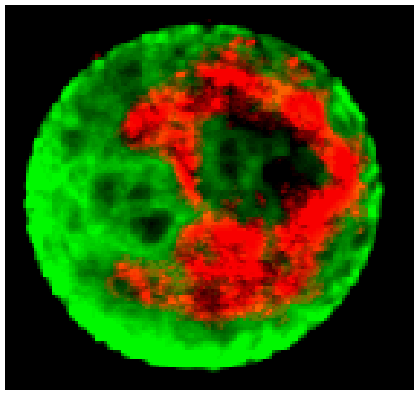
Confocal Fluorescence/ Raman Microscopy

Integrated Flour/Raman Image



Multivariate Curve Resolution

Chemical Image



Light Microscopy

Each pixel in the image is a combination of 3 (RGB) colors (morphology, refractive properties)

Spectral/Hyperspectral Imaging

Each pixel in the image is a spectrum relating to chemical and/or molecular structure within

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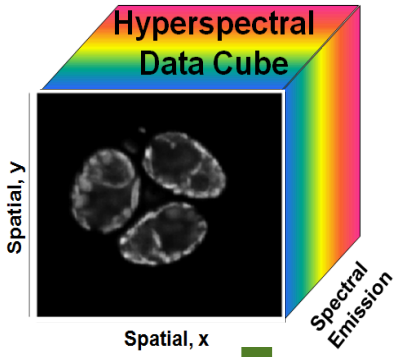
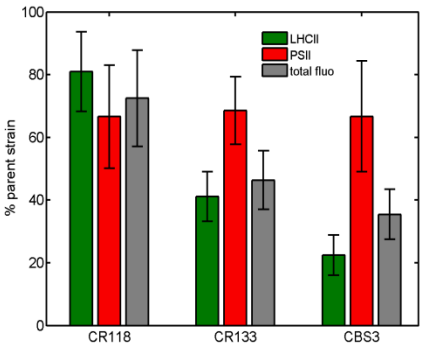
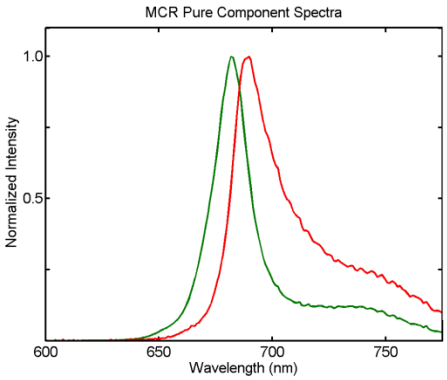
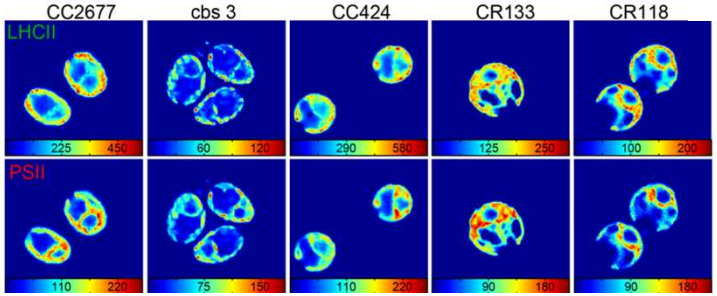
Hyperspectral Imaging of Single Cells

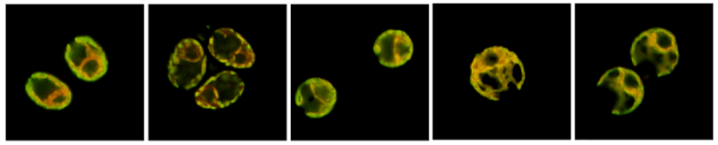
- *Identity of pigments*
- *Location, relative abundance*
- *Spatial relationship*
- *Single cell statistics*











C. reinhardtii antennae mutants, work in preparation for joint publication with the Sayre lab.

Outline

- Introduction to fluorescence and Raman microscopy
  - Principles & technology
  - Advantages for photosynthetic organisms
- Spectral image analysis
  - Multivariate techniques
  - Advanced strategies
- Highlights of current PARC Research in Timlin lab

- Summary & Future Directions

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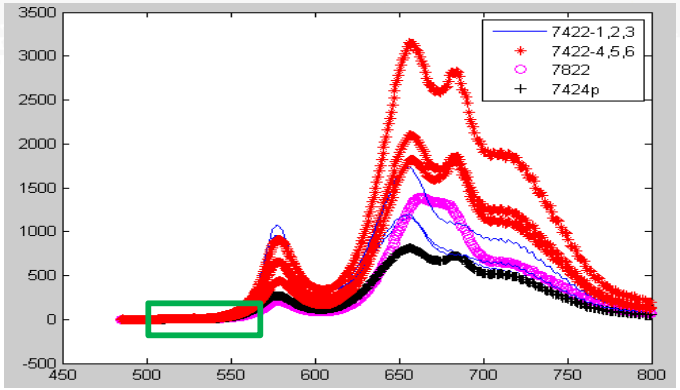
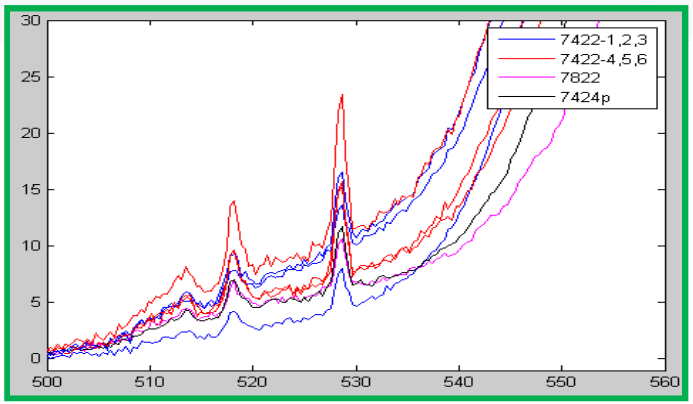
Fluorescence vs. Raman for Bioenergy Applications

Non-destructive, label-free, live-cell friendly, diffraction-limited resolution in 3D





Fluorescence Emission

- Emission from an excited state
- Excitation λ dependent
- Many important molecules have endogenous fluorescence
- Broad spectral features
- *Energy transfer system → high degree of spectral overlap, efficient excitation with a single laser*

Raman Scattering

- Scattering due to molecular vibrations
- Excitation λ independent
- Narrow spectral features, signature can be very specific
- Resonant vs. non-resonant
- *Carotenoids and lipids*

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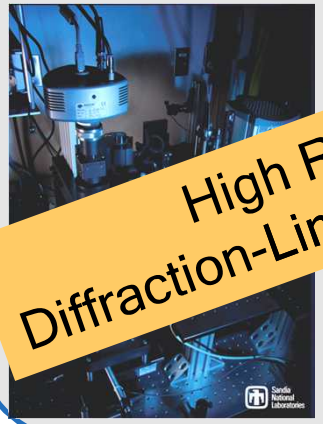
Technology Available

SNL's Hyperspectral Confocal Microsc



- 488 nm excitation
- 60x (1.4 N
- L
- 00-7900 cm^{-1}
- ectral resolution = 35 - 100 cm^{-1} (1-3 nm)
- Acquisition rate = ≤8300 spectra/s

Sinclair, et. al., Applied Optics, 45, 6283-6291 (2006).

High Read-out Rate, Diffraction-Limited Spatial Resolution

WiTec alpha300R

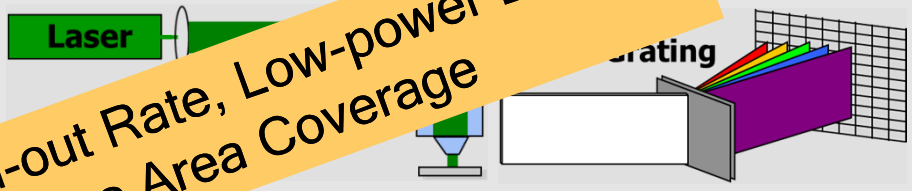
- 532 nm excitation
- 100x (0.9 NA) dry objectiv
- Lateral resolution =
- Axial resoluti
- Spec 700 cm^{-1}
- ution = 1 cm^{-1}
- ition rate = 100 spectra/s

http://www.witec.de/products/raman/alpha300-r/

Exquisite Spectral Resolution

SNL's Hyperspectra e Scanner

- 532 nm excitation
- 10x, 40x or 100x dry objective
- Lateral resolution = 1 µm
- Axial resolution = ~2-6 µm
- Spectral range = 500-1
- Spectral resolu
- Acquisition



Christensen & Morris, Applied Spectroscopy, 52, 1145-1147 (1998) & Sinclair, et. al., Applied Optics, 43, 2079-2089 (2004)

High Read-out Rate, Low-power Density Large Area Coverage

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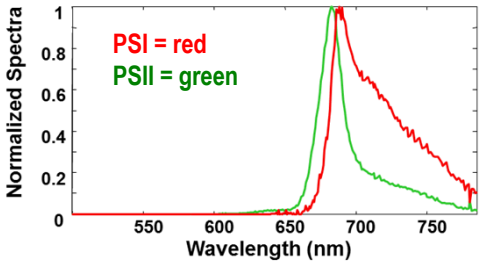
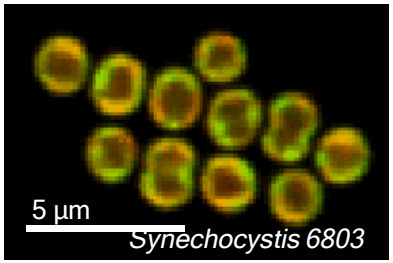
Examples of Chemical Imaging in Photosynthesis Research

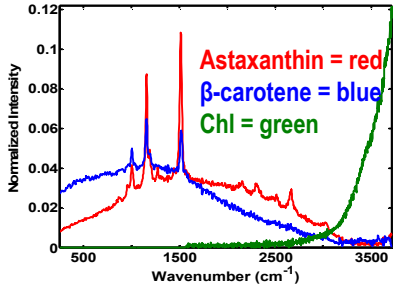
Hyperspectral Confocal Fluorescence Microscopy

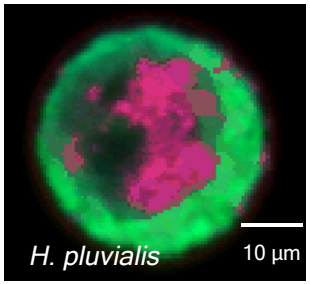




Subcellular localization, discrimination, and quantification of photosynthetic pigments

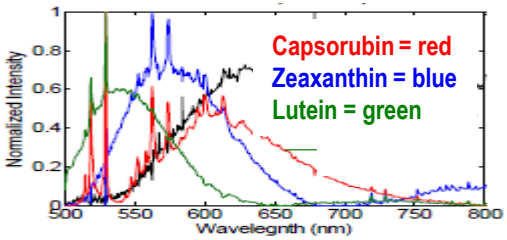
Combined Hyperspectral Confocal Raman & Fluorescence Microscopy

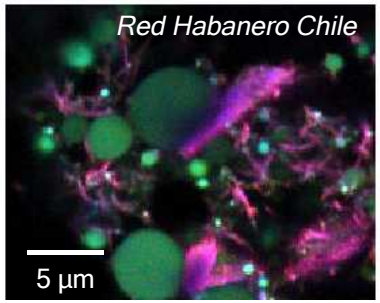




Subcellular localization, discrimination, and quantification of carotenoids and chlorophylls

Hyperspectral Confocal Raman Microscopy





Subcellular localization, discrimination, and quantification of carotenoid, lipids, and precursors

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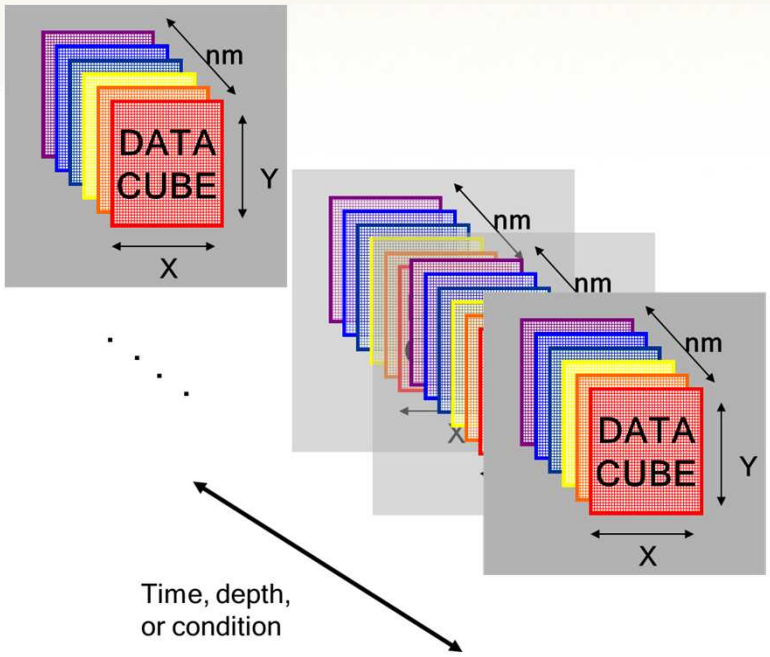
The Hyperspectral Data Cube



How do you get from hundreds of thousands of highly overlapped spectra to chemical information?

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Spectral Image Analysis Methods

Required knowledge

Univariate methods

→ Band integration, peak height, peak positions

→ Isolated bands, no spectral interference

Multivariate methods

- Unmixing methods
  - → CLS
  - → Least-squares prediction based
  - → *A priori* knowledge required
- Factor analysis methods
  - → PCA, SIMPLISMA, self modeling curve resolution/multivariate curve resolution
  - → Data defines
  - → No *a priori* knowledge of spectral shapes/pure pixels

Mathematical complexity, accuracy

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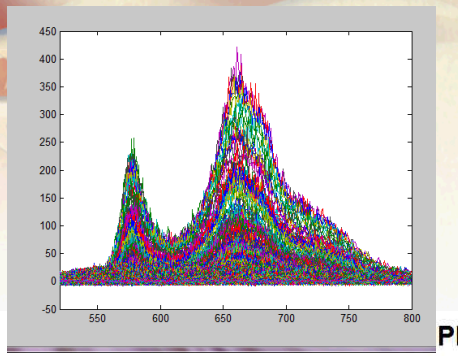
Multivariate Curve Resolution (MCR) Example



Hyperspectral Data Cube

Spatial, y

Spatial, x

Spectral Emission

Phycoerythrin

AllophycocyaninB

Carotenoid

Phycocyanin

Chl

How much is present? Where is it located?

What is fluorescing?

Preprocess

PCA

MCR

- Calibrate wavelength axis
- Remove cosmic spikes

- Weight data for Poisson noise
- Estimate # of components
- Initial guess spectral signatures

- Constrained alternating least squares analysis
- Linear additive model, $D = CS^T$

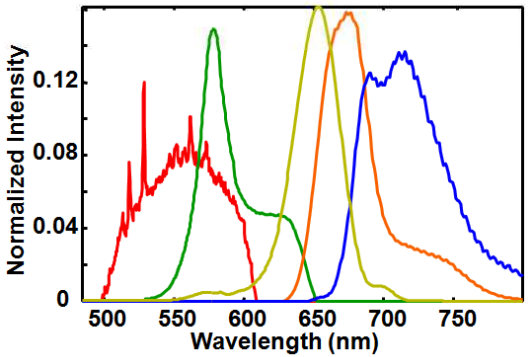


Figure 2. Mathematical isolation of independently varying chemical species is accomplished using a fast multivariate curve resolution algorithm with robust constraints. Example shown: hyperspectral imaging of *endogenous* pigments in the cyanobacterium *Cyanothece* sp. PCC 7822.

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Haaland, et al., Proc. SPIE, Vol. 4959, 55 (2003) ; Jones, et. al., J Chemom, 22:482-490 (2008); Jones, et. al., J Chemom, 117:149-158 (2012)

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Analysis: The Importance of Experimental Design

- Components that co-vary can not be isolated independent of one another
- Net analyte signal is more important that per pixel signal to noise
- Different models can highlight different aspects of a data set

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Analysis: Advanced Strategies

- Well characterized instrumentation
- Selective ROIs
- Composite data

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Current PARC Research

- Phycobilisome degradation under nitrogen starvation conditions
- Light/heat induced bleaching in Symbiodinium
  - Poster “Resolving highly overlapped pigment emissions in living *Symbiodinium* with hyperspectral Imaging and multivariate analysis”
- Pigment dynamics in response to light quality
- Carotenoid composition in avian retinas

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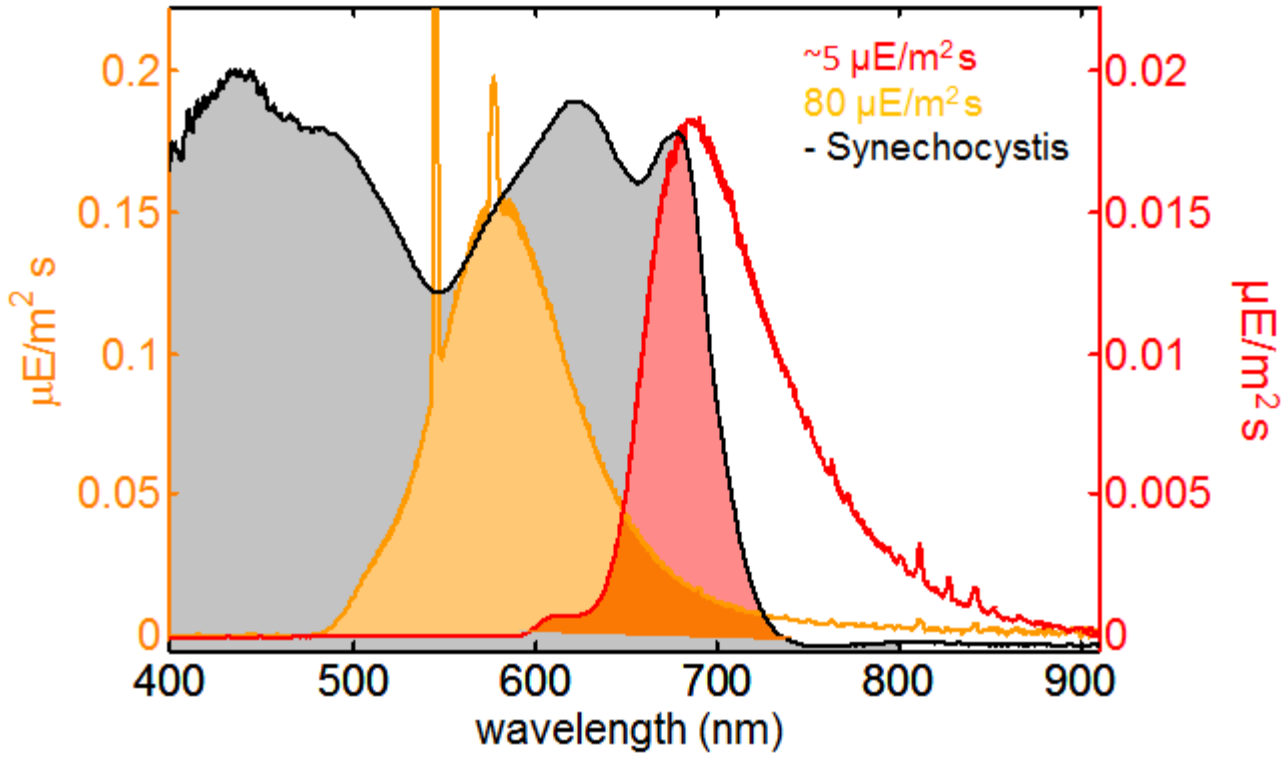
Global Pigment Dynamics in Response to Light Quality

- Cyanobacteria: *Acarychloris marina, Cyanothece, Spirulina platensis*
- Grown under red or yellow light



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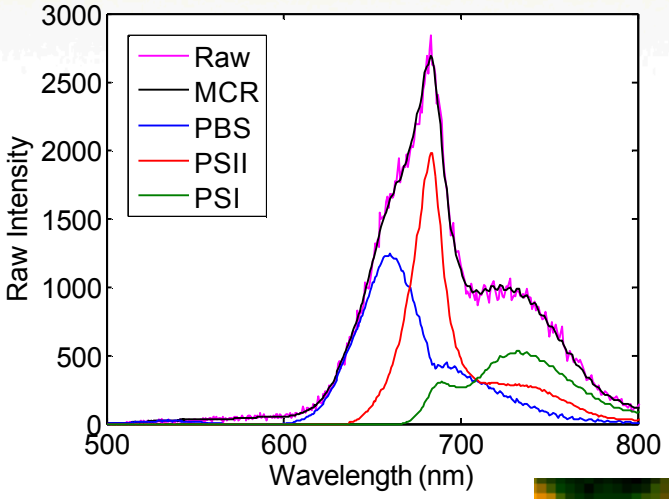
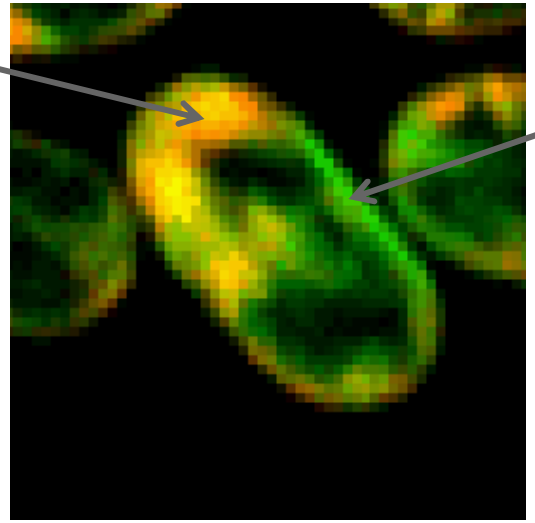
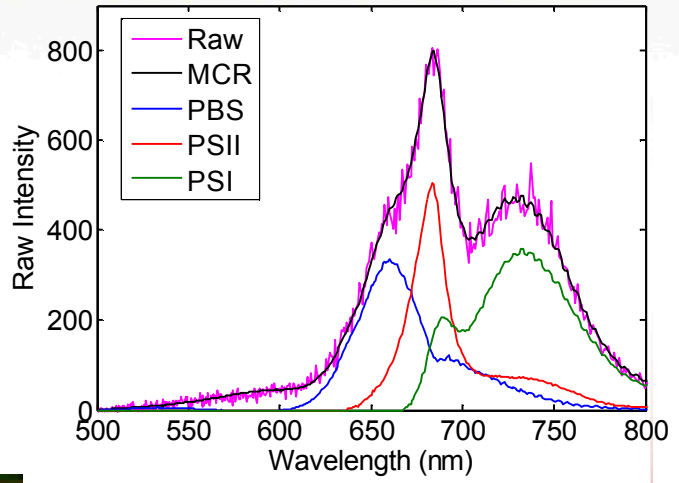
Cyanothece







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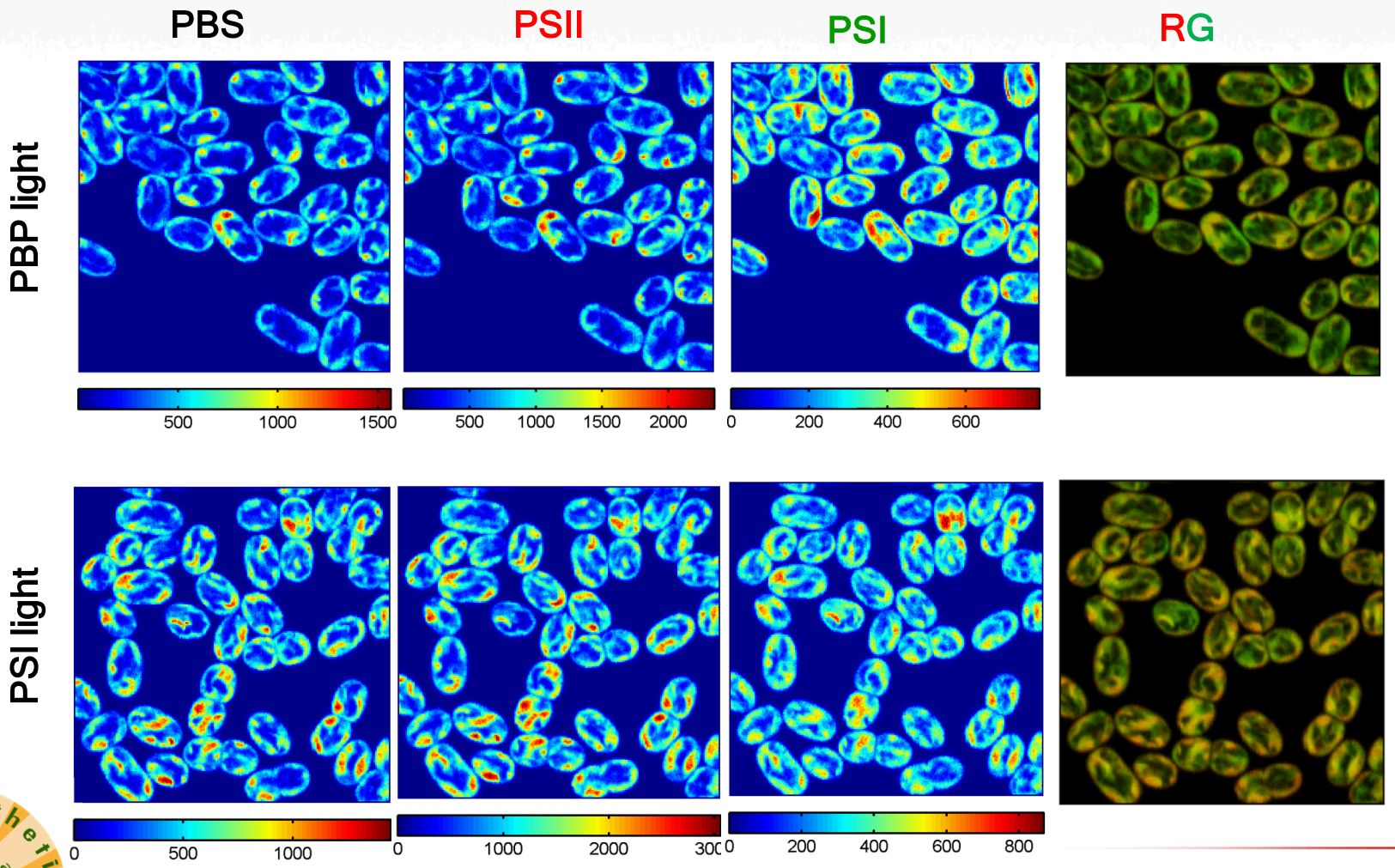
Cyanothece



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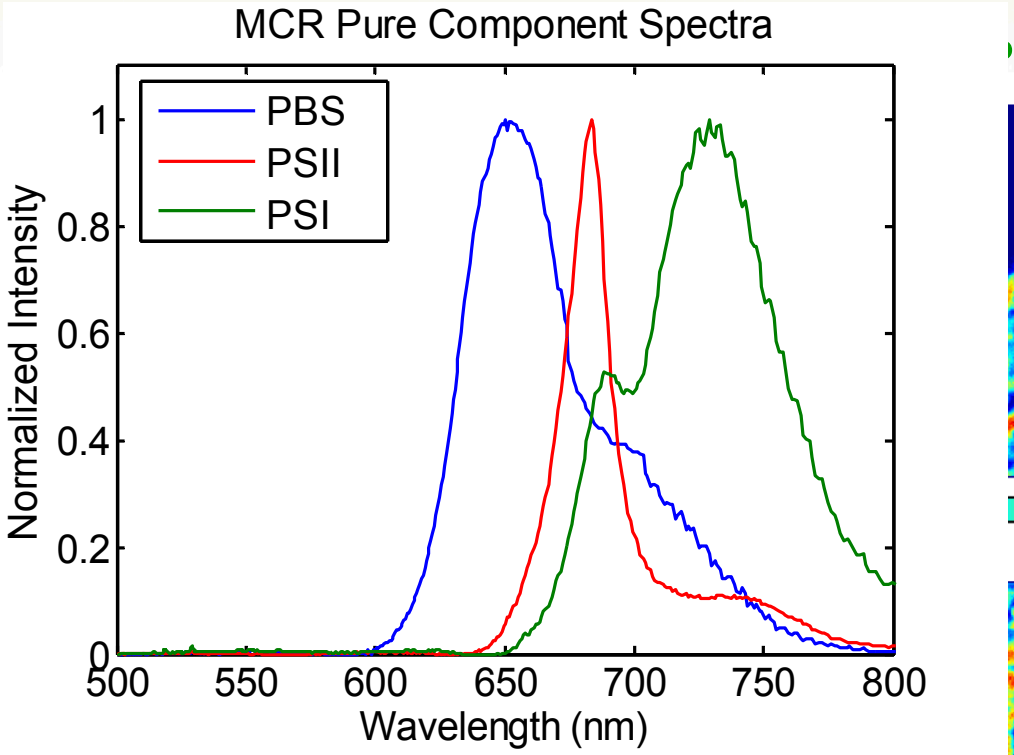
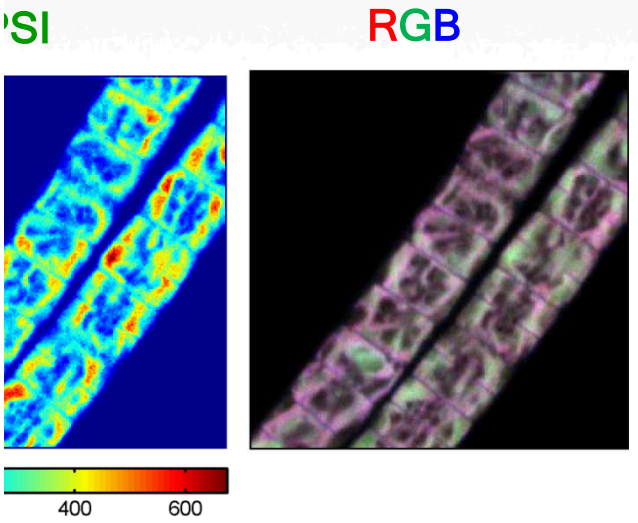
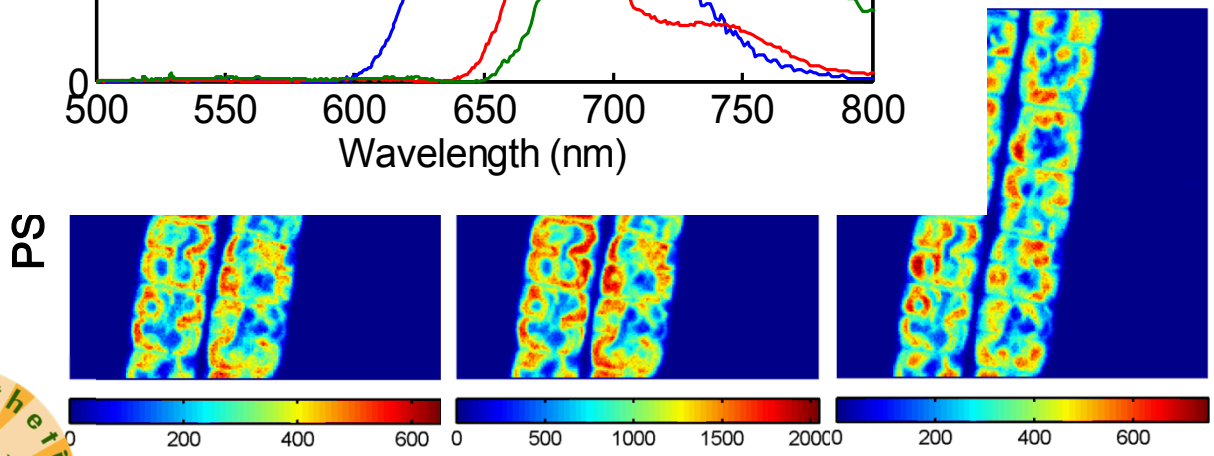
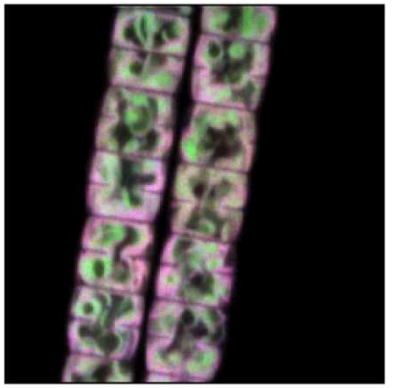
S. platensis









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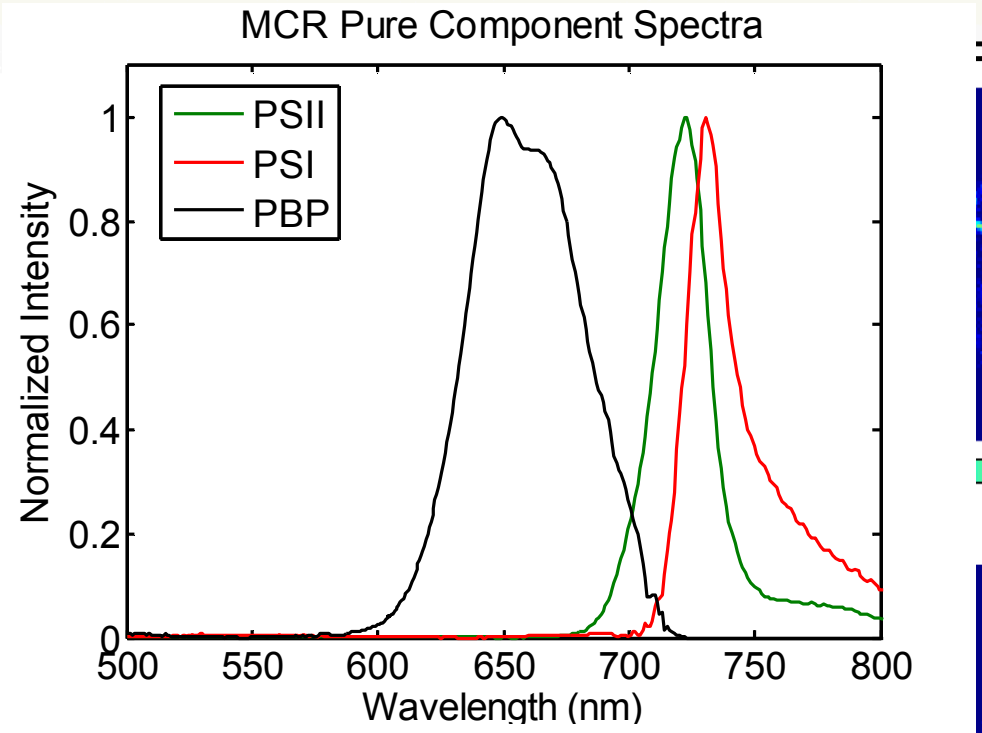
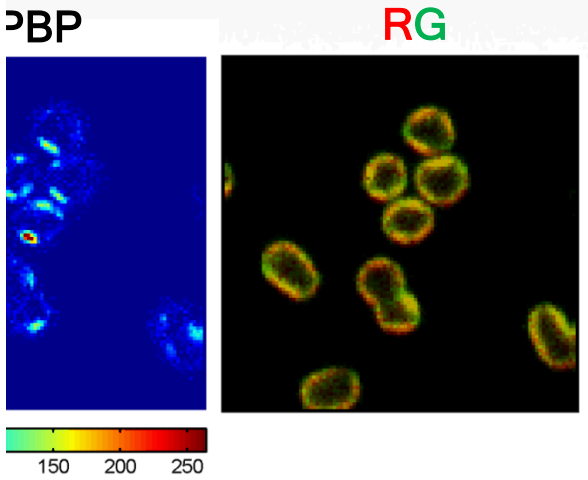
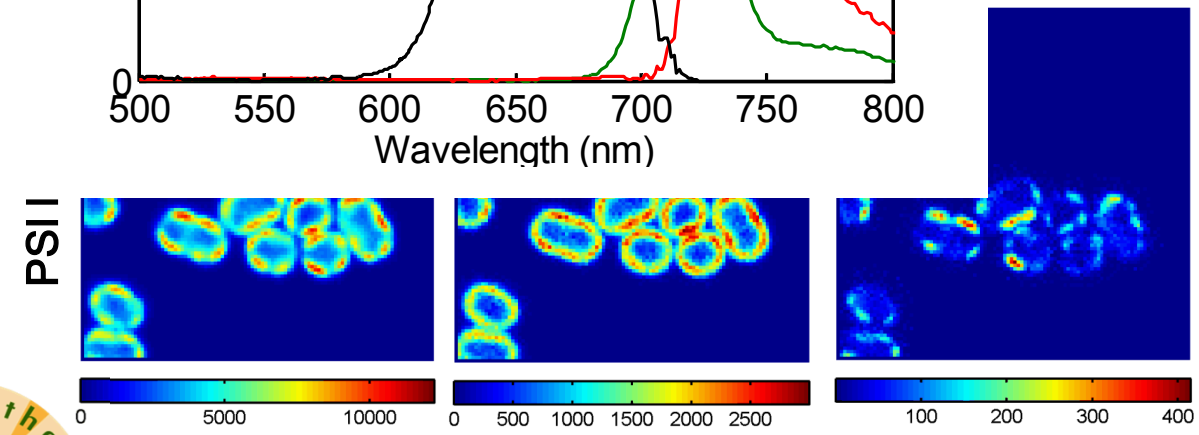
A. marina







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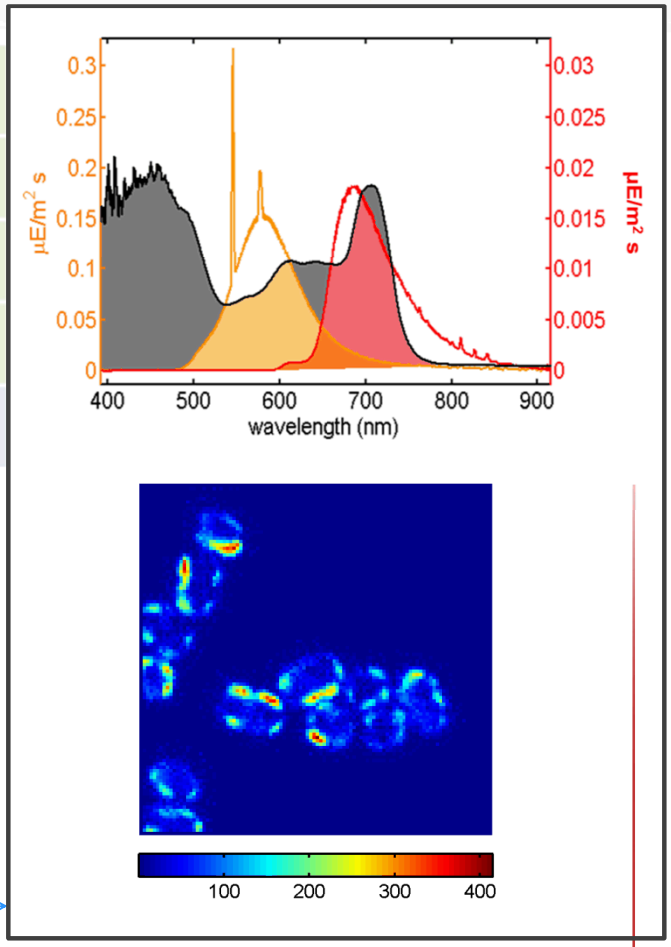
Differential Response Across Species

	A. marina	*Cyanothece*	*S. platensis*
ΔPBS	116%	18%	127%
ΔPSII	39%	52%	129%
ΔPSI	154%	-1%	31%

Change in abundance from PBS to PSI



- Far red light: disruption in the linear electron flow from PSII to PSI; organisms compensates by synthesizing more PSII and PBS to attempt to restore balance

- *A. marina*: long wavelength chlorophyll responds as if high light and low light

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Conclusions

- Global pigment dynamics can be probed in living cells with hyperspectral confocal fluorescence and Raman microscopy
- Open questions remain …
  - Functional implications: lower signal could be less coupling or less abundant, couple with biochemical assays
  - Real-time analysis
- On the horizon …
  - Excitation-emission spectral imaging
  - Hyperspectral imaging-based cell sorting

Future areas: high-throughput screening for phenotype, population dynamics in microbial communities, characterization of biohybrid, bio-inspired systems

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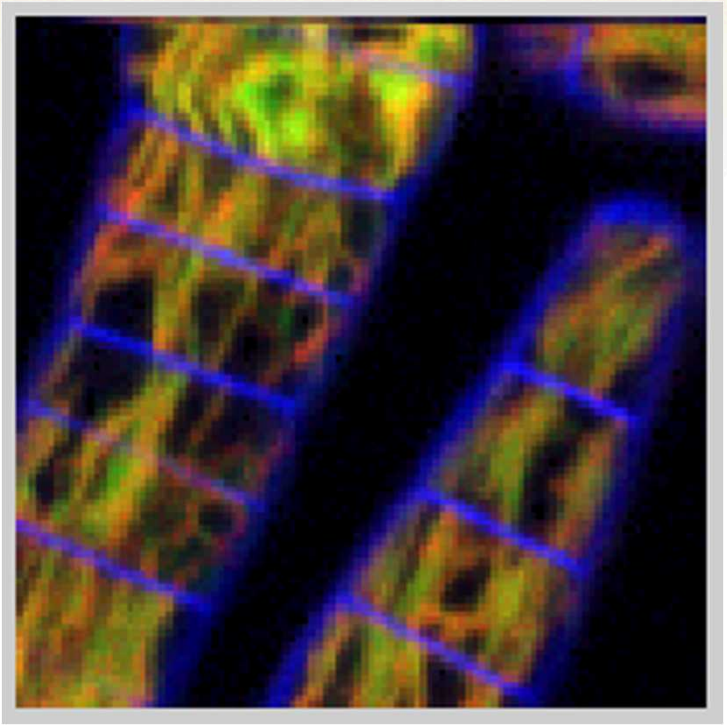
Acknowledgements

- Aaron Collins
- Kylea Parchert
- Anne Ruffing



- Sangeeta Negi
- Sayre Lab, NM Consortium

- Michelle Liberton
- Pakrasi Lab, Wash U

- James Kilcrease
- O'Connell Lab, NMSU

IMAGING/ANALYSIS SUPPORT

- Michael Sinclair
- Thomas Beechem
- Howland Jones
- David Haaland
- Michael Keenan
- Mark Van Benthem
- Omar Garcia
- Michelle Raymer

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Extras

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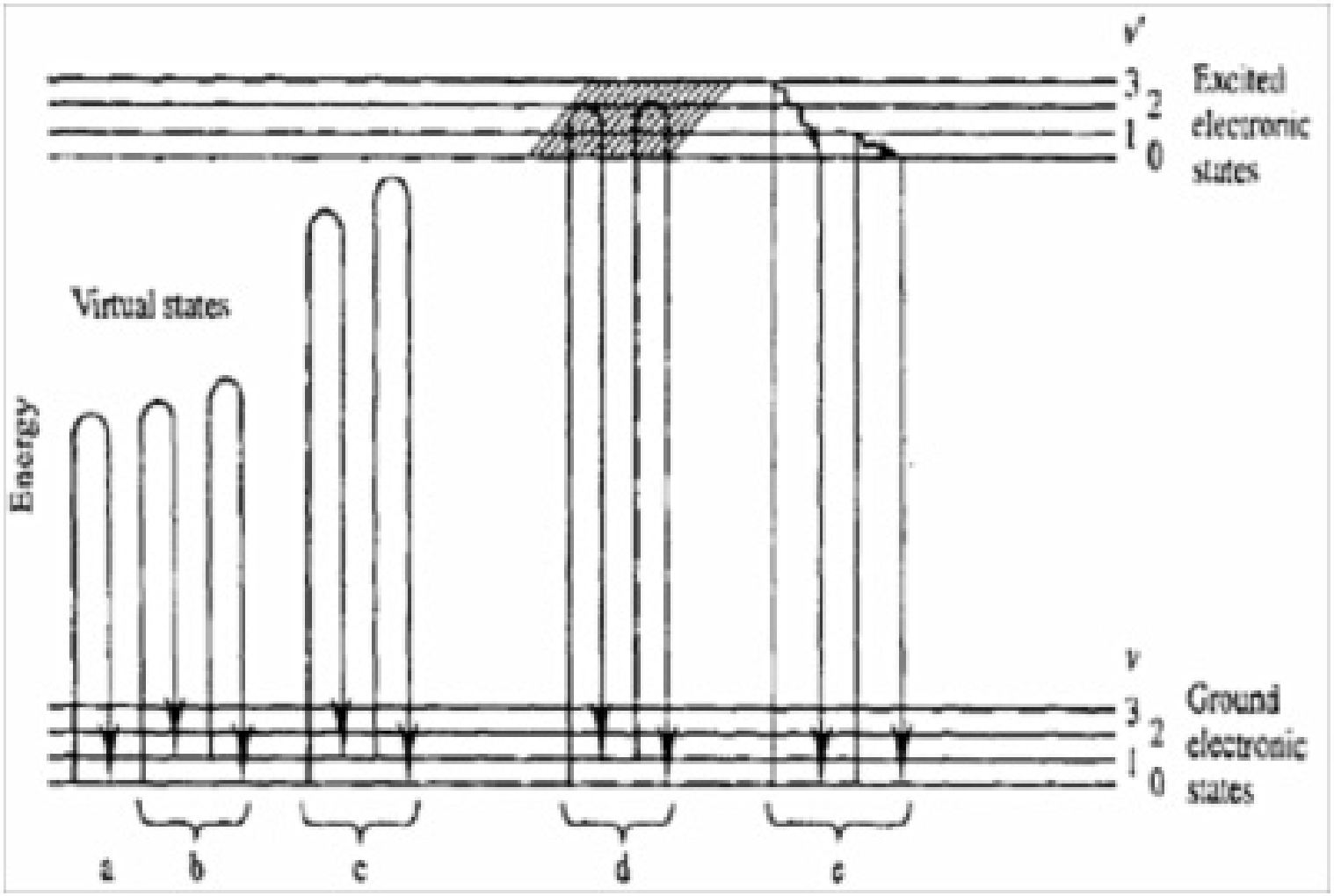
Interactions with Light



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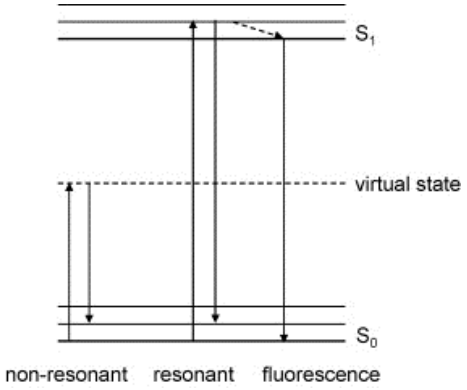
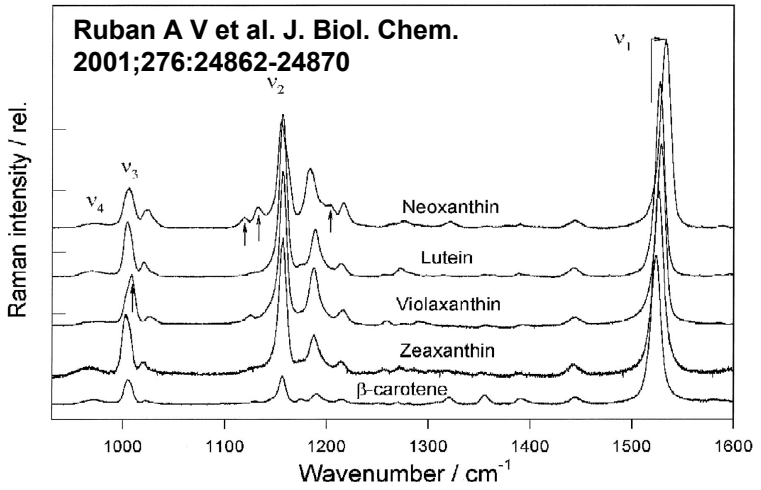
Raman Spectroscopy and Spectral Imaging of Carotenoids

- Resonant Raman vs. non-resonant Raman





ν_1: C = C stretching vibrations
ν_2: C-C stretches coupled to C-H in-plane bending or C-CH_3 stretching
ν_3: CH_3 in-plane rocking vibrations
ν_4: C-H out-of-plane bending modes

- Carotenoid biogenesis has varied applications
  - Bioenergy, environment, human health
- Non-destructive, live-cell friendly
- RR-based spectral imaging is particularly exciting because of the ability to discriminate, quantify, and localize carotenoids *in situ*.

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