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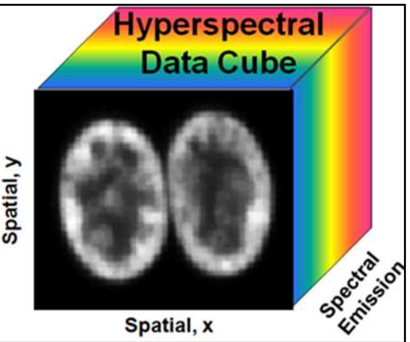


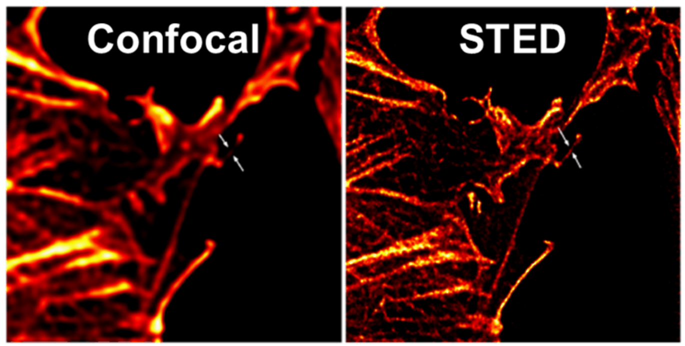


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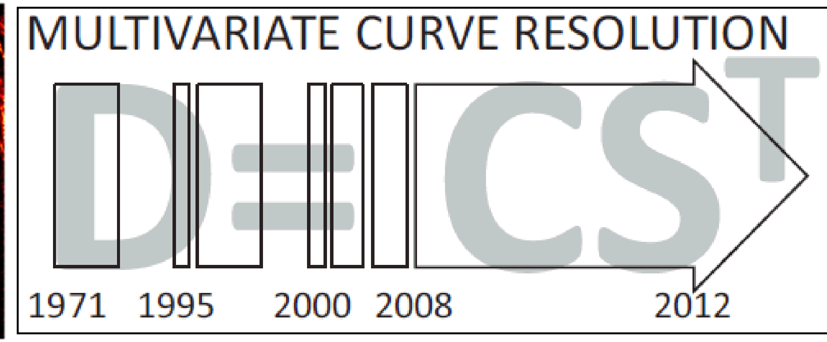



Ruckebusch, C. and L. Blanchet (2013). Analytica Chimica Acta **765: 28-36.**

# Super-resolution Hyperspectral Microscopy and Image Analysis


Stephen M. Anthony


April 18, 2016

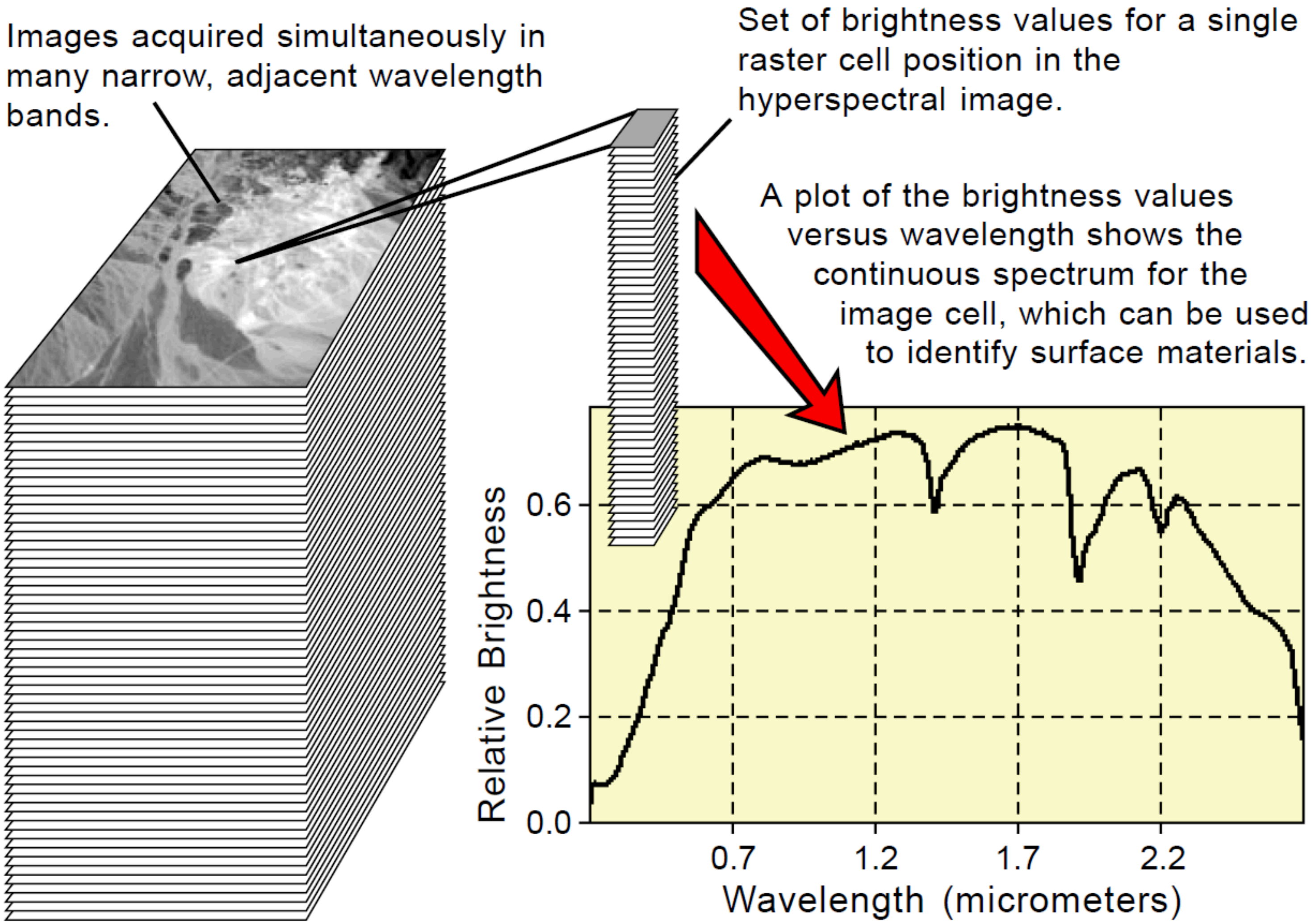
U.S. DEPARTMENT OF ENERGY

NNSA National Nuclear Security Administration

# Outline

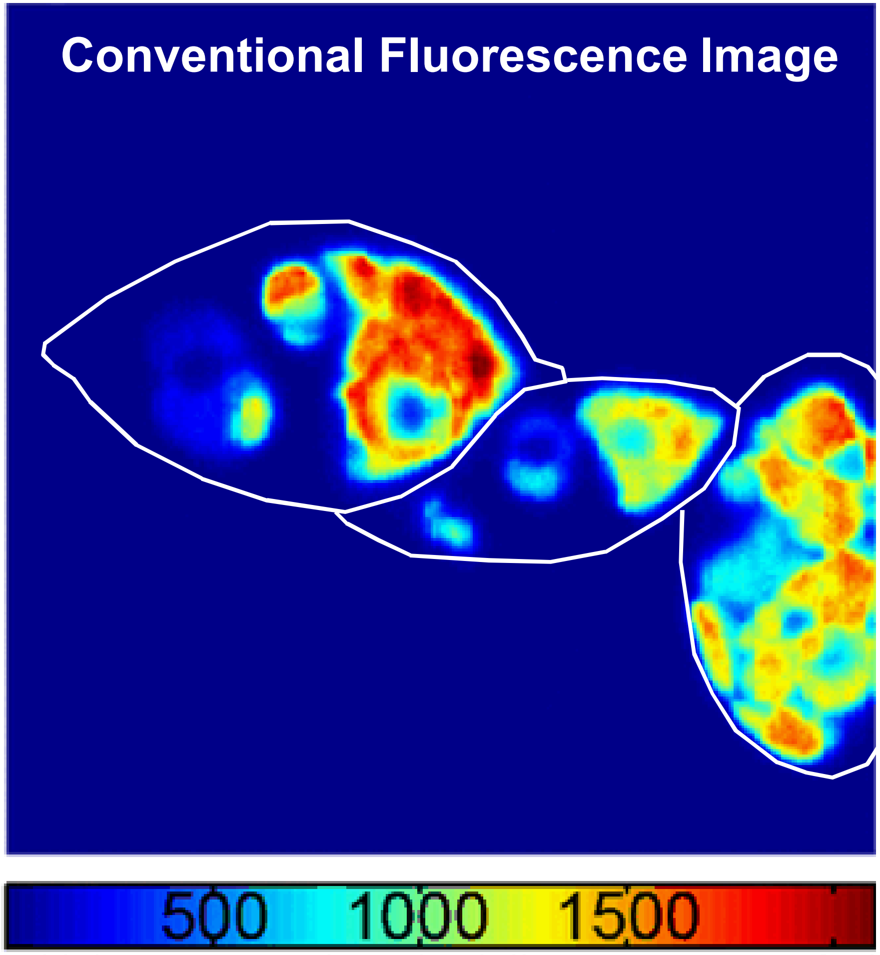
- **Hyperspectral Microscopy** – What is it and what are its benefits?
- **Super-resolution Microscopy** – What is it and why did it win a Nobel prize?
- **Hyperspectral STED Microscopy** – Combining hyperspectral and super-resolution microscopy.
- **Multivariate Curve Resolution (MCR)** – Extracting information from hyperspectral datasets.

# Introduction to Hyperspectral Imaging




Smith, R. B. (2012) Introduction to Hyperspectral Imaging. Microimages
http://www.microimages.com/documentation/Tutorials/hyprspec.pdf

# Why Use Hyperspectral Imaging

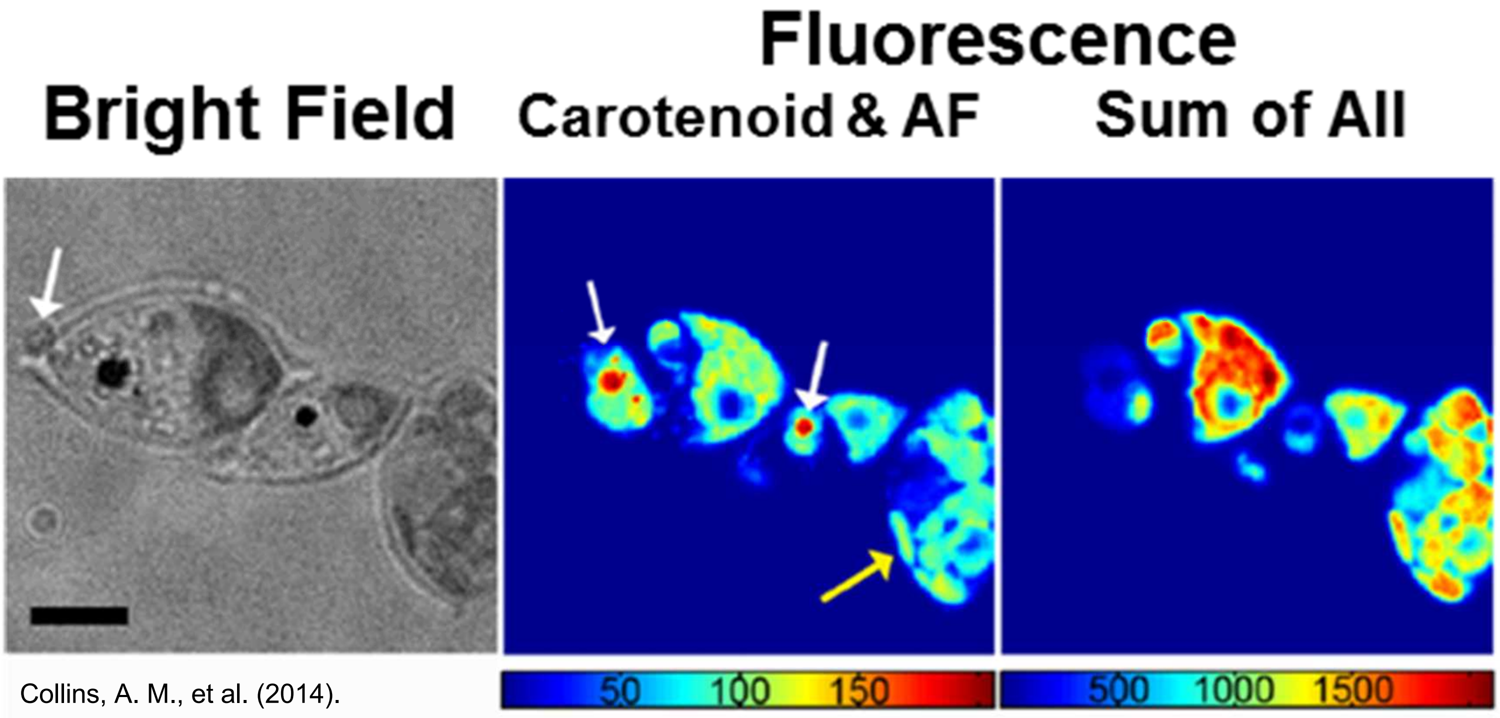



Adapted from Collins, A. M., et al. (2014). "Host Cell Pigmentation in Scenedesmus dimorphus as a Beacon for Nascent Parasite Infection." Biotechnology and Bioengineering **111(9): 1748-1757.**

Image of the endogenous fluorescence from *S. dimorphous* (algae) undergoing parasitic infection by *A. protococcarum*.

- Approximate cell borders are hand-drawn in white.
- Two of the cells contain parasitic vacuoles.
- **Can you spot the parasitic vacuoles?**

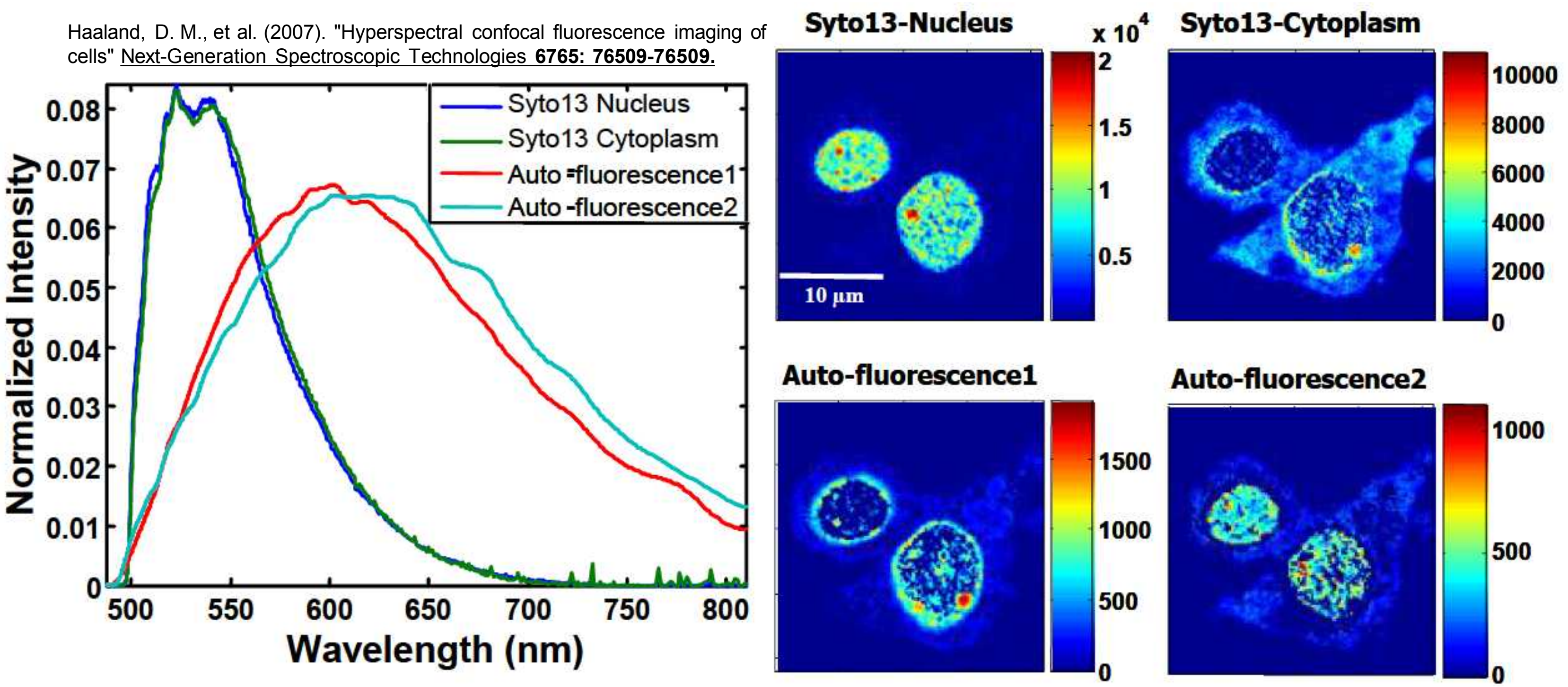
# Why Use Hyperspectral Imaging




- Parasitic vacuoles (white arrows) are easily spotted using the combined carotenoid and autofluorescence signal.
- Spotting them is nearly impossible when examining all the fluorescence together.
- **Hyperspectral imaging reveals otherwise hidden features.**

# Why multispectral is not enough

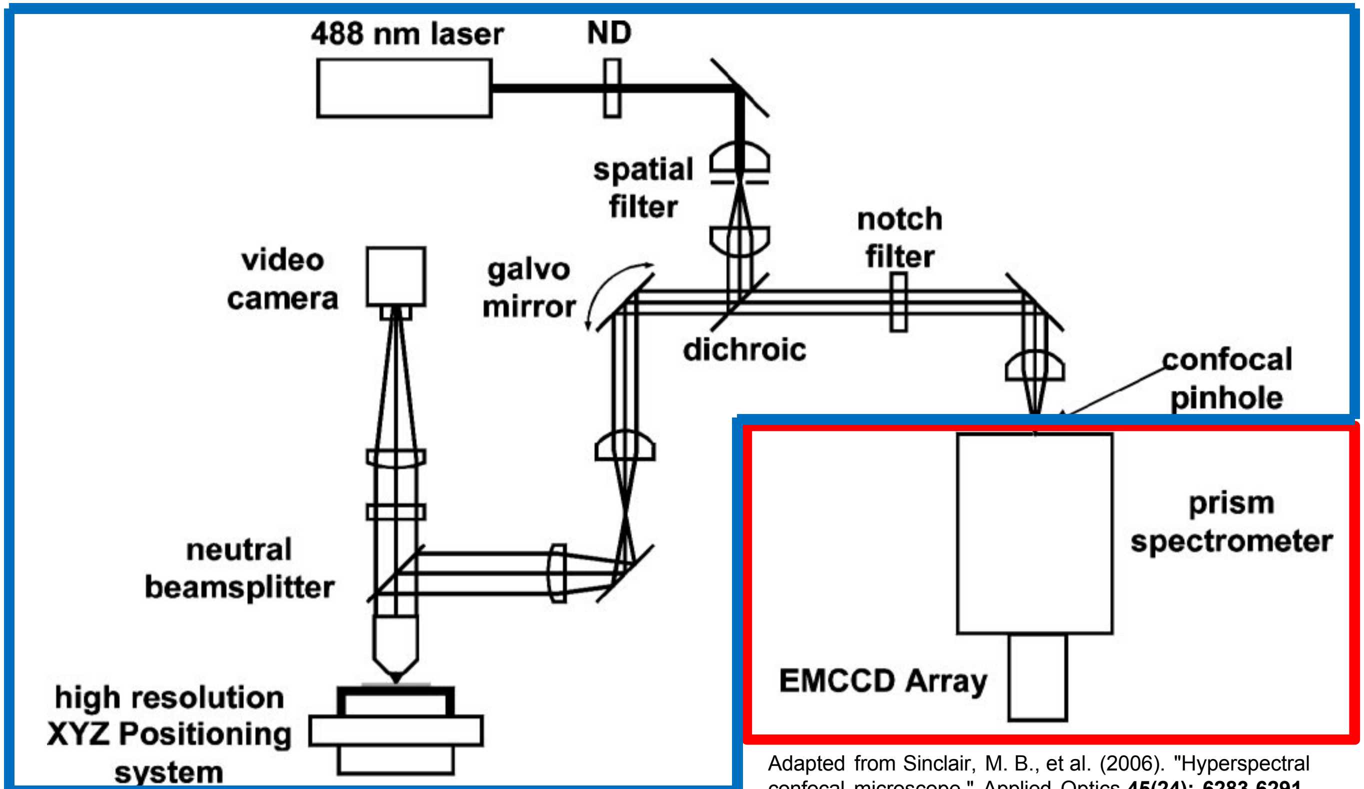
Haaland, D. M., et al. (2007). "Hyperspectral confocal fluorescence imaging of cells" Next-Generation Spectroscopic Technologies **6765: 76509-76509.**




Left) Fluorescence spectra for two Syto 13 and two autofluorescence emission components. Right) Relative concentration of the components' spatial distributions in mouse macrophage cells (Raw 264.7).

- Multispectral imaging (e.g. filter-based microscopes) would only distinguish Syto 13 from autofluorescence – two components.
- **Hyperspectral imaging can distinguish nearly identical spectra.**

# How to Build a Hyperspectral Microscope




Adapted from Sinclair, M. B., et al. (2006). "Hyperspectral confocal microscope." Applied Optics **45(24): 6283-6291.**

Schematic diagram of Sandia's hyperspectral confocal microscope

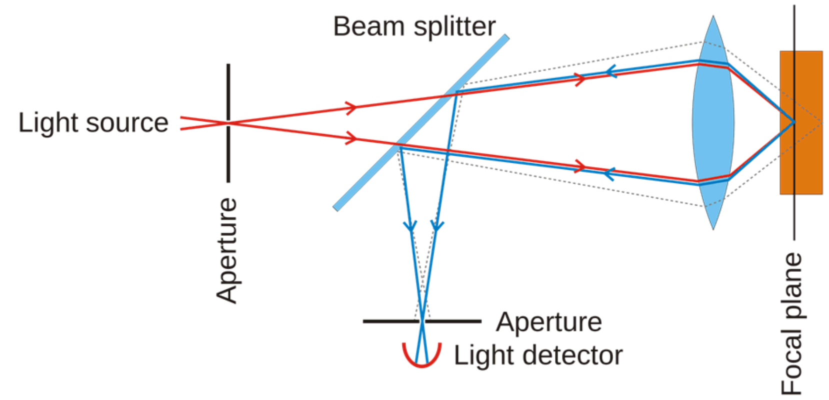
**Hyperspectral Confocal Microscope =**

**Confocal Microscope + Spectrometer**

# Why use a confocal microscope?

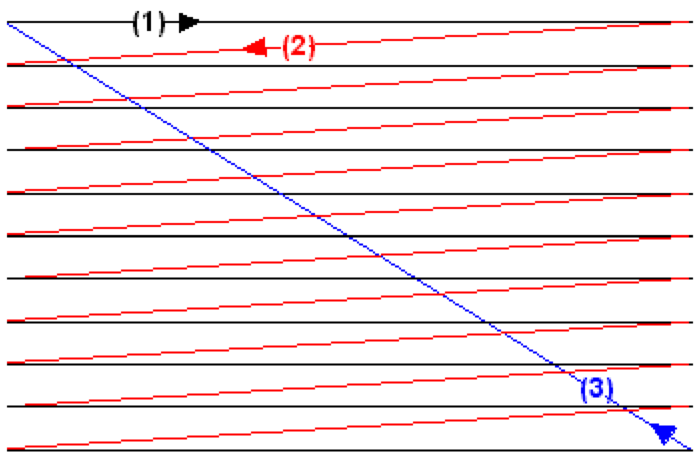
- Hyperspectral data cubes are 3-dimensional (image) or 4-dimensional (movie)
- Cameras (CCD or CMOS) can only record 2D images or 3D movies
- Confocal microscopy is a point scanning technique – conventional confocal only requires a point detector
- **A standard spectroscopic camera is sufficient for hyperspectral confocal**

## Principal of Confocal Microscopy




https://en.wikipedia.org/wiki/File:Confocalprinciple_in_English.svg

## Raster Scanning




http://encyclopedia2.thefreedictionary.com/raster+scan

# Hyperspectral Microscopy Takeaways

**Hyperspectral microscopy:**

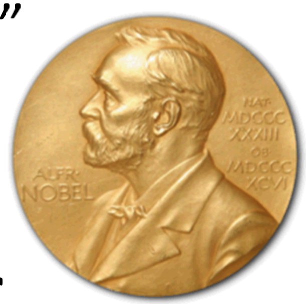
- **Adds an additional, spectral dimension to conventional microscopy**
- **Can distinguish between nearly identical spectra**
- **Allows detection of features which cannot be seen with conventional microscopy**

# Outline

- **Hyperspectral Microscopy** – What is it and what are its benefits?
- **Super-resolution Microscopy** – What is it and why did it win a Nobel prize?
- **Hyperspectral STED Microscopy** – Combining hyperspectral and super-resolution microscopy.
- **Multivariate Curve Resolution (MCR)** – Extracting information from hyperspectral datasets.

# Super-resolution Microscopy

The Nobel Prize in Chemistry in 2014 was awarded "for the development of super-resolved fluorescence microscopy."



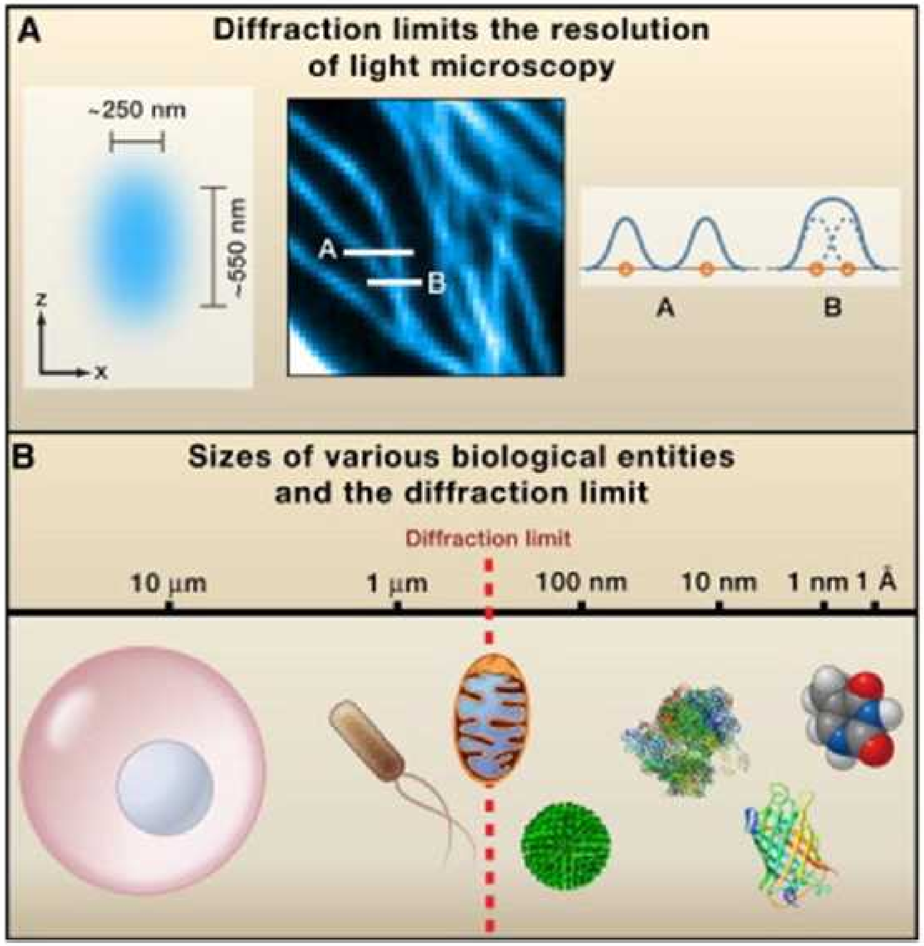

**"For a long time optical microscopy was held back by a presumed limitation: that it would never obtain a better resolution than half the wavelength of light. Helped by fluorescent molecules the Nobel Laureates in Chemistry 2014 ingeniously circumvented this limitation. Their ground-breaking work has brought optical microscopy into the nanodimension."**

Nobel Prize Press Release

Won by: Eric Betzig, Stefan W. Hell, & William E. Moerner

# Diffraction Limit

- Conventionally, resolution was limited by Abbe's Criterion: $D = \lambda/(2*NA)$
- Super-resolution microscopy bypasses the diffraction limit
- Two different methods super-resolution methods were developed – Stimulated Emission Depletion (STED) & Photoactivated Localization Microscopy (PALM)




http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/advanced-chemistryprize2014.pdf

# Visualizing Super-resolution

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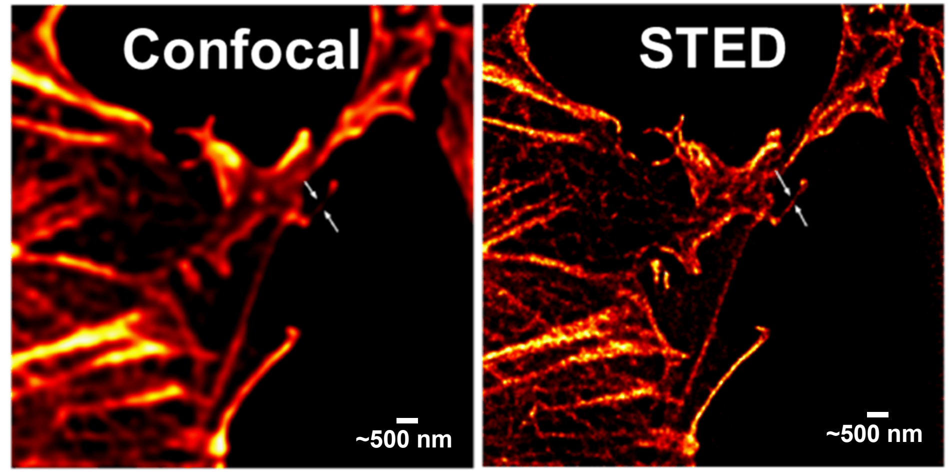
- PALM and STORM (stochastic optical reconstruction microscopy) are very similar techniques
- Both STORM/PALM and STED images show dramatic improvements in resolution
- While producing similar images, the processes for acquiring STORM/PALM and STED images are quite different

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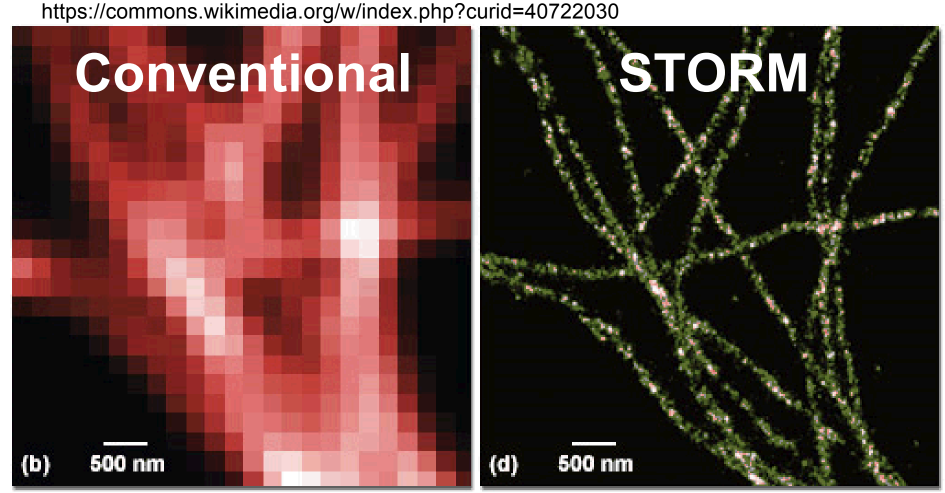
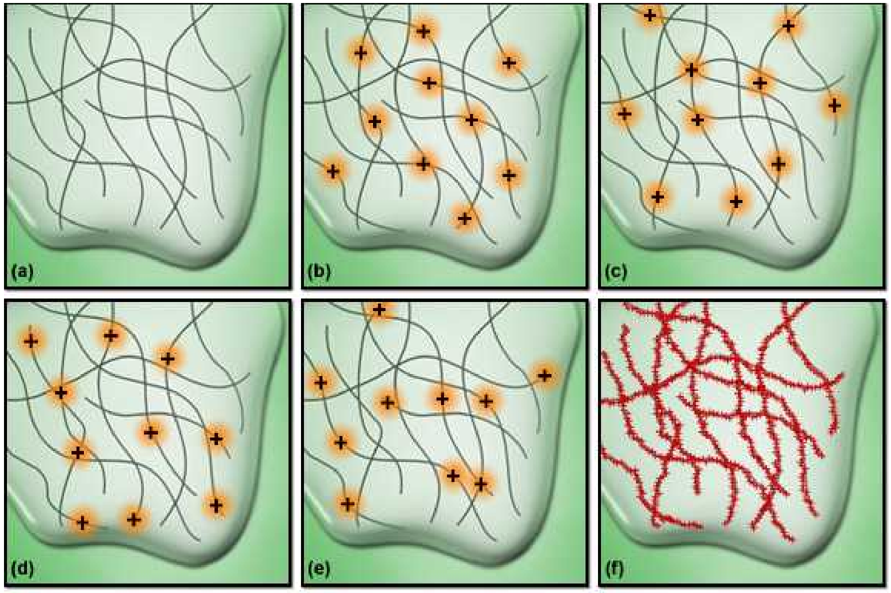



Adapted from http://www.microscopyu.com/articles/superresolution/stormintro.html

# How STORM/PALM Works

**Basic Principle of STORM**




http://www.microscopyu.com/articles/superresolution/stormintro.html

- **Acquire multiple images where each image contains a subset of the fluorophores.**
- **The multiple images combine to make a single STORM image.**

Advantages:

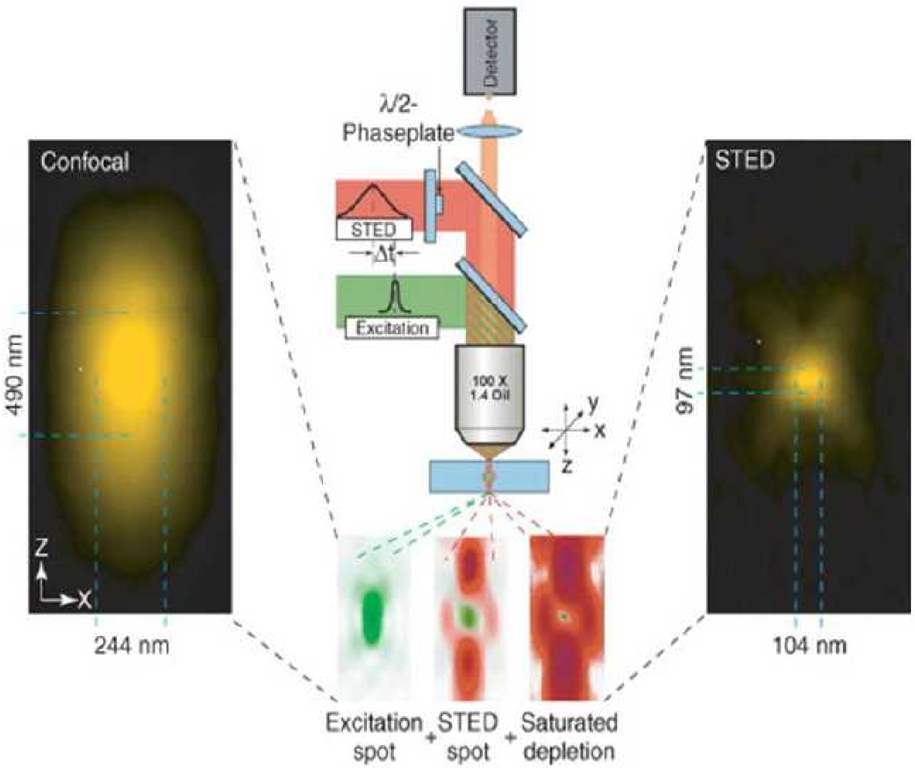
- No special optics – works with existing microscopes
- Better resolution (<10 nm)

Disadvantages:

- Acquiring many images limits time resolution
- Often requires special labels or buffer solutions
- Wide-field technique – **not easily compatible with hyperspectral imaging**

# How STED Works

### Basic Principle of STED




http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/advanced-chemistryprize2014.pdf

**Like confocal imaging with two co-aligned beams – one excitation beam and one depletion beam.**

## Advantages:

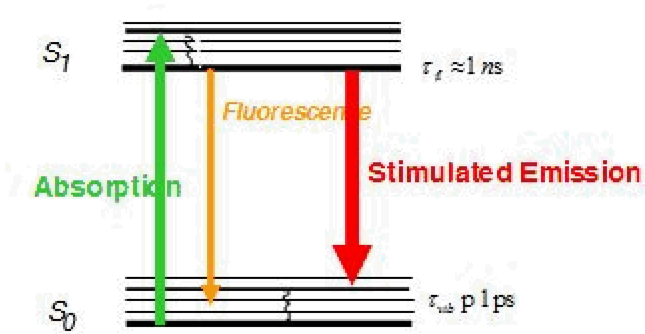
- Point-scanning technique – **can make hyperspectral**
- Compatible with wide range of fluorophores
- Improves both lateral and axial resolution

## Disadvantages:

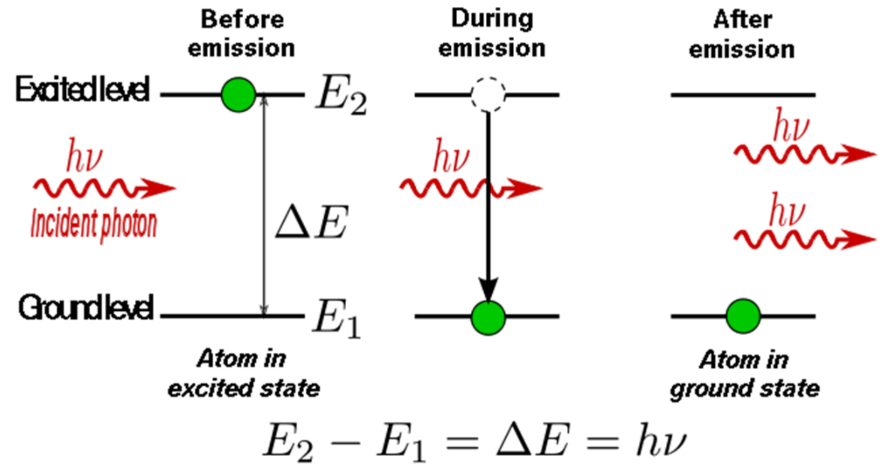
- Point-scanning limits time resolution
- Requires complicated alignment – cannot simply use existing microscope

# Stimulated Emission Details

### Basic Principle of Stimulated Emission

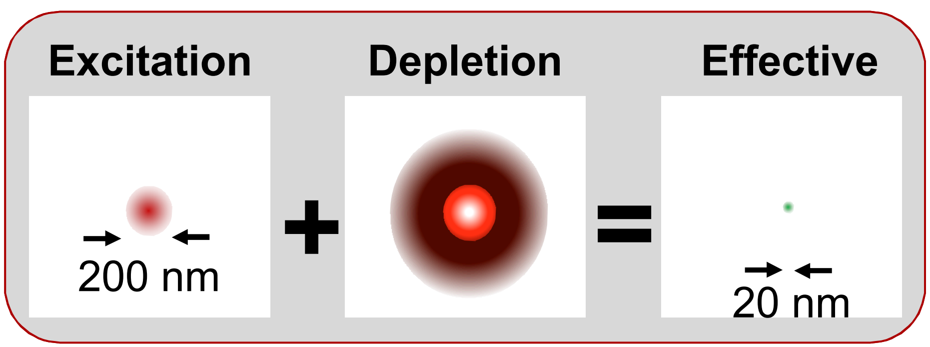



https://en.wikipedia.org/wiki/File:STED_Jablonski.jpg




By V1adis1av - Own work, GFDL, https://commons.wikimedia.org/w/index.php?curid=3983414

### Generating the STED Point Spread Function (PSF)




**Neither beam PSF can exceed the diffraction limit, but the effective PSF can!**

# SR Microscopy Takeaways

**Super-resolution (SR) microscopy:**

- Bypasses the diffraction limit
- Can obtain images with resolution <50 nm
- Can monitor protein interactions

**STORM/PALM:**

- Offers SR microscopy with some advantages
- Not easily compatible with hyperspectral imaging

**STED:**

- Point-scanning SR microscopy technique
- **Compatible with hyperspectral imaging**

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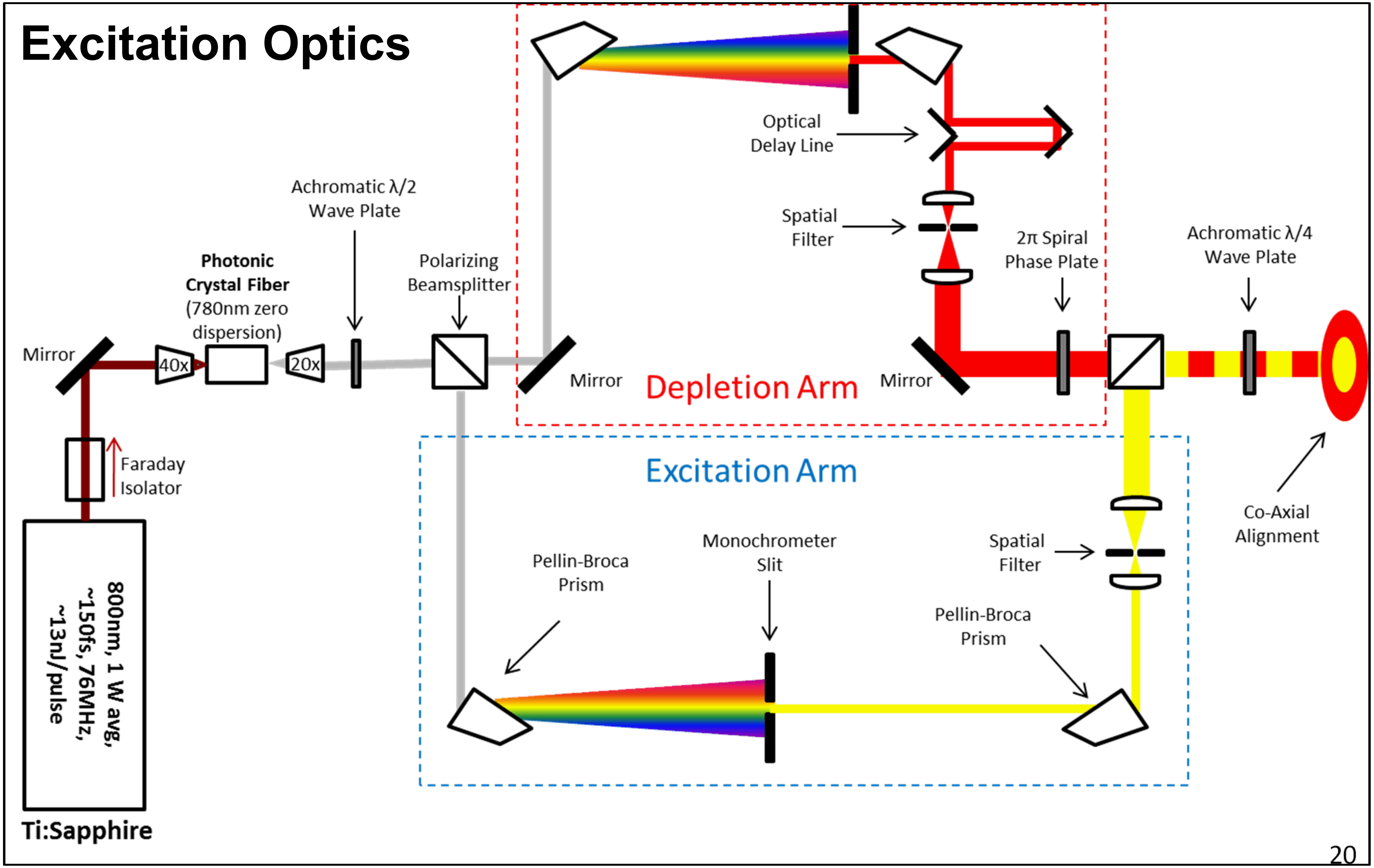
# Why Hyperspectral STED

- First hyperspectral super-resolution microscope
- Of super-resolution techniques, STED is most compatible with hyperspectral imaging
- Combines the advantages of super-resolution and hyperspectral microscopy
- Super-resolution potentially allows monitoring interactions of multiple proteins at nm resolution – requires the ability to distinguish multiple labels
- Hyperspectral imaging is often sensitive to changes in the fluorophore environment – combining with super-resolution would allow nm-mapping of the cellular microenvironment

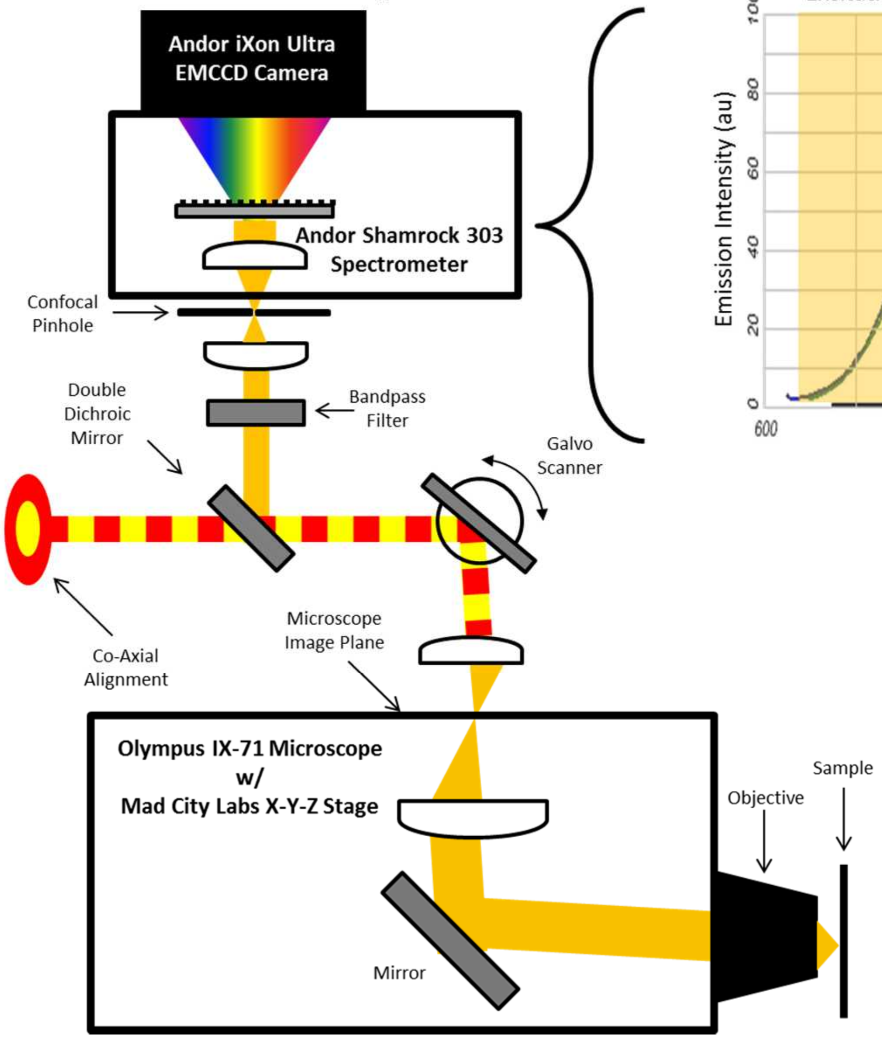
Idea credit: Timlin, J. A. and J. S. Aaron (2014). Hyperspectral stimulated emission depletion microscopy and methods of use thereof, Patent US8686363 B1

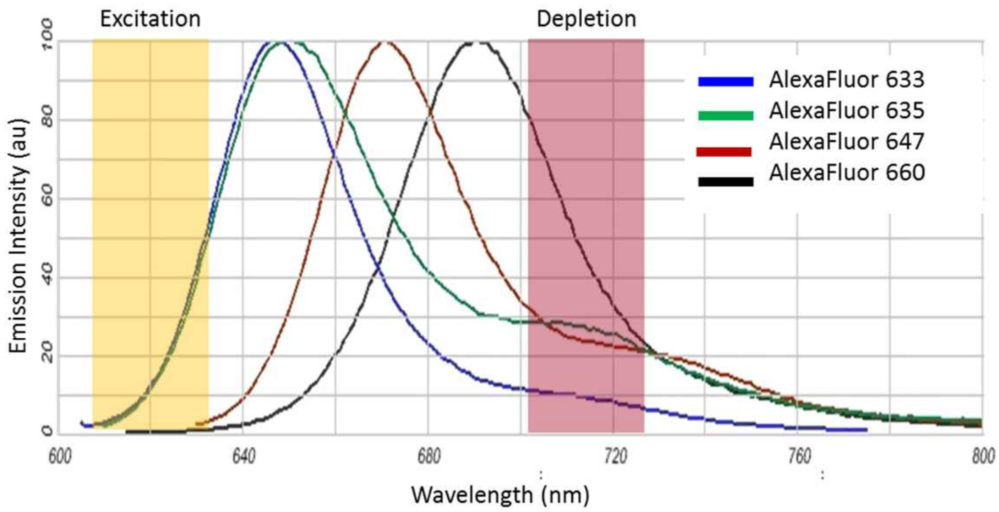
# Building a Hyperspectral STED



# Building a Hyperspectral STED

## Detection Optics




### Design Considerations

- Tunable wavelength for both excitation and depletion beams
- Can be optimized for any STED fluorophore with exchange of a single optic (the dichroic)

# Hyperspectral STED Takeaways

## Sandia's Hyperspectral STED:

- Will be the first hyperspectral super-resolution microscope
- Combines the advantages of super-resolution microscopy with hyperspectral imaging
- Greatly facilitates using multiple different labels to observe interactions within cells (e.g. protein interactions)
  - Capable of distinguishing small spectral differences
  - Tunable beam wavelength allows a broader selection of possible dyes

# Outline

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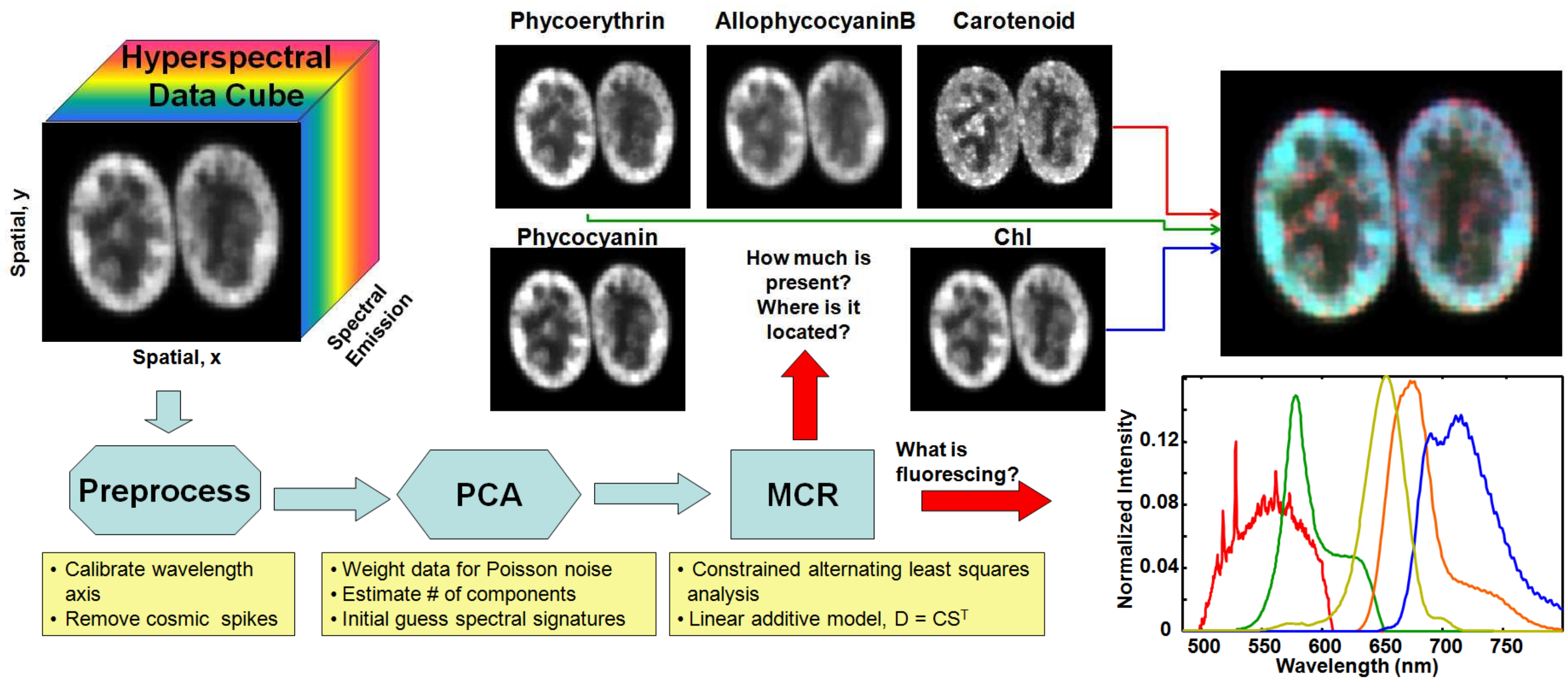
# Data Analysis is Critical

## Overview of Analysis Pipeline




MCR simultaneously solves for both the concentration and the spectral components without *a priori* info.

# Common Spectral Image Analysis Methods

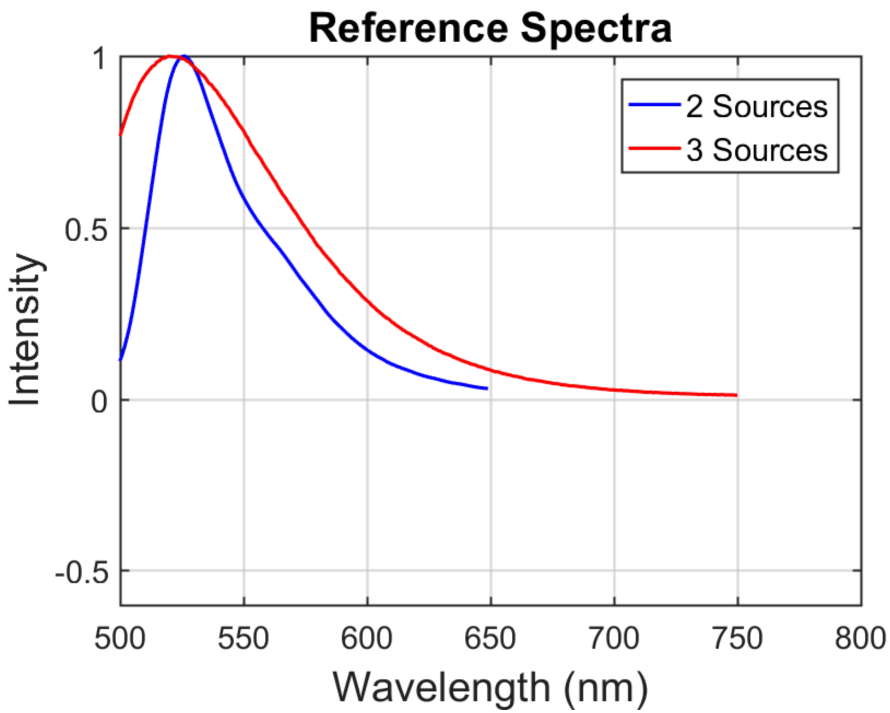
Required knowledge of system behavior

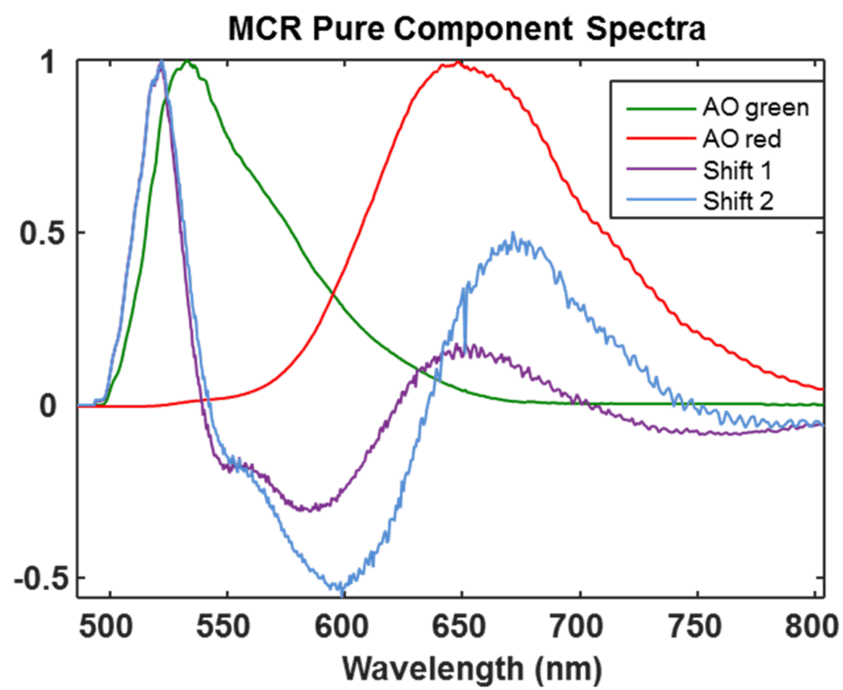
Mathematical Complexity, overall accuracy

- Univariate methods
  - → Band integration, peak height, peak positions
  - → **Isolated bands, no spectral interference**
- Multivariate methods
  - Unmixing methods
    - → Least squares prediction based
    - → ***A priori* knowledge**
      - → Spectral shapes or pure image pixels
  - Factor Analysis methods
    - → Principle components analysis (PCA) , Factor analysis, SIMPLISMA, self modeling curve resolution, multivariate curve resolution **(MCR)**
    - → Data defines
    - → **No *a priori* knowledge** of spectral shapes/pure pixels
      - → Need number of components
      - → Constraints to narrow solution space

# Why Aren't the Spectra Known?

"Acridine Orange is a cell-permeant nucleic acid binding dye that emits **green fluorescence** when bound to dsDNA and **red fluorescence** when bound to ssDNA or RNA." - ThermoFisher Scientific




**Reference spectra are not always available, and when available do not always capture the complete spectral properties.**

# MCR Assumptions

1. **Assumes a linear additive model:**

$$D = CS^T + E$$

| | |
|---|---|
| D = Data matrix | nPoints X nWavelengths |
| C = Concentrations matrix | nPoints X nComponents |
| $S^T$ = (Spectra matrix)$^{Transpose}$ | nComponents X nWavelenths |
| E = Noise (error) matrix | nPoints X nWavelengths |

**2. The # of spectral components is known or can be estimated**

# Why MCR vs. PCA?

- Three related techniques
  - Multivariate Curve Resolution (MCR)
  - Principal Component Analysis (PCA)
  - Independent Component Analysis (ICA)
- All resolve the data into pure spectral components and concentrations without a priori information
- **Different Constraints**
  - MCR – Physical and Chemical Constraints (e.g. no negative concentrations, no negative intensities)
  - PCA – Linearly uncorrelated
  - ICA – Statistically Independent

# Basic Operation

$$D = CS^T + E$$

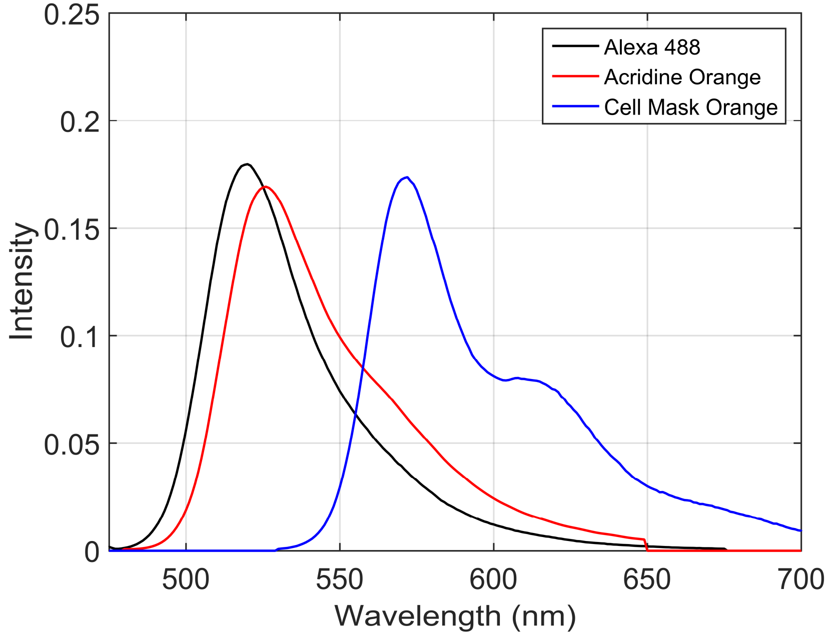
- D is known
  - If C were known, could solve for S
  - If S were known, could solve for C
- Constrained Alternating Least Squares
  1. Provide an initial guess for S (or C)
  2. Solve for C based upon current S guess, enforcing constraints
  3. Solve for S based upon current C guess, enforcing constraints
  4. Repeat steps 2 & 3
  5. **Iterations converge on solution**

# Advantages of MCR

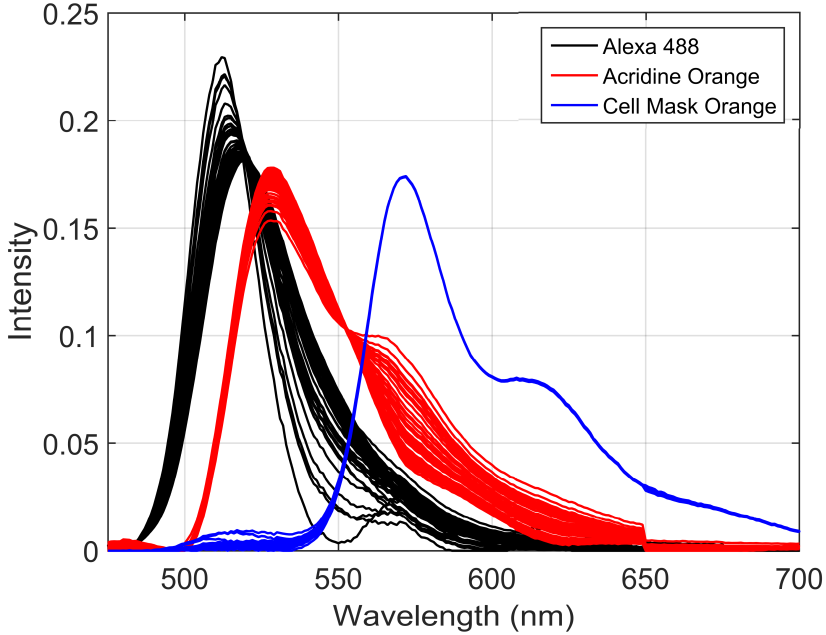
- Extracts underlying relationships from complex data sets
- No *a priori* knowledge needed
- Signals below the noise level can be detected!
- Physically meaningful constraints
  - Negative concentrations not allowed
  - Negative intensities not allowed
- Efficient algorithms developed at Sandia
  - Keenan, M. R. and P. G. Kotula (2003). Apparatus and system for multivariate spectral analysis, Google Patents.

# Ongoing Work – Reduce Rotational Ambiguity

**Pure Spectra**




**MCR Spectra (100 different runs)**

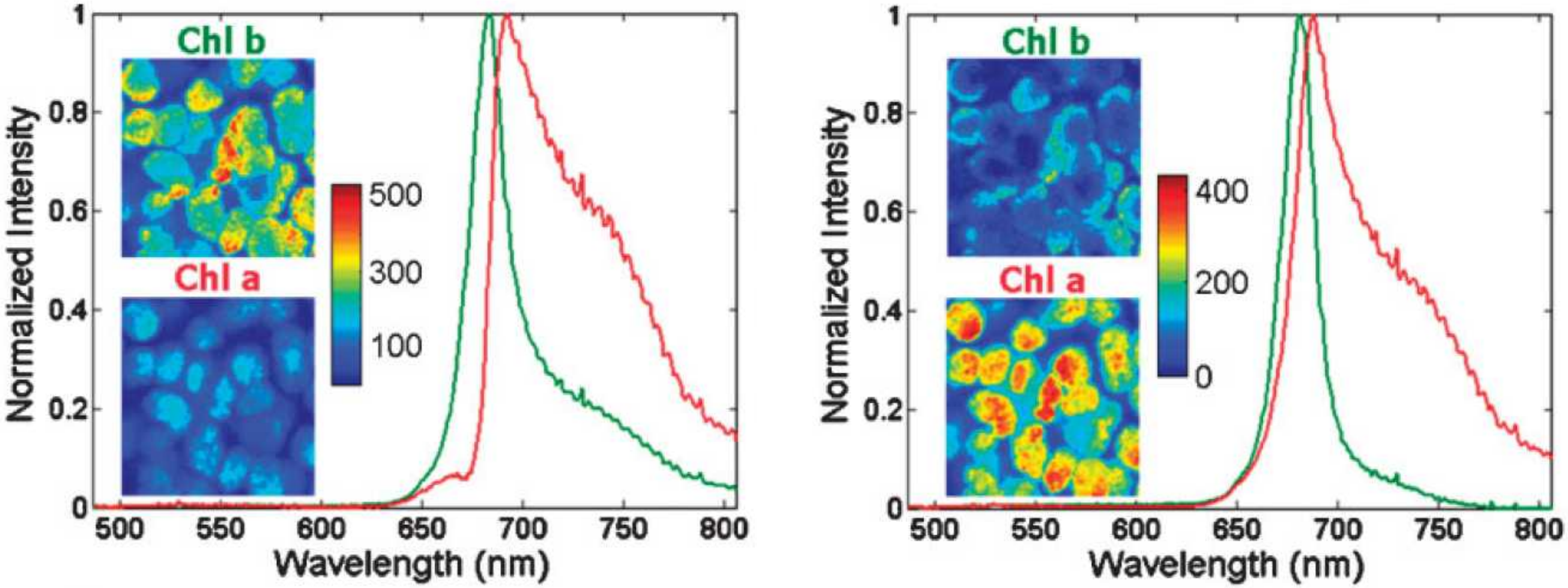



**Rotational ambiguity** – MCR may not converge or there may not be a single unique solution!

MCR results for 100 runs on a simulated data set for a 100 x 100 pixel hyperspectral image averaging ~55000 counts for each spectrum initialized with random spectra.

# Ongoing Work – Improved Weighting

**Without Weighting** | **Weighted for Poisson & Gaussian Noise**




Jones, H. D. T., et al. (2008). "Weighting hyperspectral image data for improved multivariate curve resolution results." Journal of Chemometrics **22(9-10): 482-490.**

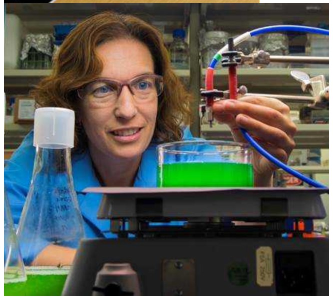
## Proper weighting makes a major difference!

Working on improving the weighting to correctly account for all sources of noise, including the pre-processing steps.

# Overall Summary

- Super-resolution & hyperspectral microscopy are valuable tools
  - Hyperspectral facilitates multicolor labeling and can reveal new features
  - Super-resolution offers the potential to monitor protein interactions at distances comparable to the protein sizes
- Hyperspectral STED will provide the first microscope combining both
- Data analysis is critical, where MCR provides the best combination of experimental flexibility and accuracy when analyzing hyperspectral data

# Acknowledgements

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Jeri Timlin – Sandia postdoc

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## Materials:

Pakrasi laboratory at Washington University, St. Louis for the *Cyanothece* in the example.

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