

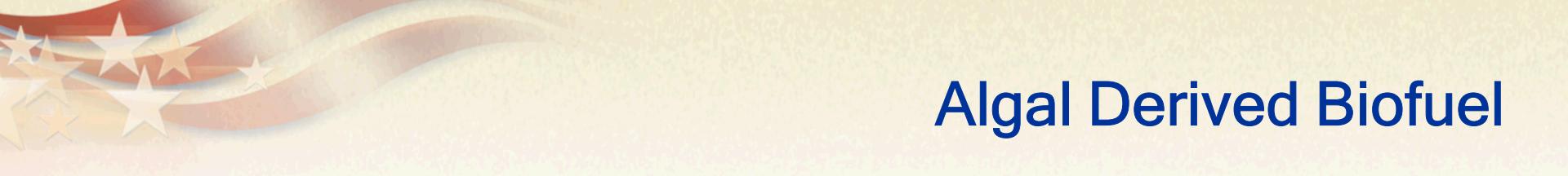
# The Power of Spectral Imaging for Algal Bioscience

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*Sandia National Laboratories is a multi-program laboratory operated by Sandia Corporation, a wholly owned subsidiary of Lockheed Martin Corporation, for the U.S. Department of Energy's National Nuclear Security Administration under contract DE-AC04-94AL85000.*

- Motivation
- Introduction to spectral imaging
  - Hardware modalities
  - Software algorithms
- Current applications
  - Hyperspectral confocal fluorescence microscopy for label-free lipid quantification
  - Hyperspectral confocal Raman microscopy to elucidate carotenoid biogenesis
- Parting thoughts



# Algal Derived Biofuel

...a critical piece in multi-faceted renewable energy puzzle



*“The problem is not making oil from algae, it is making algae with oil, actually it’s just making algae ... Need to improve current best commercial practice by over a factor of ten”* -- John Benneman co-author of “A Look Back at the U.S. DOE’s Aquatic Species Program: Biodiesel from Algae” (1978-1996)



# Why is it so hard to grow algae *at scale*?

- Open pond culture is most economical
  - Environmental fluctuations (multiparametric)
  - Escalated temporal response
  - Underlying response of organism to a stressor is species specific
  - Weak knowledge of effect of single stressors and interaction effects are almost completely unknown

Sensitive, selective, automated methods for early detection of fluctuations in algal communities.

*But, major gaps in knowledge of fundamental algal biology limit our ability to “engineer” a solution*

# Spectral Imaging: Spatially and Temporally Resolved Biochemical Response of an Organism to Its Environment

## Light Microscopy

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

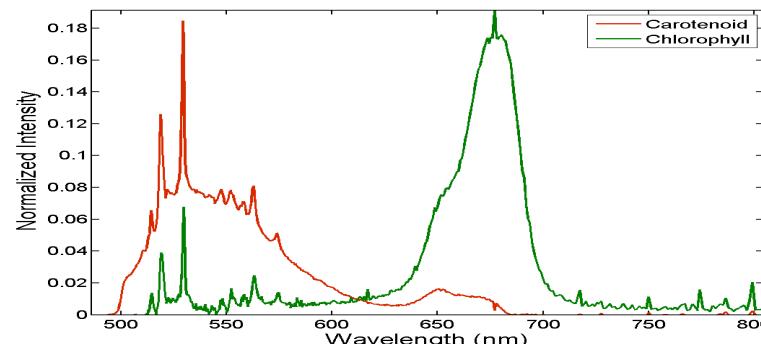
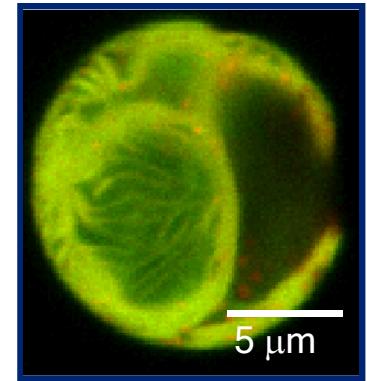
Pretty picture →



## Spectral Imaging

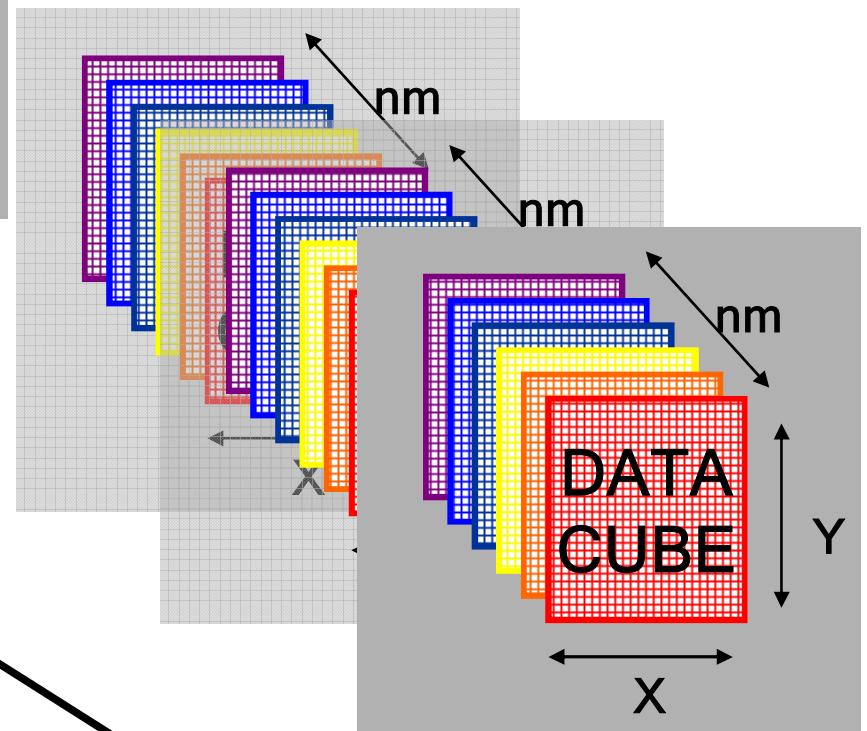
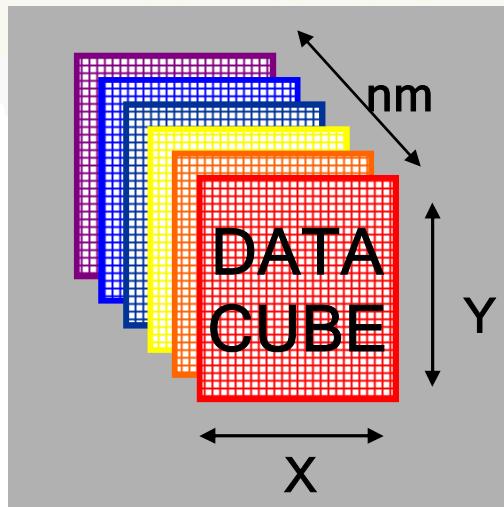
Each pixel in the image is a spectrum relating to chemical and/or molecular structure within

Chemical Information →



Dunaliella salina

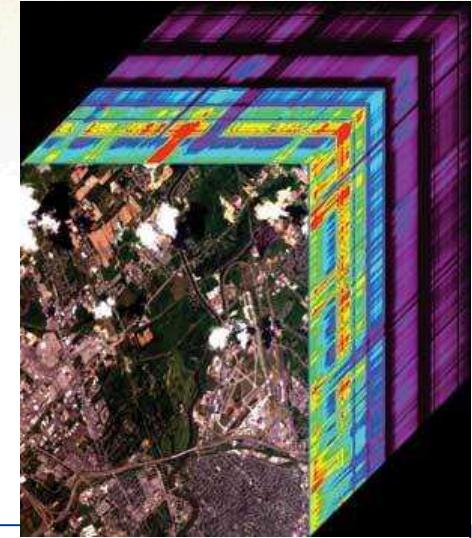
# The Spectral Image Cube



Time, depth,  
or condition

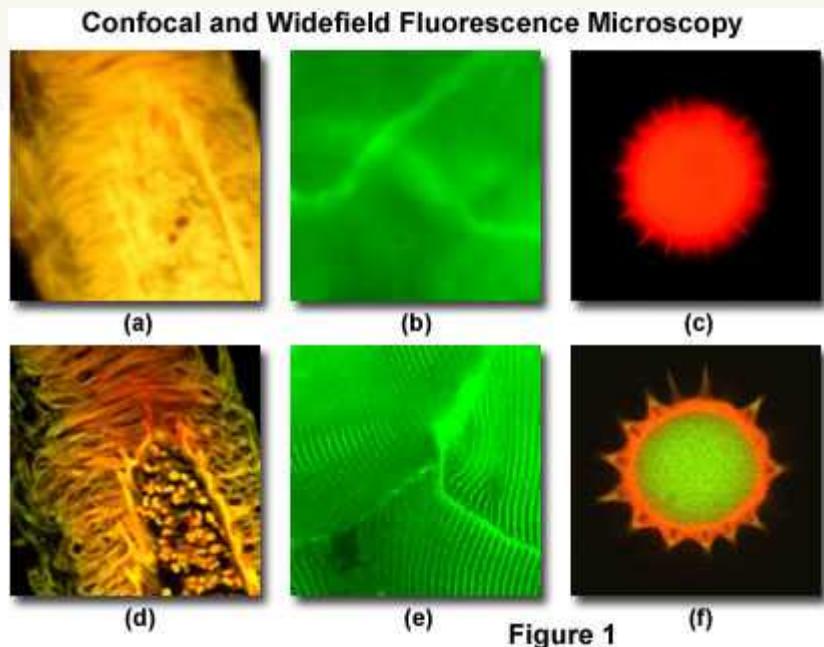
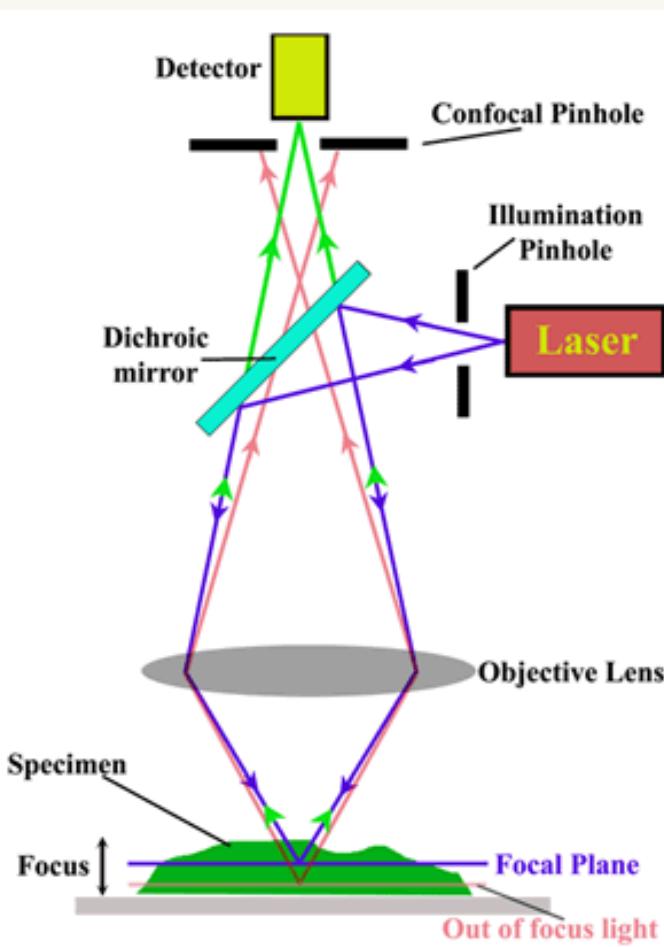
# The Many Faces of Spectral Imaging

- Spectral Imaging
  - Variety of modalities (Fluorescence, IR, Raman, XRF, MS, etc)
  - Variety of implementations (Frequency scanning, sample scanning, interferometer-based, etc)
  - Nm to km
- Categories of Spectral Imaging
  - Multispectral (2-10 spectral channels)
  - Hyperspectral (tens to thousands of spectral channels)
  - Hyperspectral imaging coined in 1990's in Remote Sensing. This is still area of great importance at SNL and worldwide
- Focus - Hyperspectral Confocal Fluorescence & Raman Microscopy (AKA - Chemical Imaging)



An airborne hyperspectral image, with its spectrum shown as a colored stack beneath each pixel  
(Source of data: NASA Jet Propulsion Laboratory)

# Confocal Imaging: Primer



- Increases accuracy and interpretation
- Enables optical sectioning on transparent samples
- Reduces signal-to-noise (longer scan times, higher laser powers)

Images courtesy of <http://www.olympusfluoview.com/theory/confocalintro.html> & <http://www.microscopyu.com>



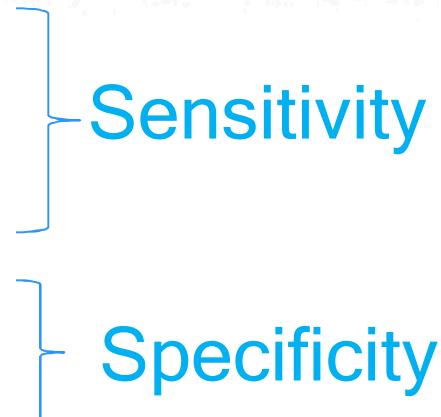
# Spectral Imaging

How do you get 512+ spectral data points  
without being 512 x slower?



# Hardware Design Principles

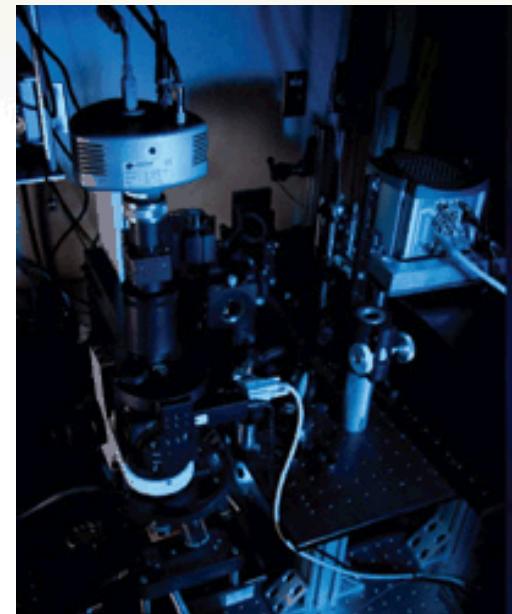
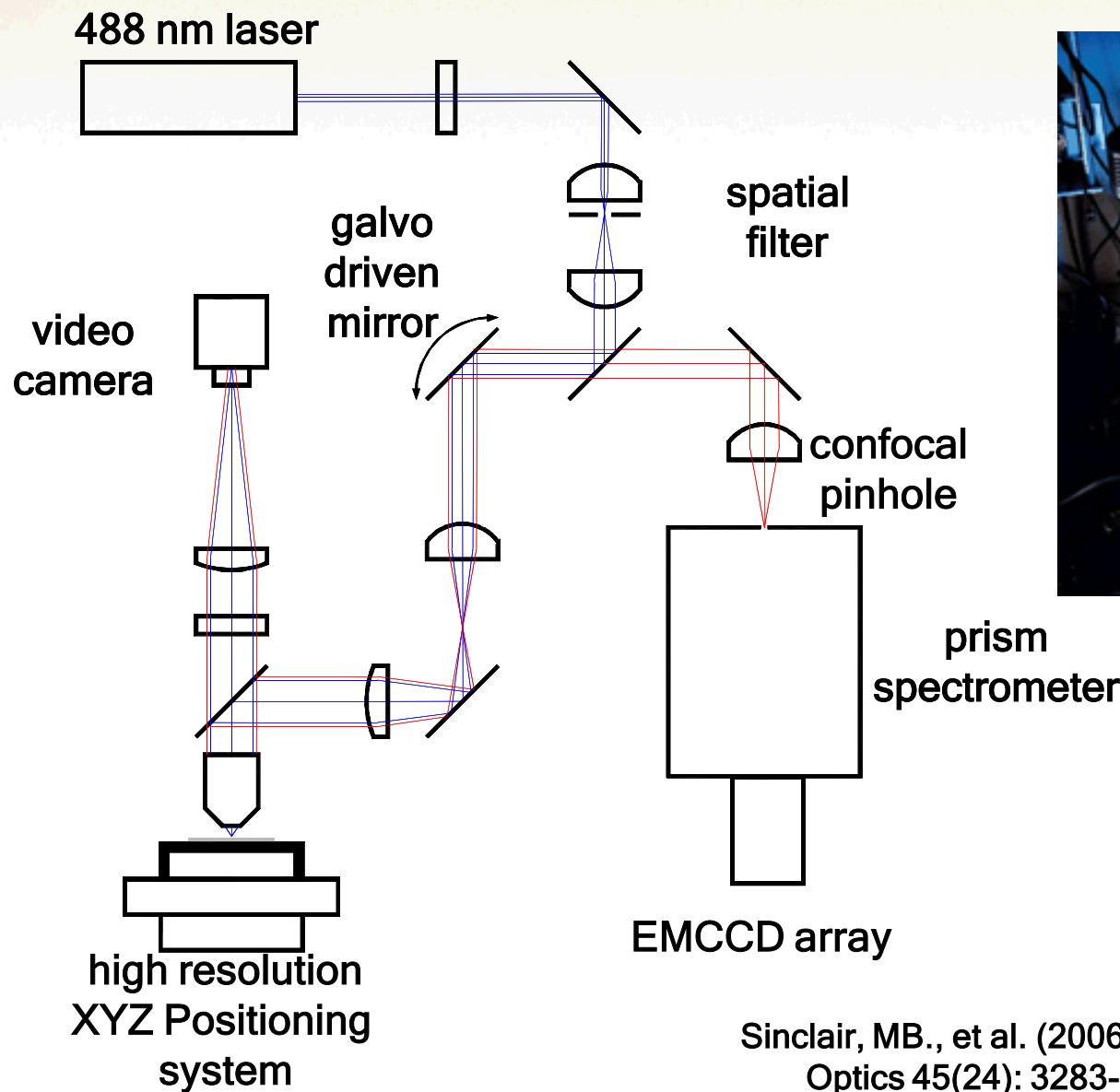
- Spatial resolution
- Speed
- Signal-to-noise
- Signal-to-background
- Spectral resolution



**Sensitivity**

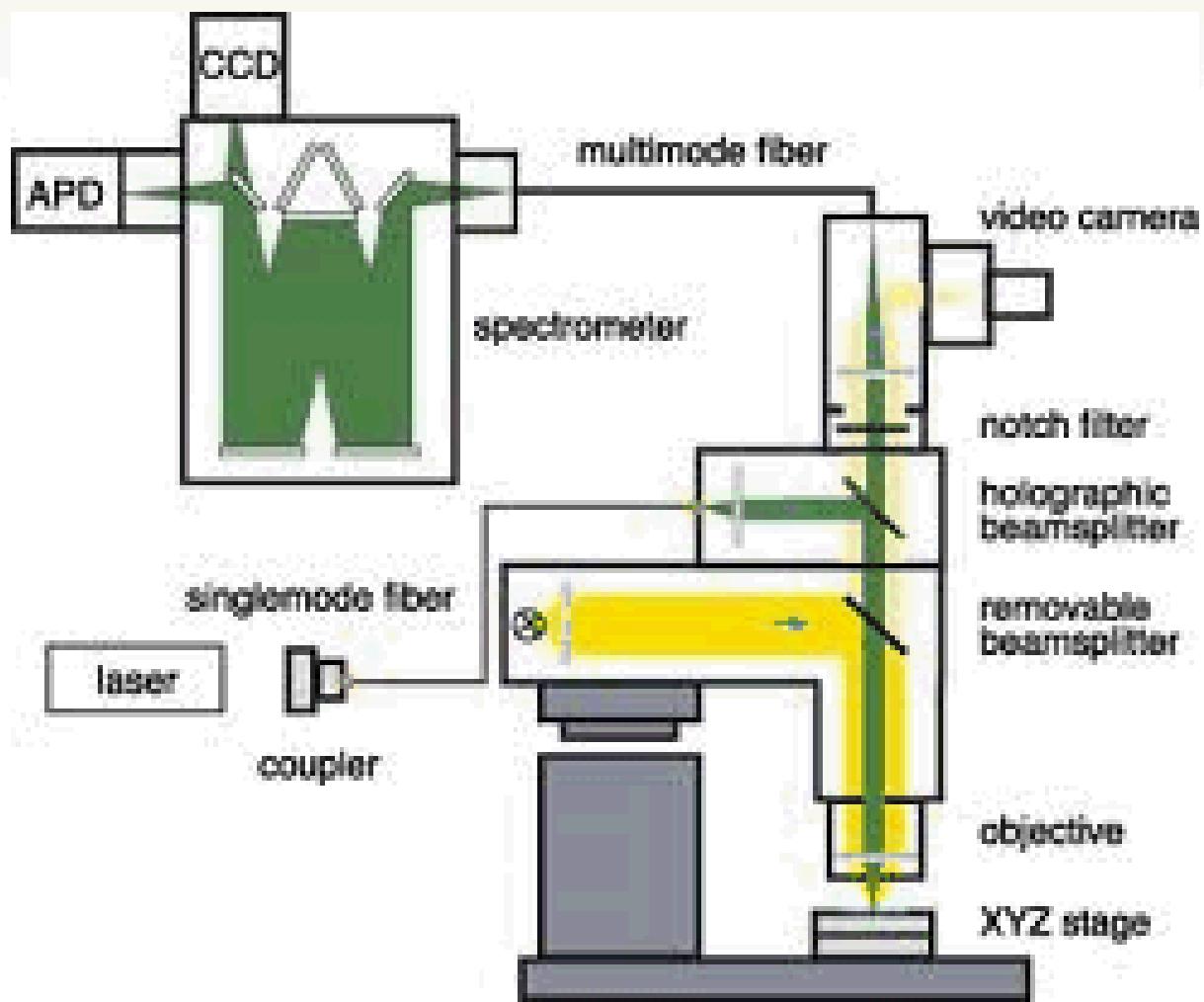
**Specificity**

# 3D Hyperspectral Confocal Microscope

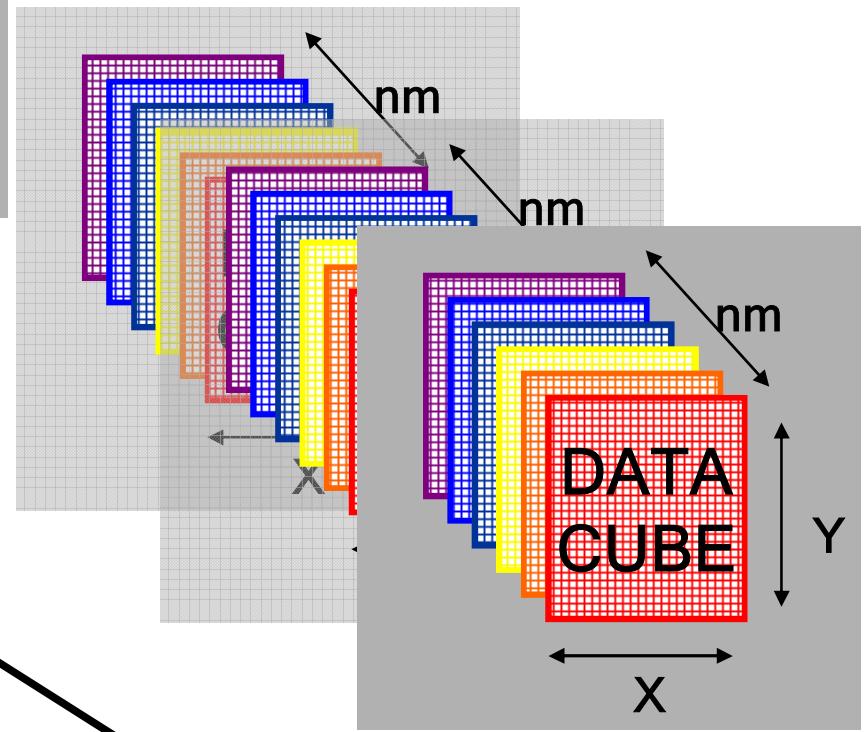
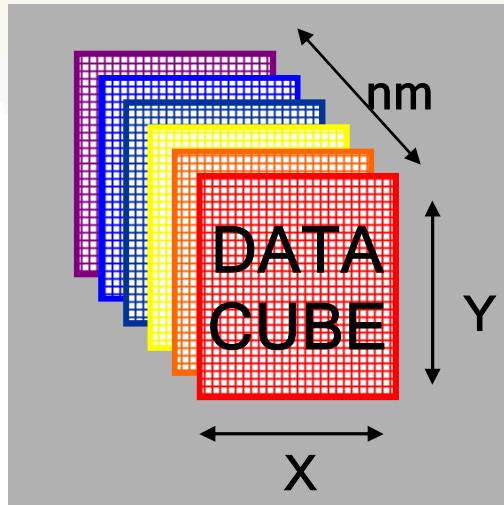


Sinclair, MB., et al. (2006). Applied Optics 45(24): 3283-3291.

# Hyperspectral Confocal Raman Microscope



# No Matter How You Collect It - There's a LOT of Data!



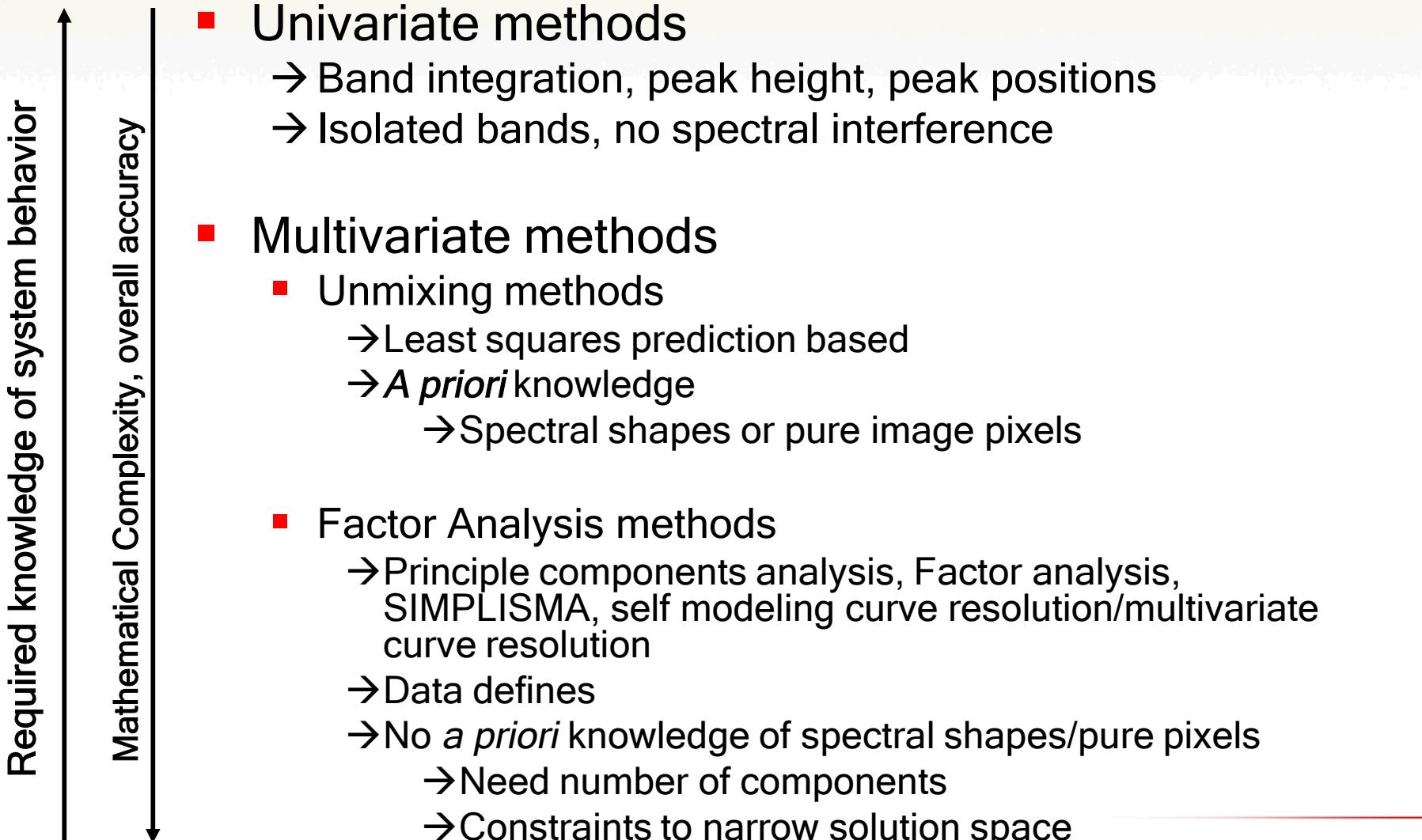
Time, depth,  
or condition



# Multivariate Spectral Image Analysis

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

# Common Spectral Image Analysis Methods





# Multivariate Curve Resolution (Constrained Alternating Least Squares)

## Advantages of MCR:

- “Discovers” & “Quantifies”
- Extracts underlying relationships from complex data sets
- No *a priori* knowledge required
- Signals below the noise level can be detected
- Physically meaningful (constraints)
  - Non-negativity, equality
- Rigorous least squares for all constraints
- Weight for noise properties
- Use new efficient algorithms developed at Sandia

## Assumptions of MCR:

- Linear additive model applies:  $\mathbf{D} = \mathbf{C}\mathbf{S}^T + \mathbf{E}$
- # of components is known/can be estimated

# Mathematical Details of MCR

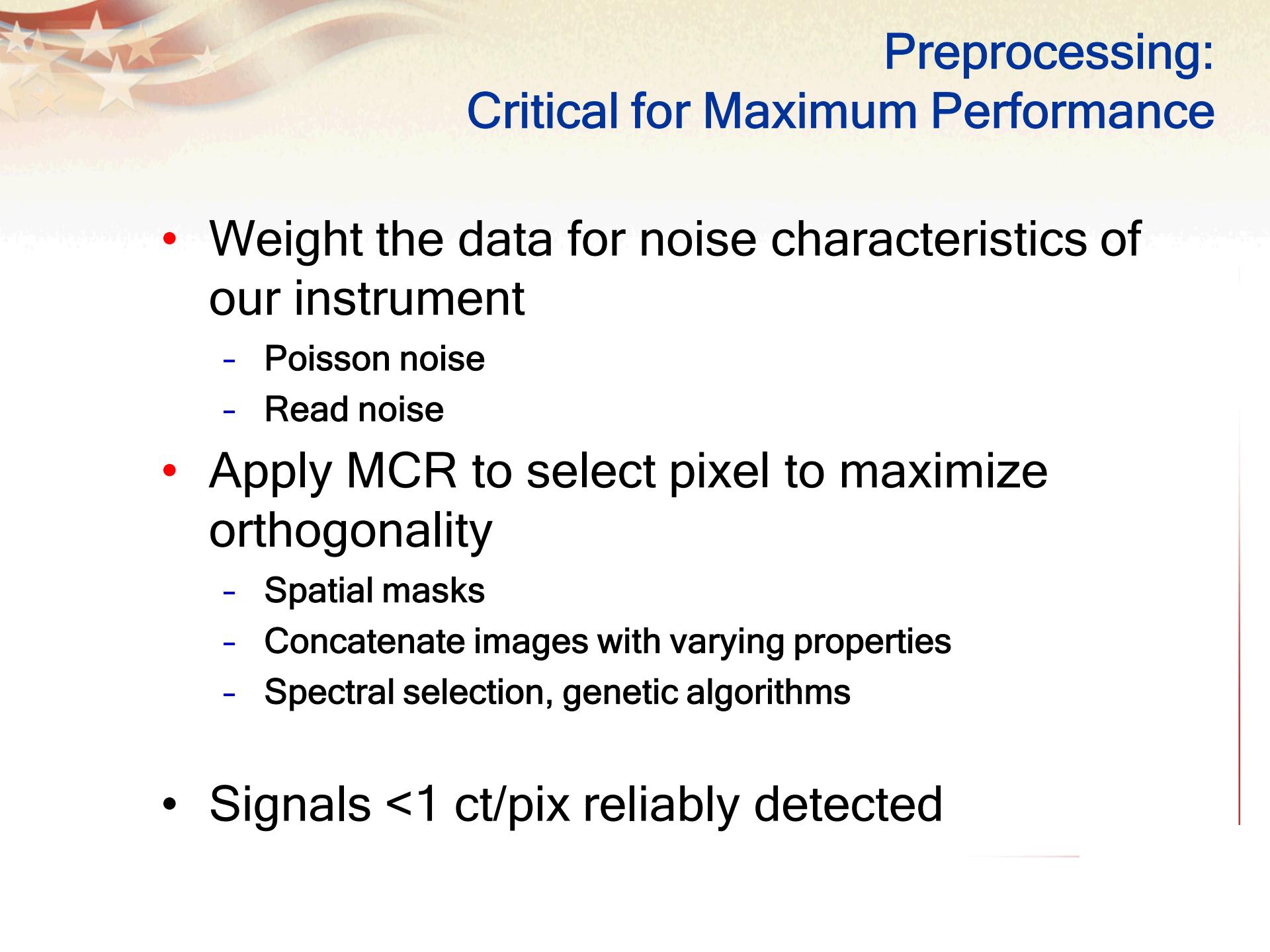
- Solve  $\mathbf{D} = \mathbf{CS}^T$  in least squares sense
  - $\hat{\mathbf{C}} = \mathbf{DS}(\mathbf{S}\mathbf{S}^T)^{-1}$  (CLS prediction)
  - $\hat{\mathbf{S}}^T = (\hat{\mathbf{C}}^T\hat{\mathbf{C}})^{-1}\hat{\mathbf{C}}^T\mathbf{D}$  (CLS calibration)
- Apply constraints to obtain realistic solutions
  - **Non-negative spectra & concentrations**
  - **Equality constraints**
  - **Rigorous least squares implementation**
  - **Use new efficient algorithms developed at Sandia**
- Iterate prediction and calibration steps until converged

**D** represents intensities of all spectra in image

**C** is concentration matrix for all spectral components

**S** is pure-component spectra from image

$\hat{\cdot}$  is least squares estimate



# Preprocessing: Critical for Maximum Performance

- Weight the data for noise characteristics of our instrument
  - Poisson noise
  - Read noise
- Apply MCR to select pixel to maximize orthogonality
  - Spatial masks
  - Concatenate images with varying properties
  - Spectral selection, genetic algorithms
- Signals  $< 1$  ct/pix reliably detected

# MCR Example

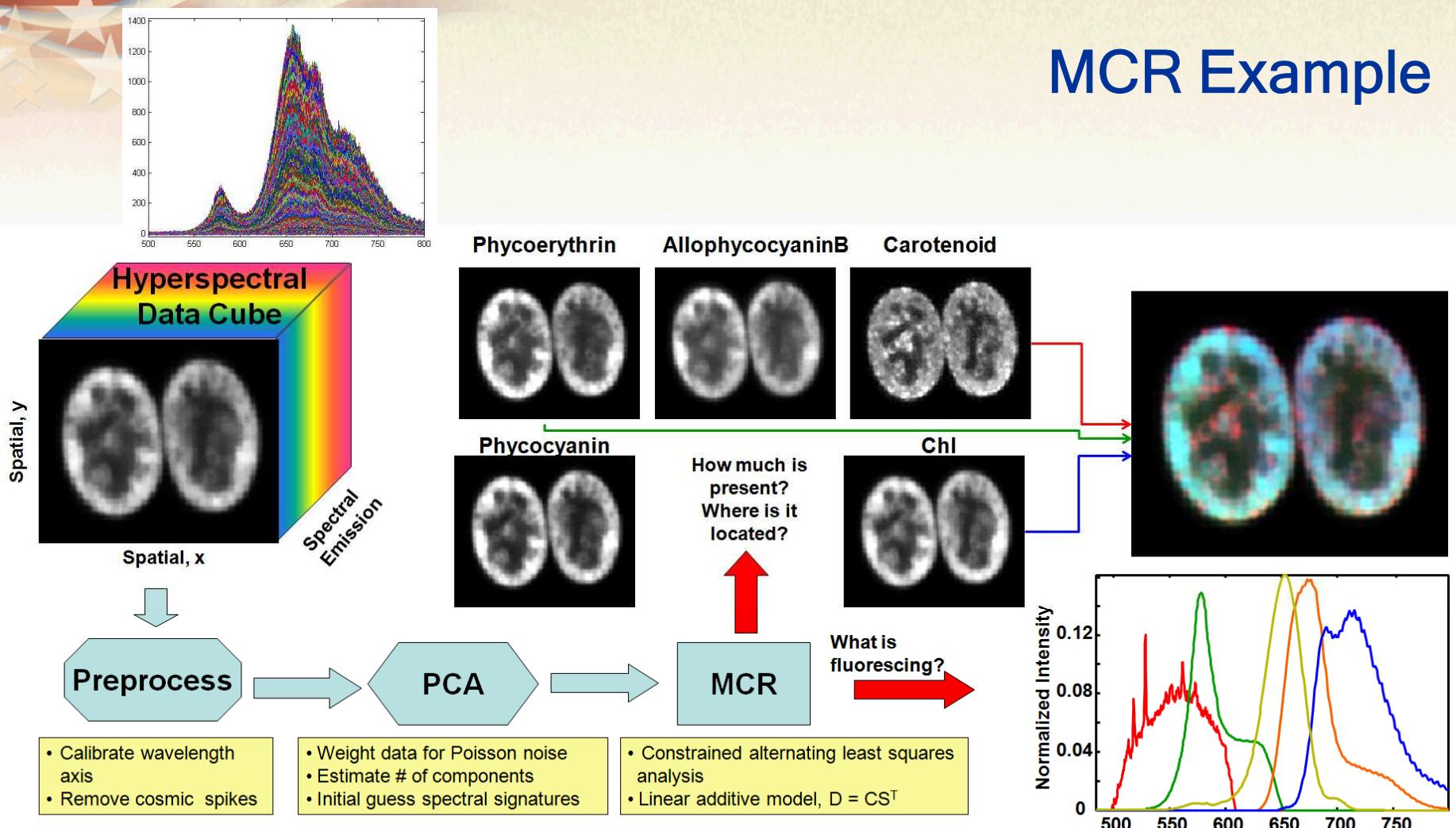


Figure 2. Mathematical isolation of independently varying chemical species is accomplished using a fast multivariate curve resolution algorithm with robust constraints. Example shown: hyperspectral imaging of endogenous pigments in the cyanobacterium *Cyanothece* sp. PCC 7822.



# Spectral Imaging in Algal Bioscience

What are we learning that we didn't know two  
yrs ago?

# Label-free in-situ Monitoring of Algal Lipid Production

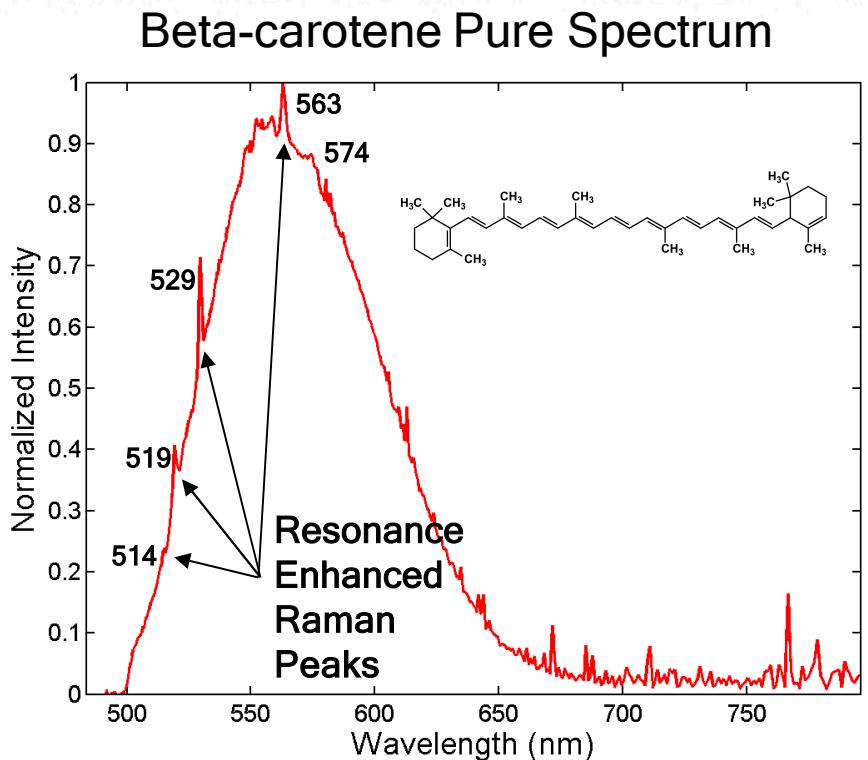
- Problem
  - Nile red stain is the current state of the art for measuring lipid using fluorescence spectroscopy
  - Not ideal for in-situ monitoring of algal cells at the microscopic level
    - Compromises the health of the cell → Toxic
    - Non-uniform staining
    - Low specificity
- Solution
  - Carotenoid is soluble in lipid
    - We see a spectroscopic signature for Carotenoid?
    - Can we use this signature to monitor lipid production?
  - Develop a methodology to use native spectroscopic signatures to monitor lipid production with spatial and temporal resolution

**Spectral Imaging**

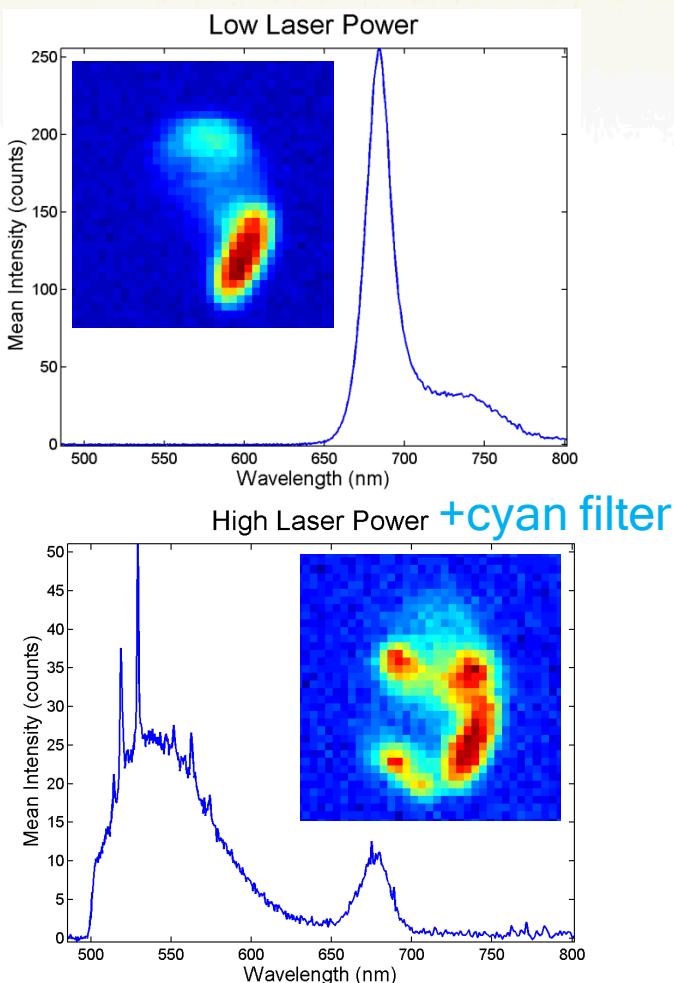


\*\*Resonance enhancement effect

# Carotenoids are Visible with Hyperspectral Confocal Fluorescence Microscopy

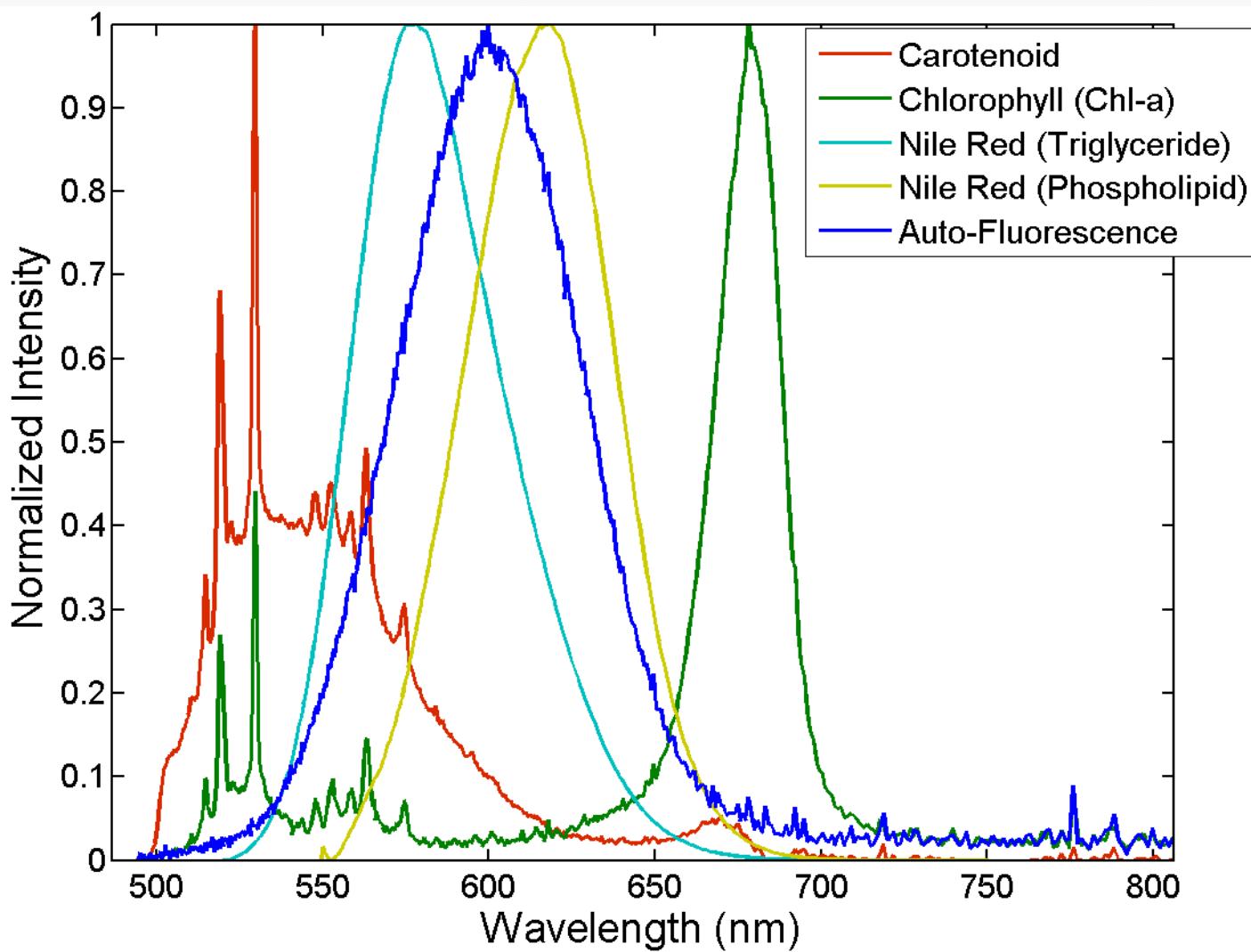


Beta-carotene in Zeiss Immersol 518F  
(low fluorescence immersion oil)



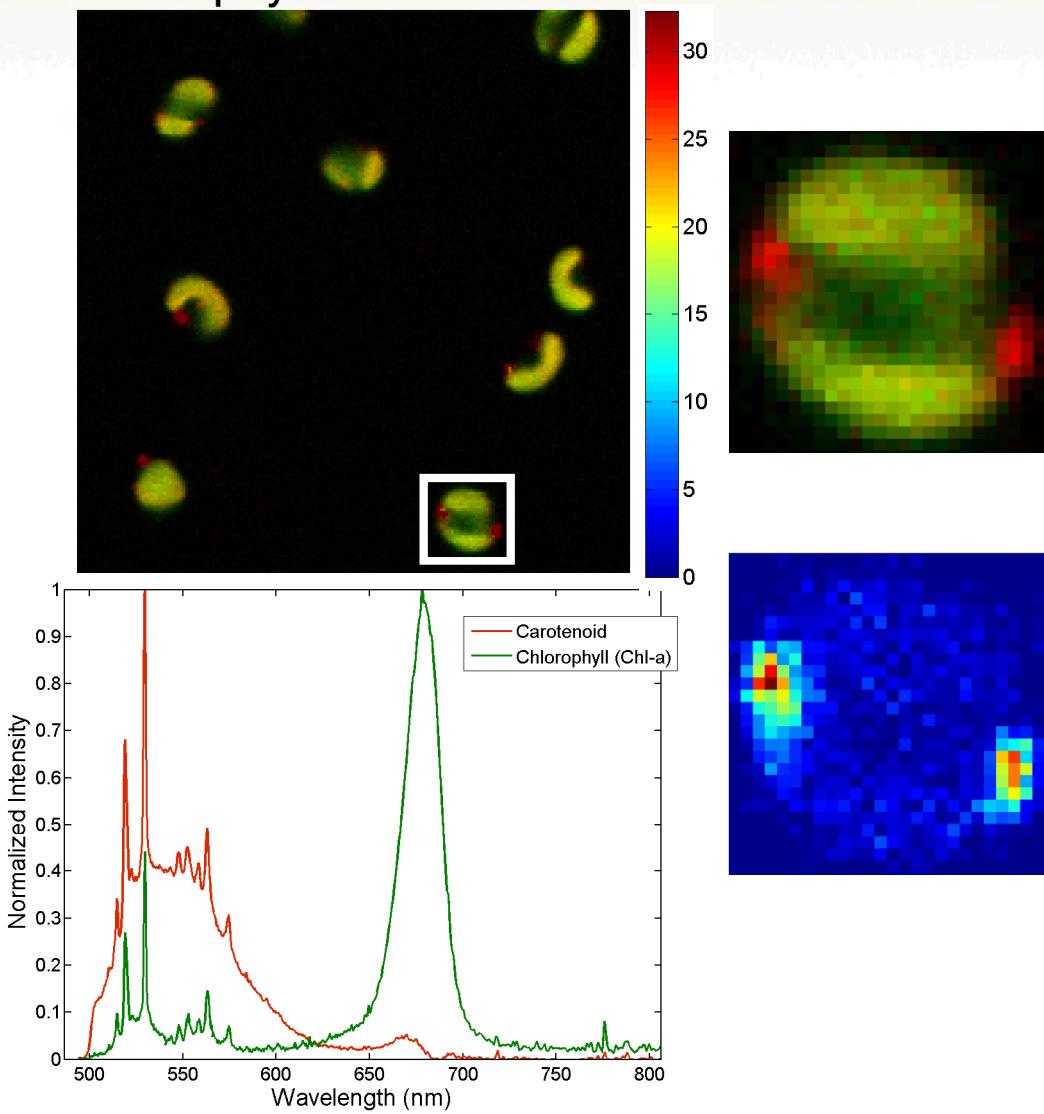
Carotenoid pigments are in the thylakoids of the chloroplast and are solvated by the produced lipid.

# MCR Spectral Components

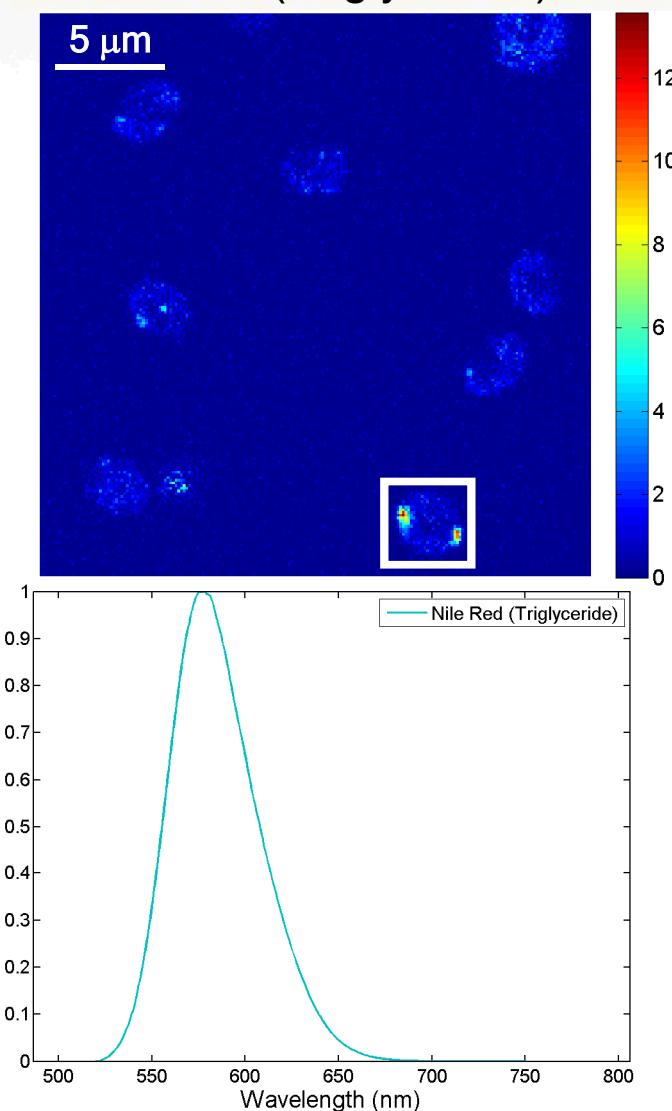


# Spatial Correlation of Lipid Carotenoid with Nile Red

Chlorophyll and Carotenoid

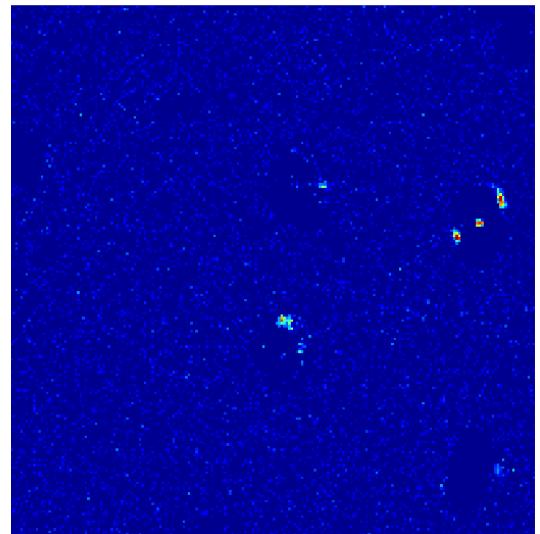
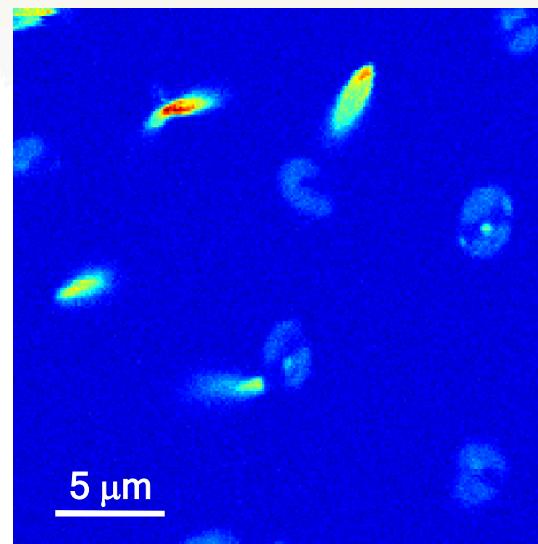
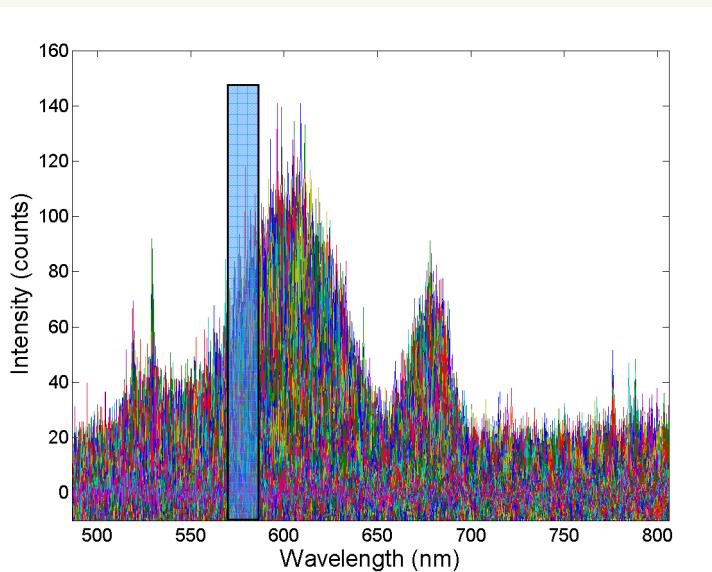


Nile Red (Triglyceride)



# What a filter-based fluorescence measurement might see...

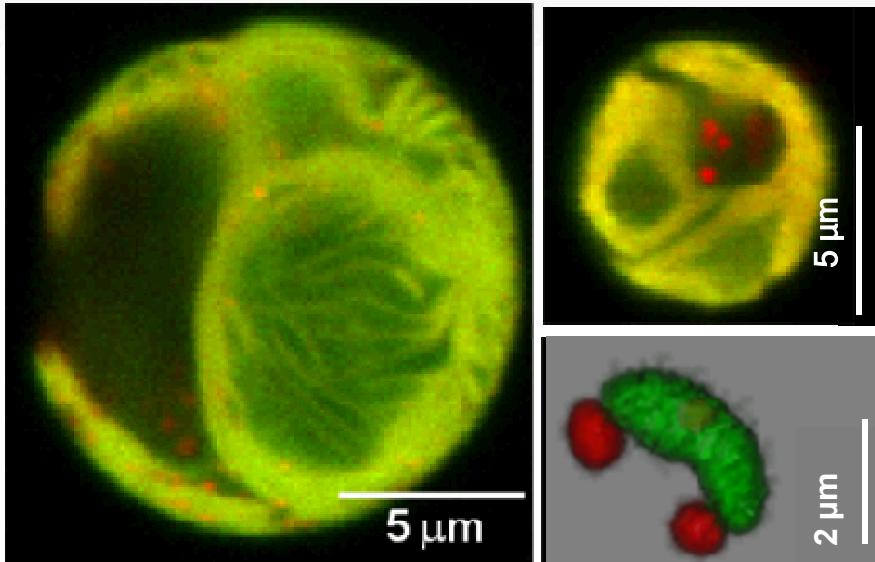
Assume the best case scenario with a narrow band filter for the Nile Red (Triglyceride).



Hyperspectral Imaging Results

Label-Free Lipid Determination

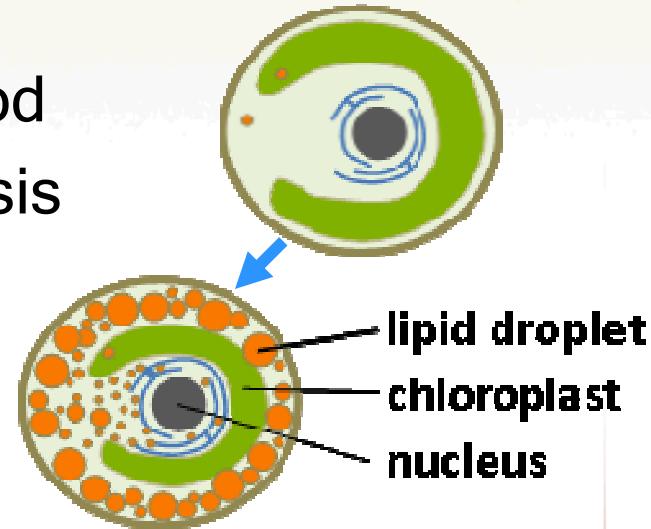
# Label-free Detection of Lipid in 2 or 3Dimensions



**Fig 1.** Compares and contrasts two biofuels production strains: *Nannochloropsis salina* and *Dunaliella salina* and the model organism: *Chlamydomonas reinhardtii*. Spectral images of these shown clockwise from lower right. Chloroplast is marked by Chl a (green) while the lipid bodies are identified by lipid-soluble carotenoid (red).

# Hyperspectral Confocal Raman Microscopy of Carotenoids

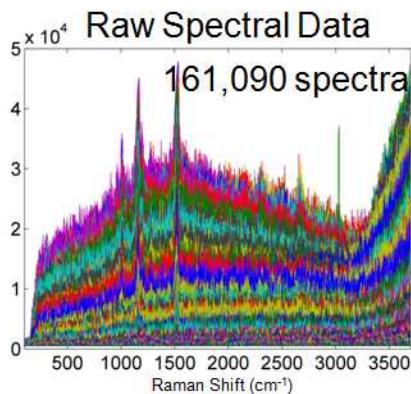
- TAG production is not **\*fully\*** understood
- Intimately tied to carotenoid biosynthesis
- Carotenoid composition varies
  - In response to environmental conditions
  - Throughout lifecycle of cell
  - Between species/strains
- Full picture absolutely requires chemical, spatial, and temporal resolution



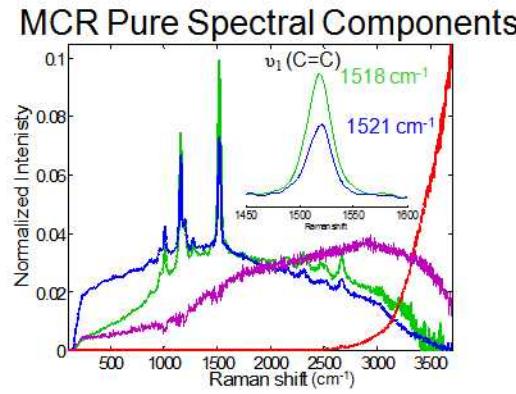
## Spectral Imaging

\*\*Raman spectral imaging of carotenoids is enabled by resonance enhancement effect

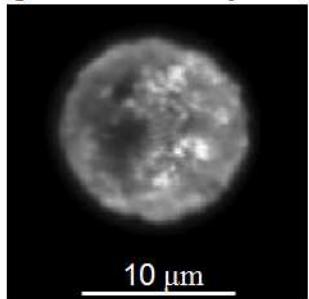
# Subcellular Localization of Carotenoids in Living Cells



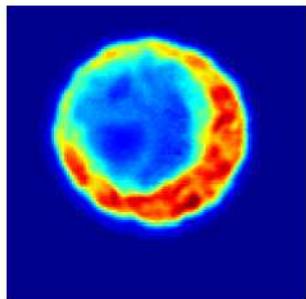
MCR



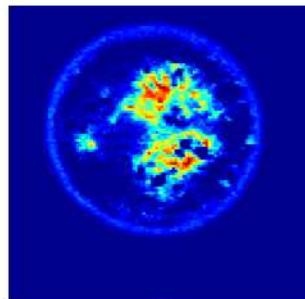
Integrated intensity image



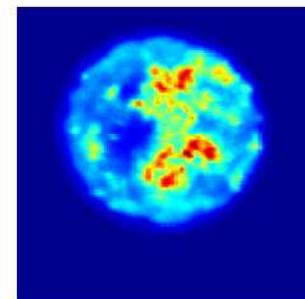
Chl



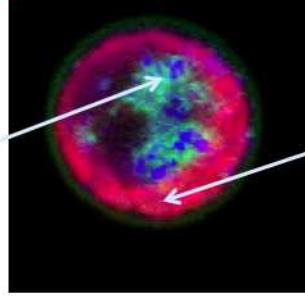
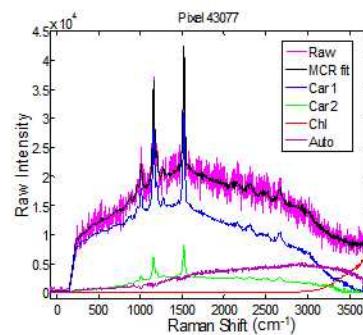
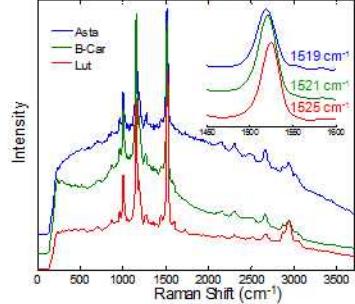
Carotenoid 2



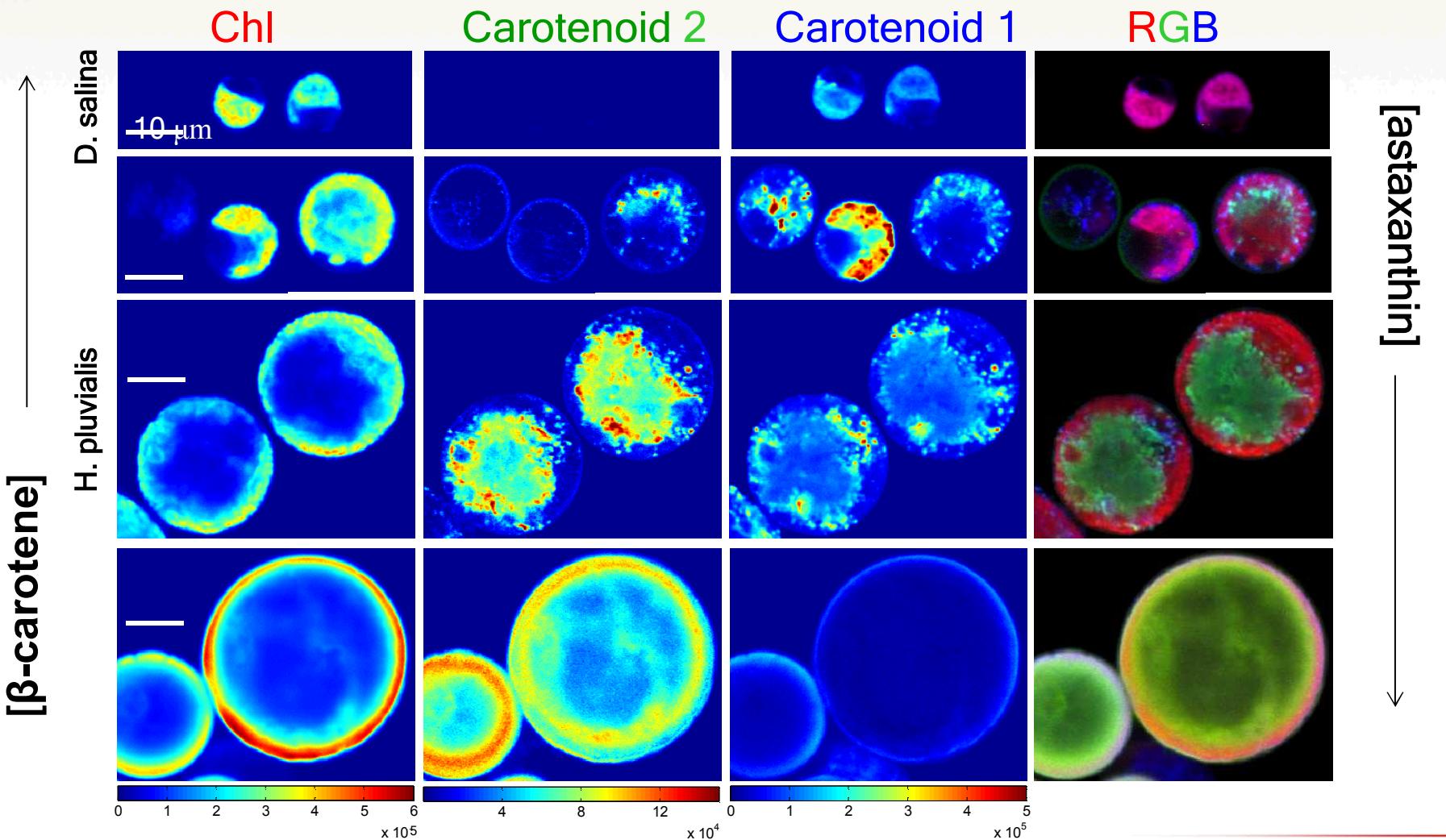
Carotenoid 1



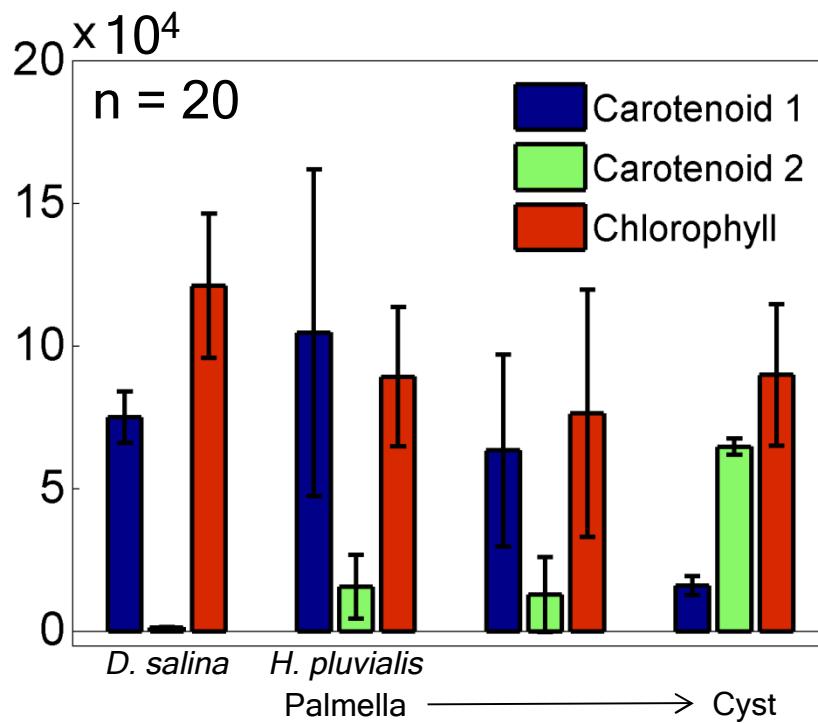
Raman spectra of pure carotenoids

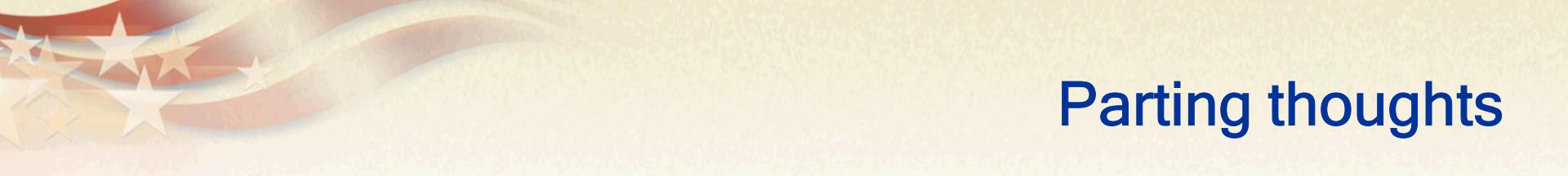


# Subcellular Localization of Carotenoids in Living Cells



# Not Just Pretty Pictures ...



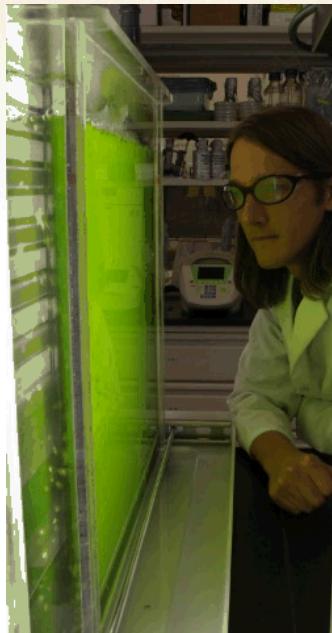


## Parting thoughts

- Spectral imaging provides chemical, spatial, and temporal resolution at the single cell level
- Raman, fluorescence, and other modalities are critical to understanding fundamental processes that govern algae growth and productivity
- Gaps in algal bioscience needs to be understood before we can realize production of algal biofuels at large scale
- *Challenges - Biology: species diversity, culture variation, algae have a cell wall, cells move*
- *Challenges - Technology: endogenous fluorophores can't be controlled, discrete vs. continuous microenvironments are hard to model, plethora of data*

## TEAM ALGAE

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