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Hyperspectral Fluorescence Imaging of Biological Systems

David M. Haaland
Sandia National Laboratories
Albuquerque, NM 87185-0895

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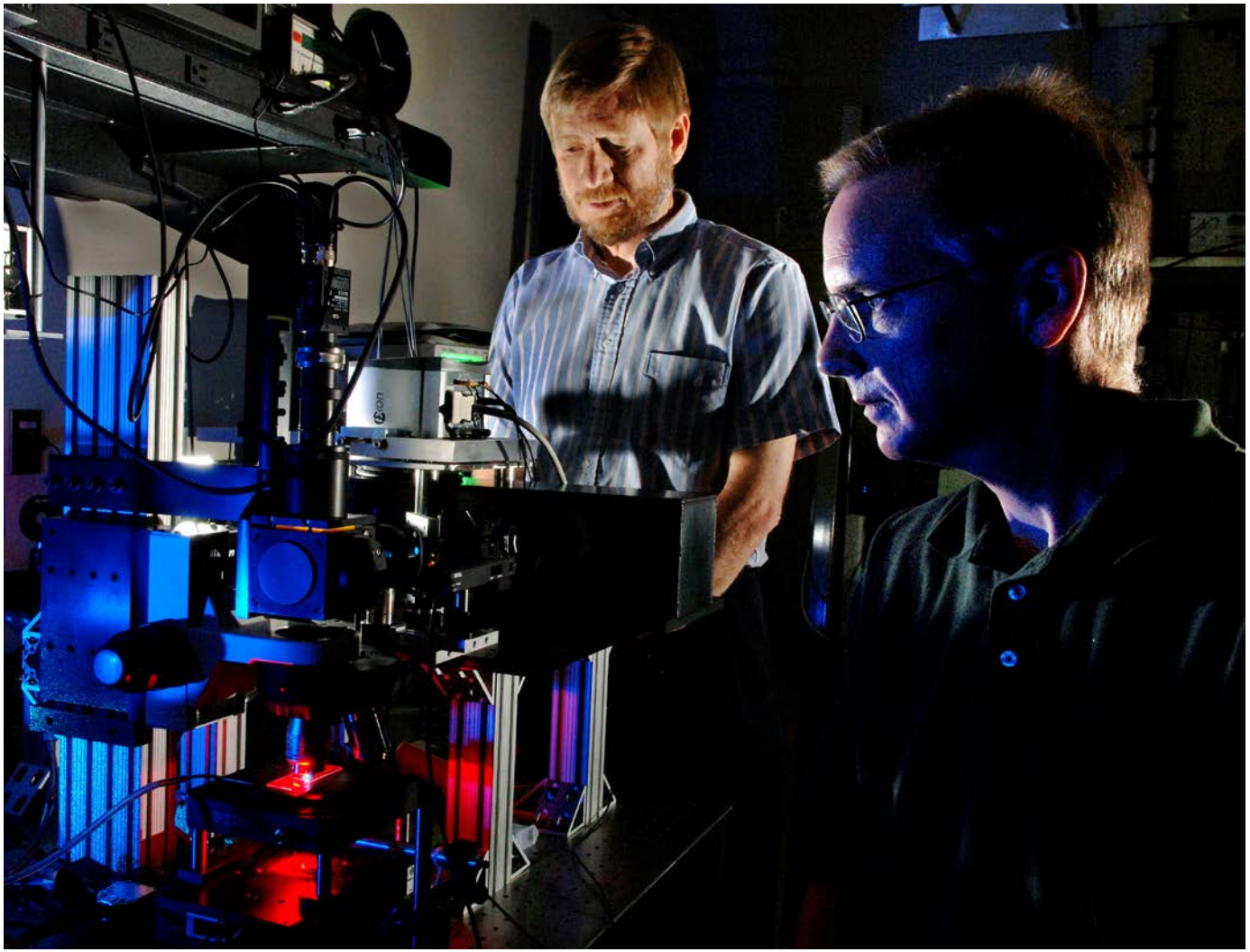
Overview

- 3D hyperspectral confocal microscope
  - Advantages of hyperspectral imaging
  - Microscope design and performance
- Multivariate curve resolution (MCR)
  - “Discover” and quantitate unknown fluorophores
  - Quantitative analysis without standards
- Biological applications
  - Imaging of photosynthetic pigments in *Synechocystis* cyanobacteria and corn leaves
  - Host-pathogen interactions - live cell imaging of HeLa and macrophage cells
  - Imaging quantum dots in solutions and live cells

Advantages of Multivariate Spectral Image Analysis

- Excitation with single laser, readout with single image scan
- Many fluorophores monitored simultaneously without cross talk (increased information per image)
- Improved accuracy, dynamic range, reliability, and understanding
- All emission sources, including autofluorescence and impurity emission, can be readily “discovered” and quantified at each pixel with implementation of Sandia’s fast and rigorous MCR algorithms.

Hyperspectral Confocal Microscope



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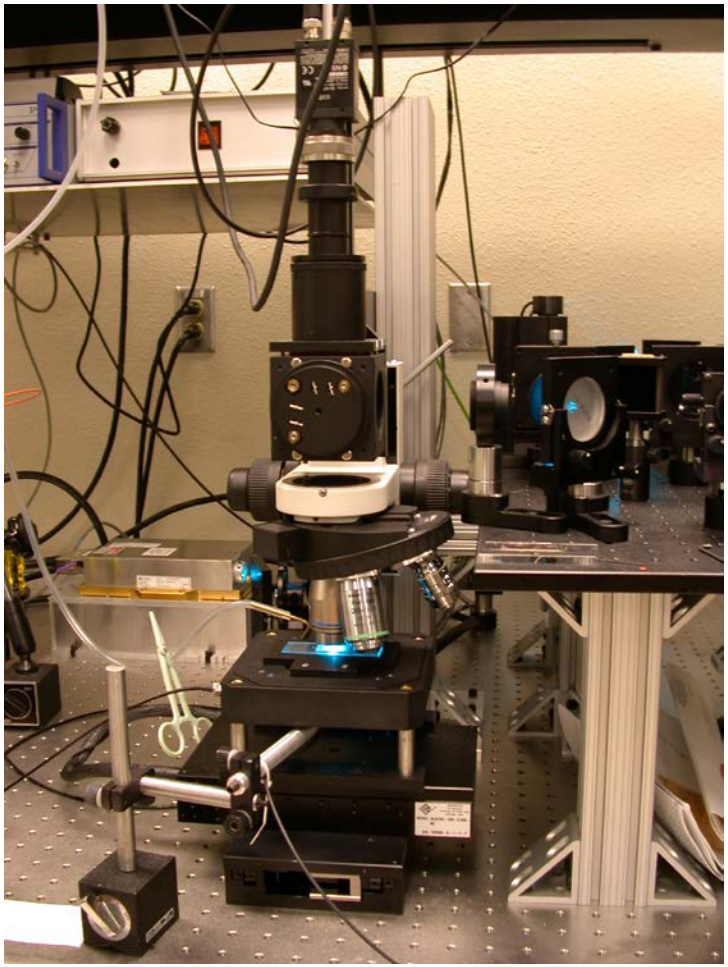
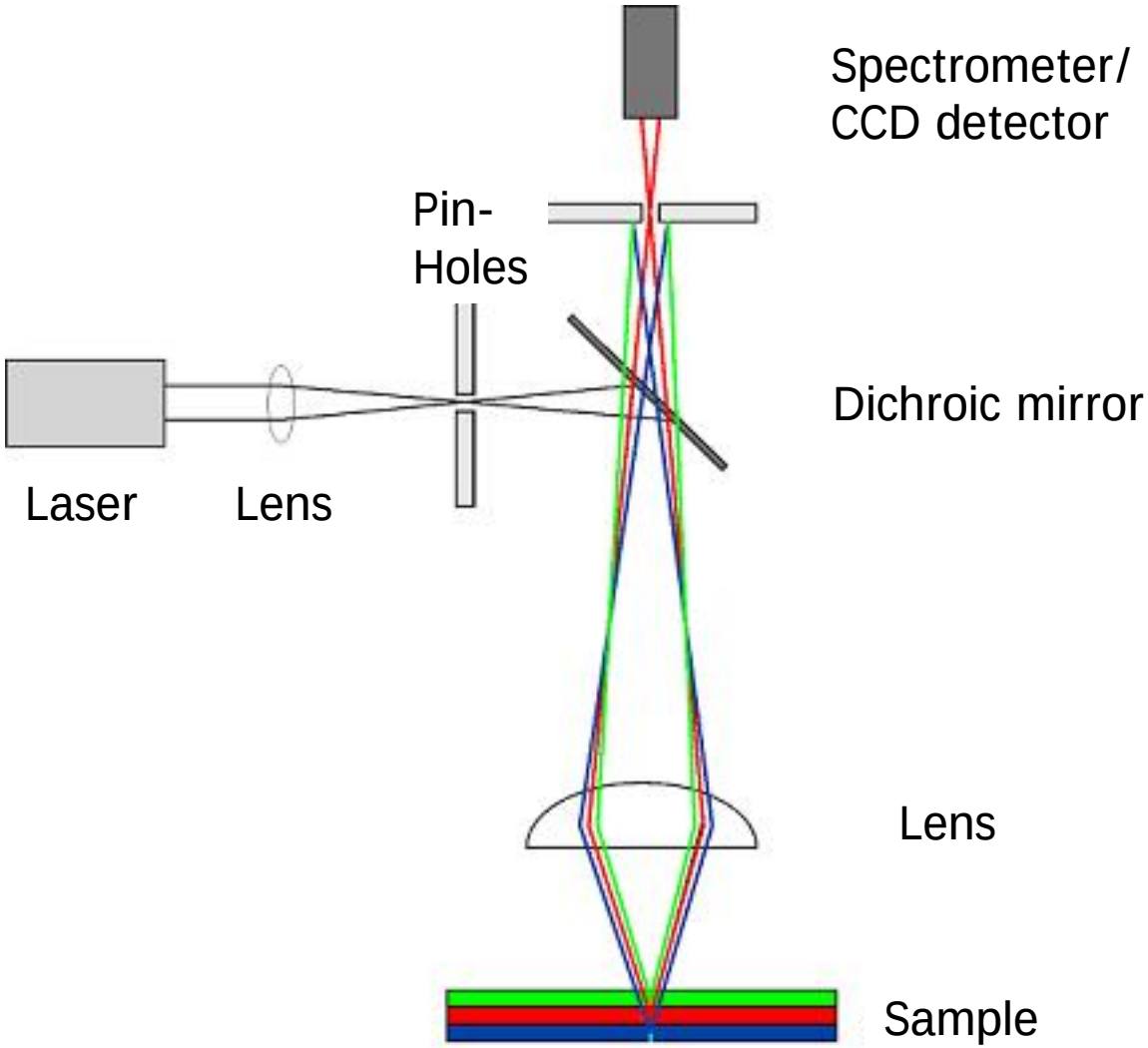
3D Hyperspectral Confocal Fluorescence Microscope

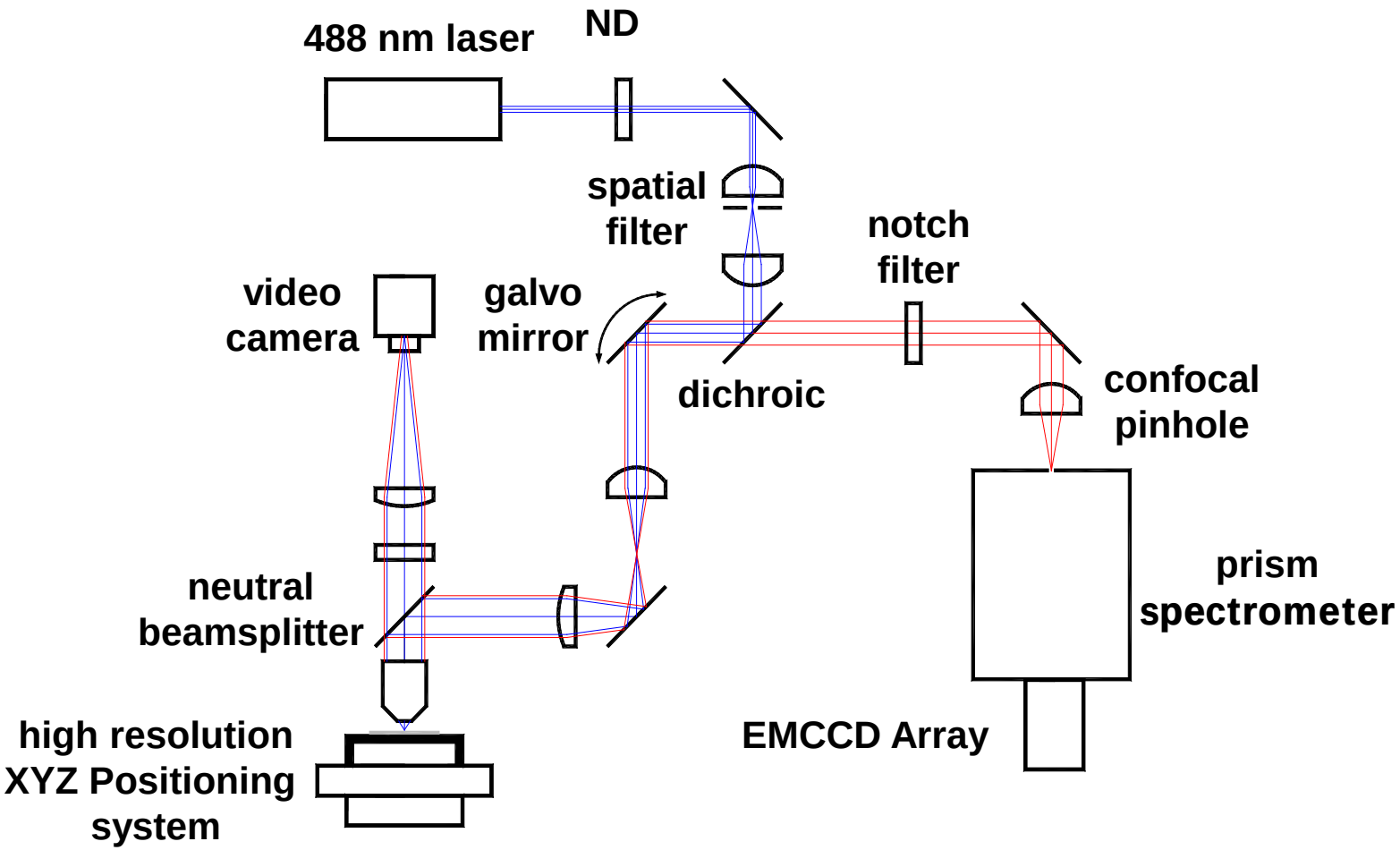
- **Fully confocal design**
  - high spatial resolution
  - optical sectioning
- **High optical throughput**
  - prism spectrometer
  - electron multiplying CCD
- **Spatial Translation**
  - 10 cm in x and y, 100 μm in z
- **Performance Specifications:**
  - 488 nm laser excitation
  - 10x, 20x, 60x, 100x objectives
  - Lateral Resolution = 250 nm
  - Axial Resolution = 600 nm
  - Spectral range 490-800 nm
  - Spectral resolution = 1 - 3 nm
  - **Acquisition rate = 8300 spectra/s**



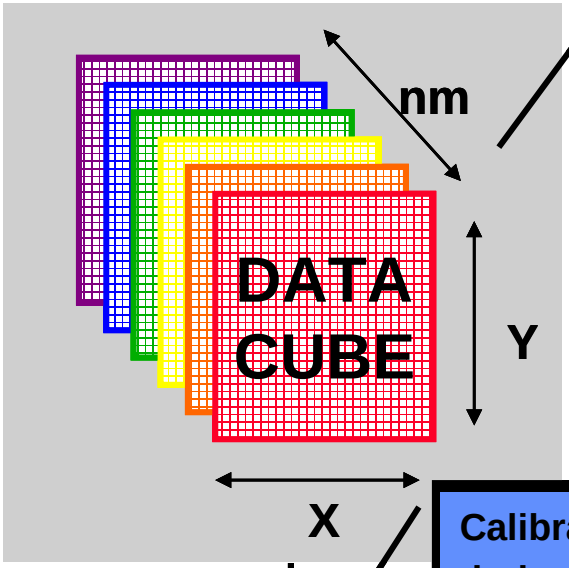
Confocal Principle



Microscope Layout



Data Analysis Flowchart



512 wavelength channels
210 x 210 x 18 voxels
~800 Mb, ~1 min

WHAT?

Spectral Components

WHERE?
HOW MUCH?

Concentration Maps

Calibrate, despike, subtract dark, weight data for noise, PCA analysis

Preprocess

Random #s, literature, PCA results – normalized to unit length

Initial Estimates

MCR

Constrained alternating least squares analysis
Linear additive model applies $D=CS^T$
# of comps known

Rigorous non-negativity & equality constraints
Efficient algorithms
Un-weight results

Multivariate Curve Resolution Used to Analyze Hyperspectral Images

- Assumption: linear additive model:

  $\mathbf{D} = \mathbf{C}\mathbf{S}^{T} + \mathbf{E}$

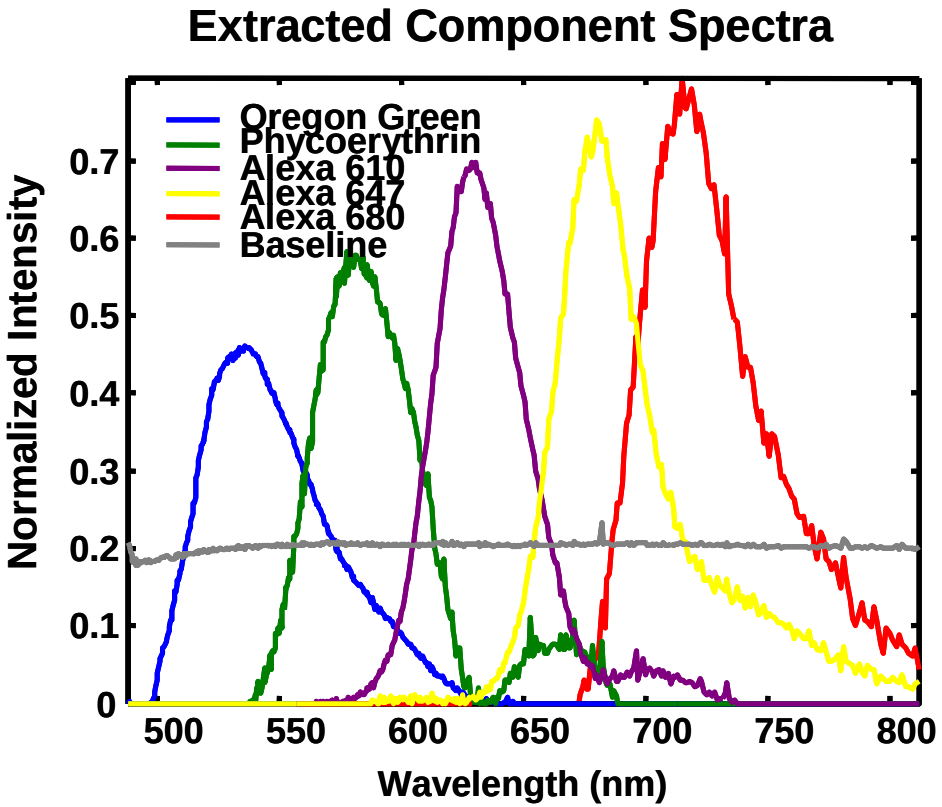
- Solve $\mathbf{D} = \mathbf{C}\mathbf{S}^{T}$ in least squares sense
  - $\hat{\mathbf{C}} = \mathbf{D}\mathbf{S}^{T+}$ (CLS prediction)
  - $\hat{\mathbf{S}}^{T} = \hat{\mathbf{C}}^{+}\mathbf{D}$ (CLS calibration)
  - Rotational ambiguity, infinite number of solutions

D represents intensities of all spectra in image
C is concentration matrix for all spectral components
S is pure-component spectra from image
^ is least squares estimate, $^{+}$ is matrix pseudoinverse

Multivariate Curve Resolution (continued)

- Apply constraints to obtain realistic solutions.
  - Constrain spectra & concentrations to be non-negative.
  - Equality constraint, e.g., all or portions of pure-emission spectrum known, some concentrations known, functional form of spectral baseline known
  - Monotonic and unimodal constraints
  - Employ rigorous least squares for all constraints.
  - Use new efficient algorithms developed at Sandia.
  - Apply weighted least squares (weighting includes Poisson and read noise sources).
- Iterate CLS prediction and CLS calibration steps until converged.

Images of Monolayer of 5 Fluorophores on 2.5 µm Silica Beads



20x objective, NA= 0.75

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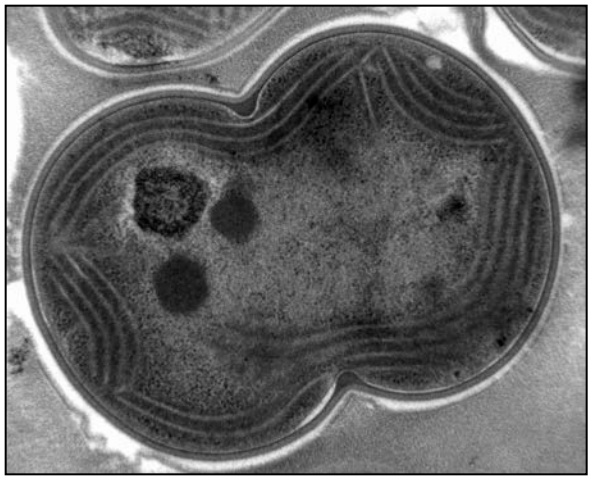
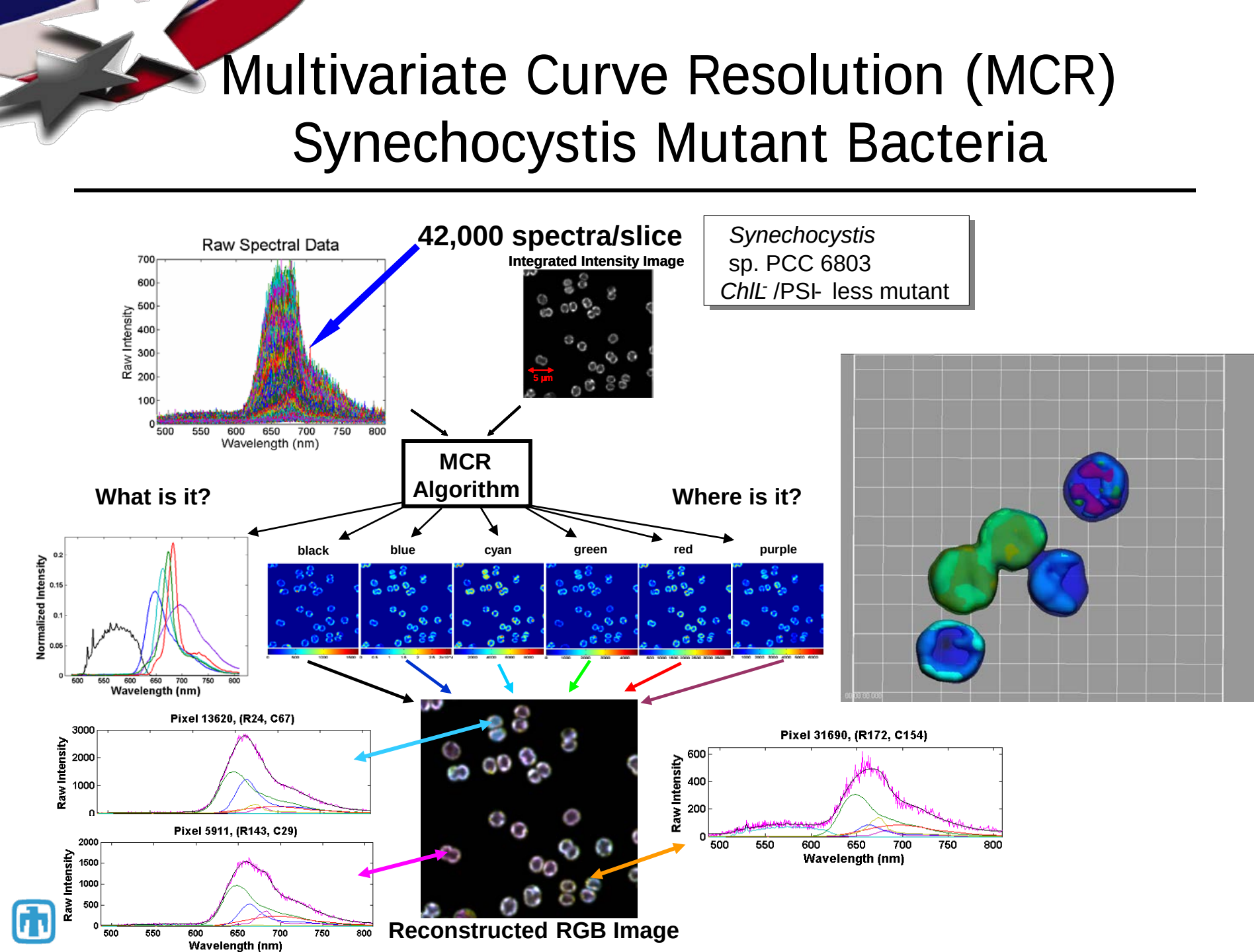
Hyperspectral Imaging of Cyanobacteria

- Responsible for 30-50% of global carbon fixation
- Main photosynthesizers in the open ocean
- Use hyperspectral imaging to identify and map photosynthetic pigments in 3 dimensions
  - Native fluorescence of *Synechocystis* sp. PCC 6803
  - Wild-type and mutant strains used to identify spectral components and map their locations in 3 dimensions

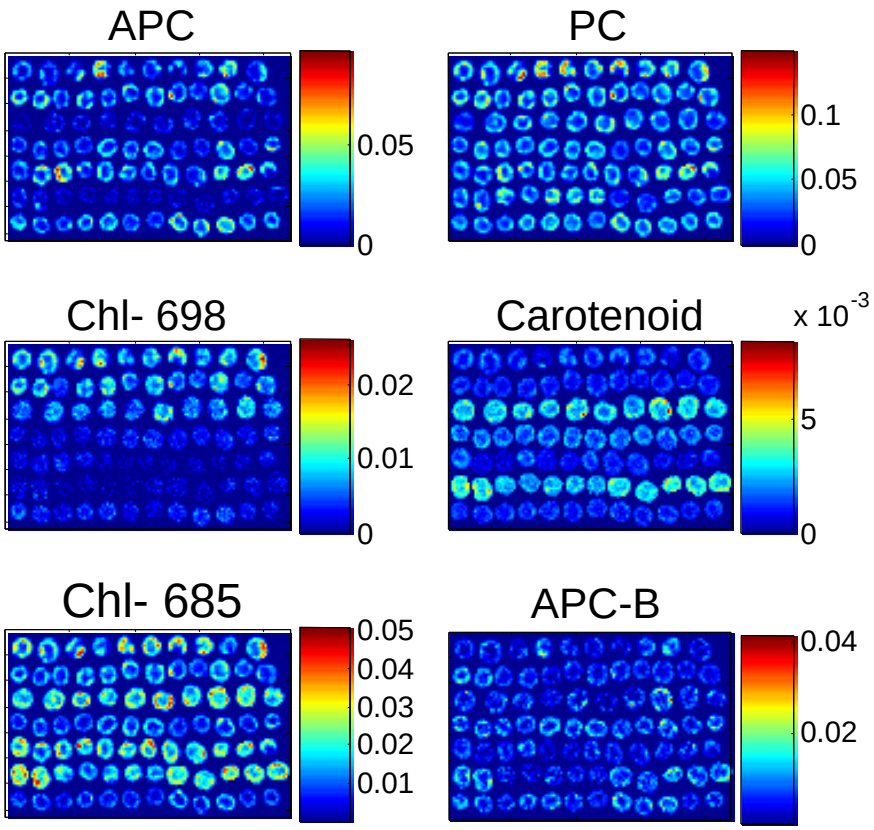


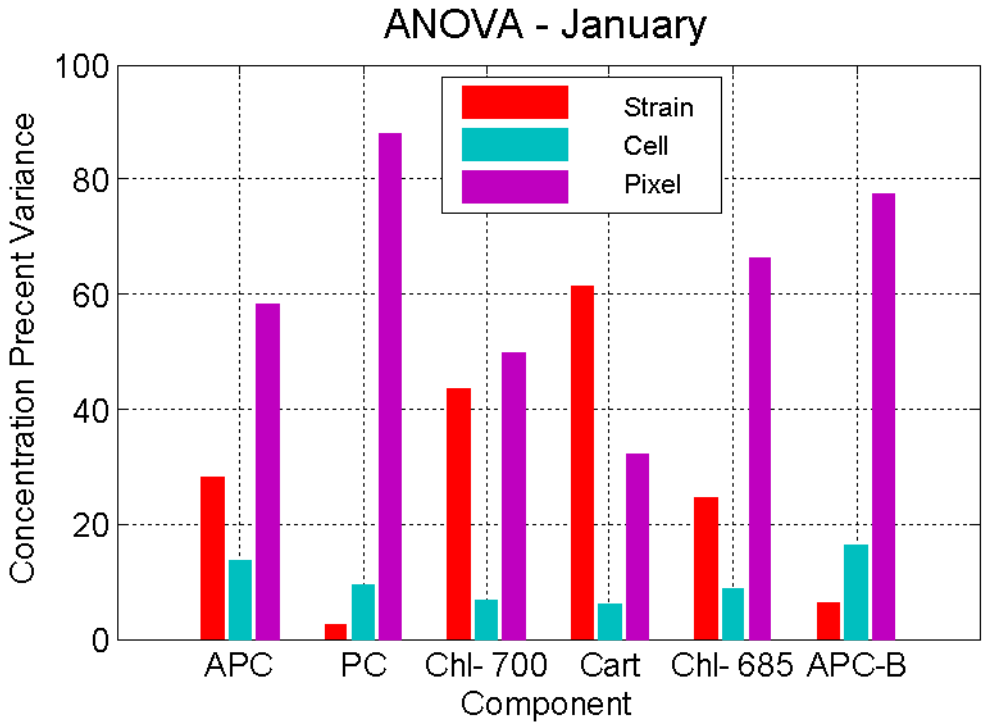
Multivariate Curve Resolution (MCR) Synechocystis Mutant Bacteria



Final MCR Concentrations and ANOVA Calculations

Normalized Concentrations





The cells within each strain are the most homogeneous.

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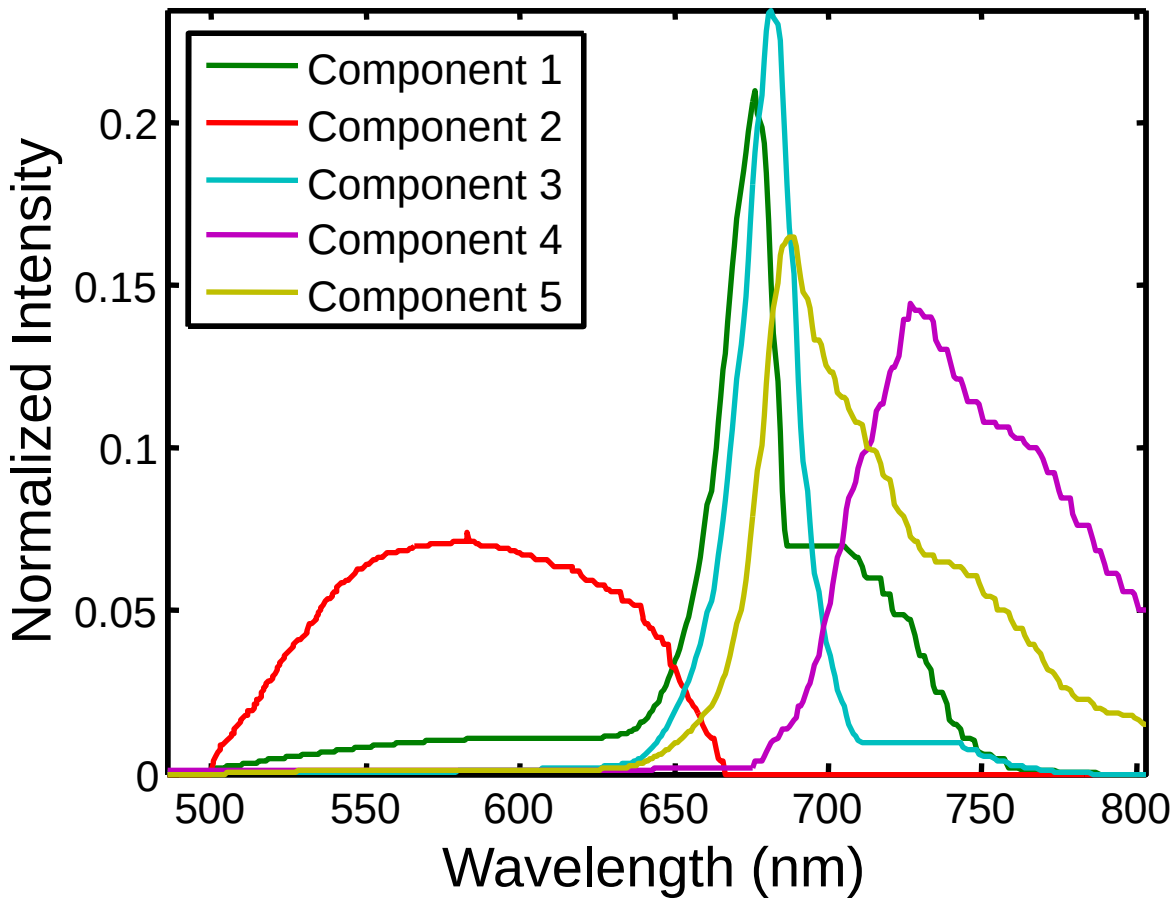
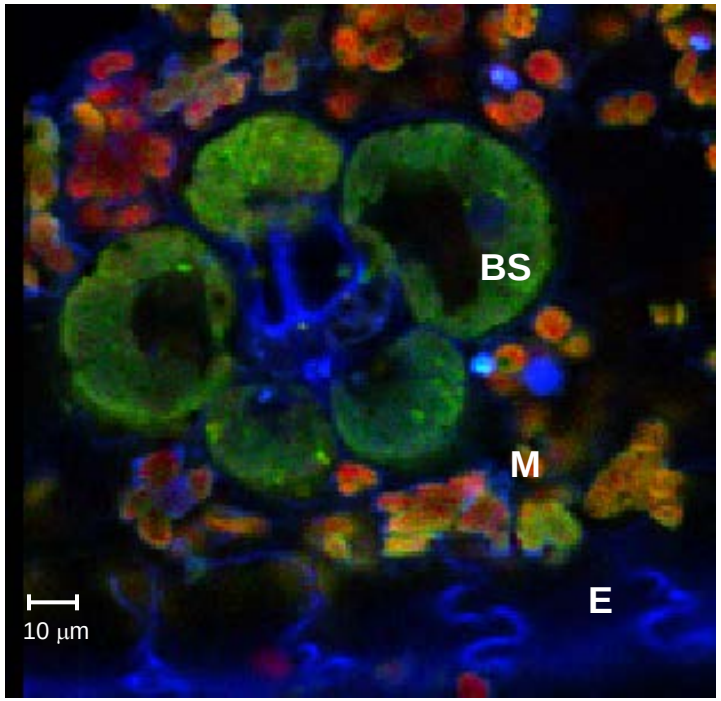
MCR Pure Emission Components from Wild-type Corn Leaf Section

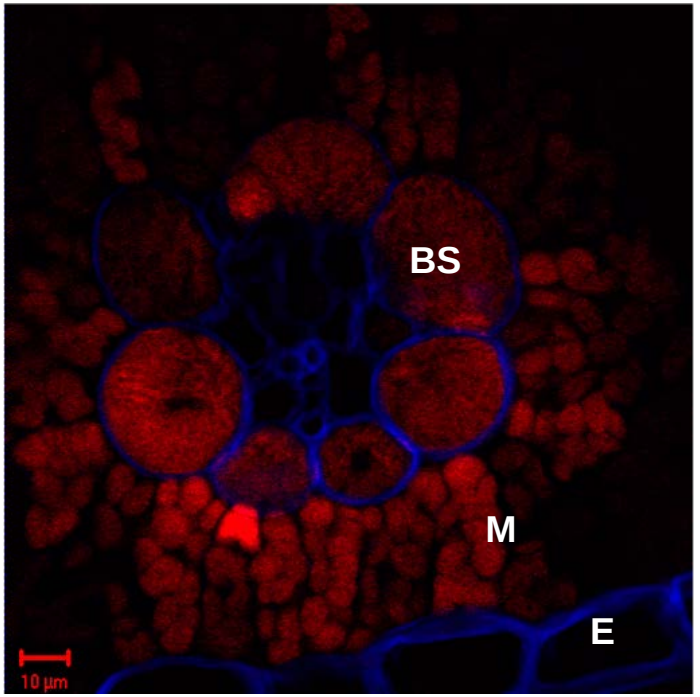


Comparison of Hyperspectral and Filter-based Confocal Microscope Images

Hyperspectral Image



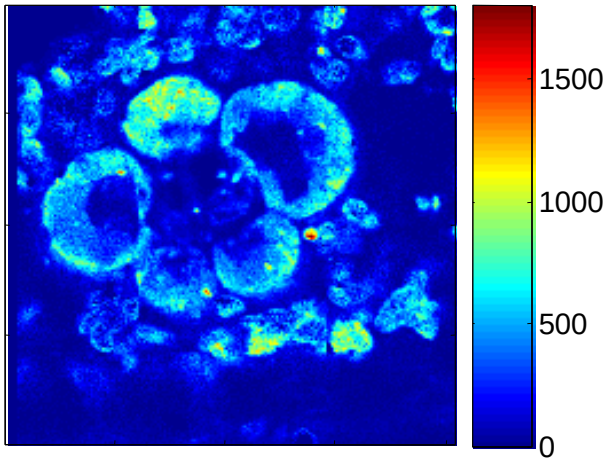
Commercial Filter-Based Image



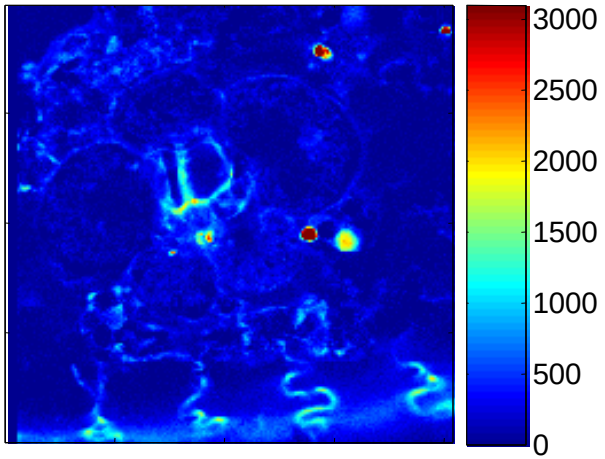
Cross-section of a corn leaf. BS, bundle sheath; M, mesophyll; E, epidermis

Relative Emission Component Intensities (Slice 3)

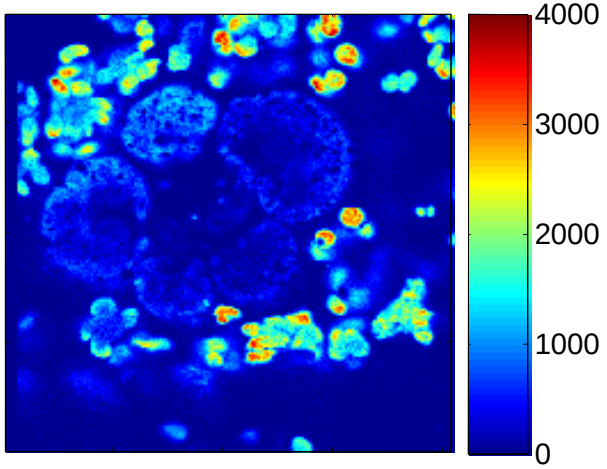
Component 1



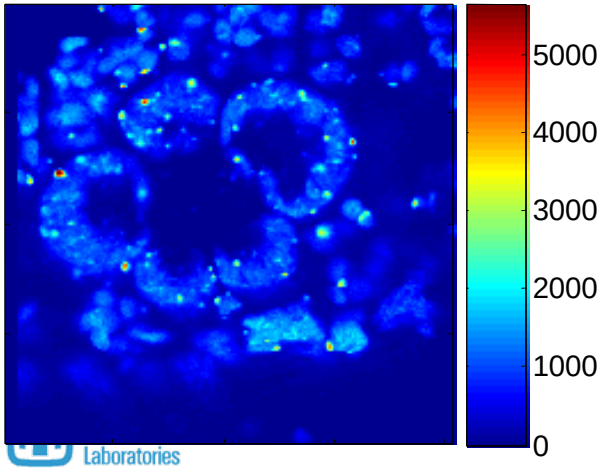
Component 2



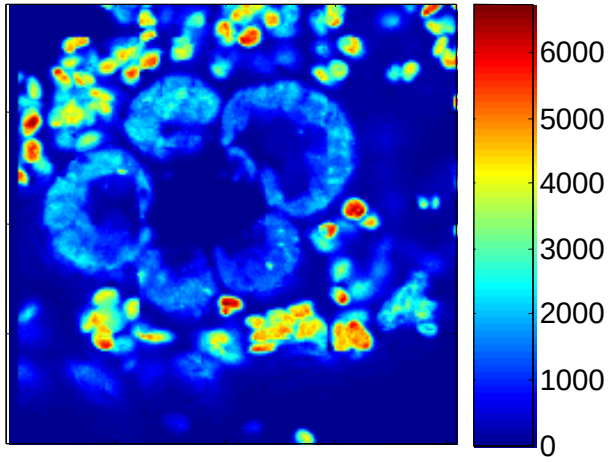
Component 3



Component 4



Component 5



Component 3 has been contrast enhanced

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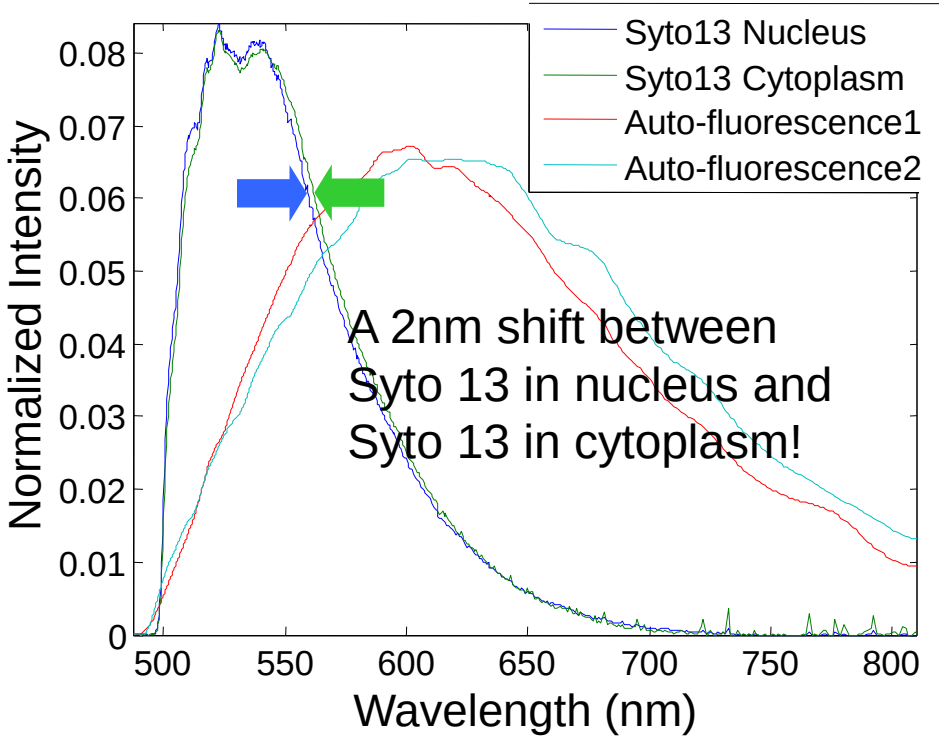
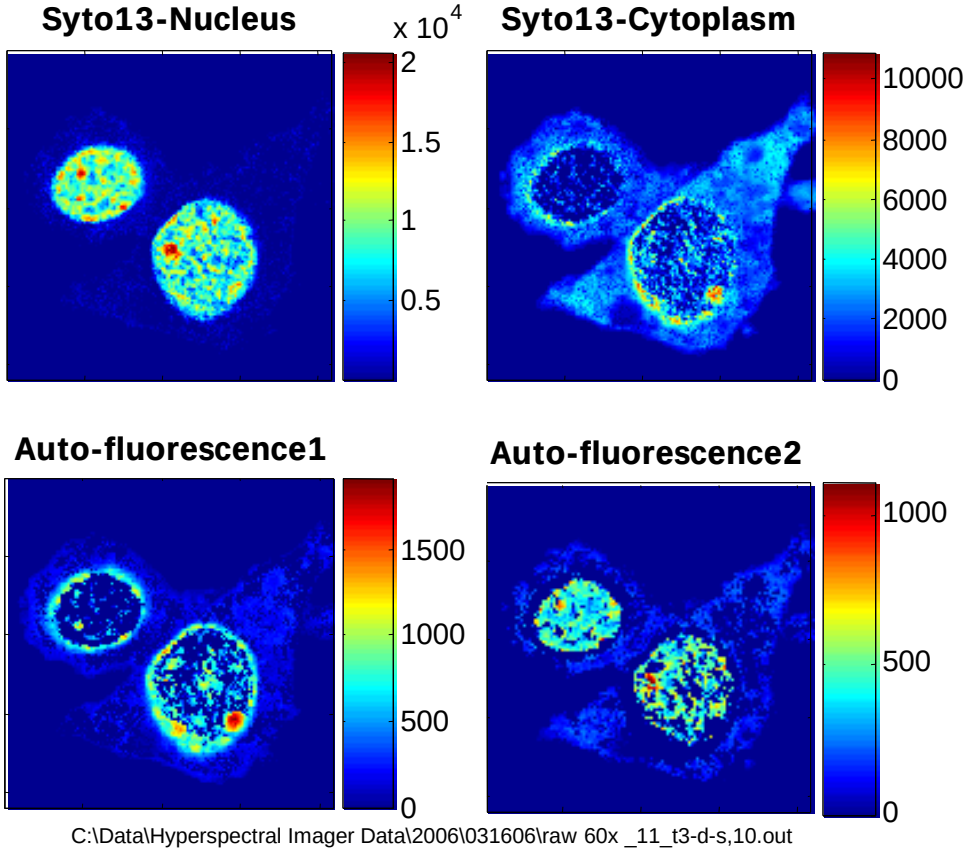
Power of HSI and MCR for Spatial Differentiation (Macrophage Cells)





Sensitive to very small spectral shifts, e.g., environmental changes

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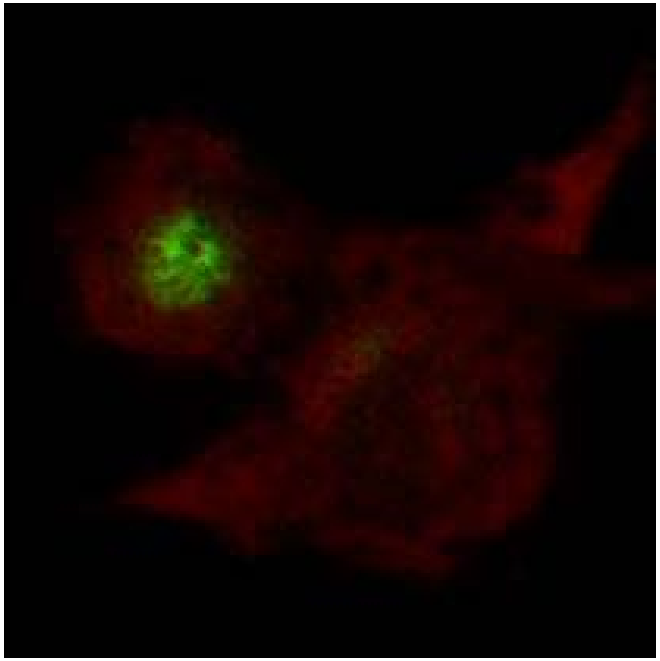
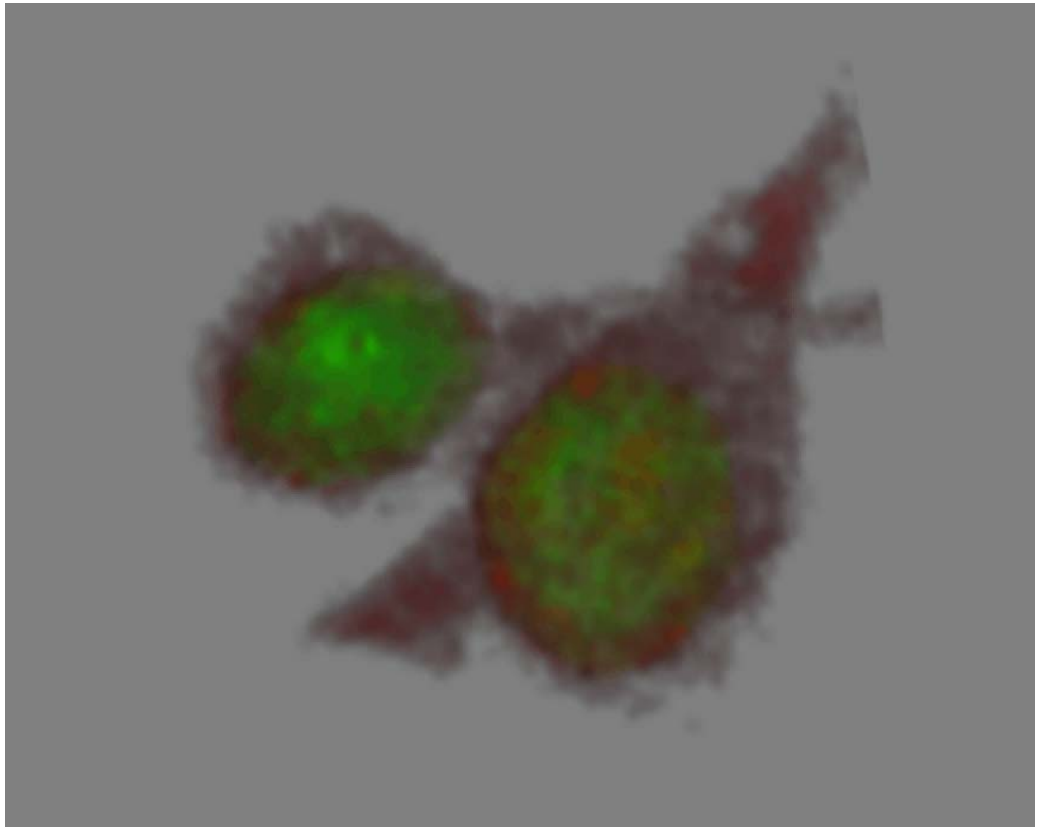
Syto 13 Stained Macrophage Cells 3D Images from MCR Analysis of HSI





HSI Volume Rendering of HeLa Cells

Image #1

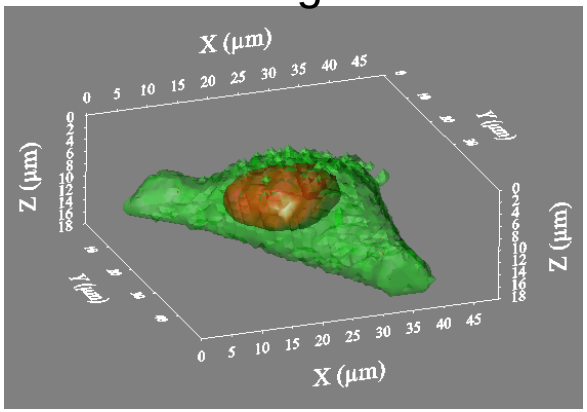


Image #2

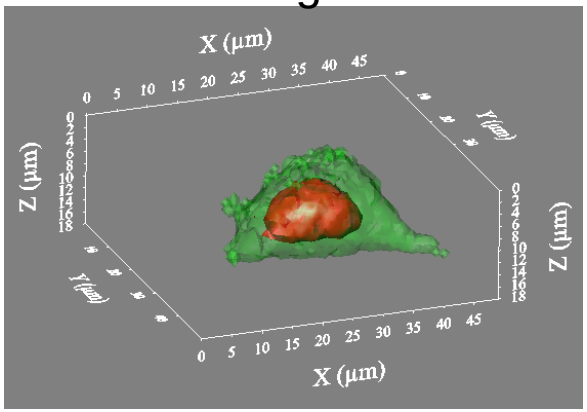


Image #3

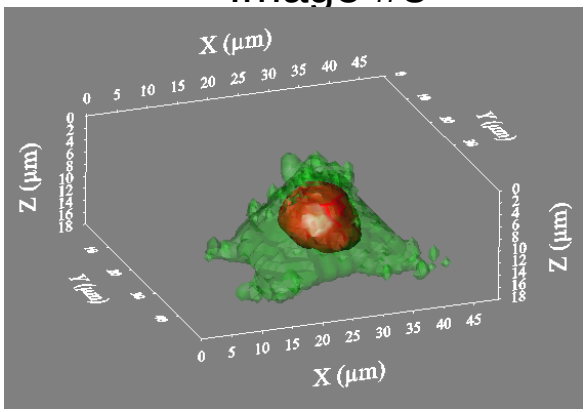
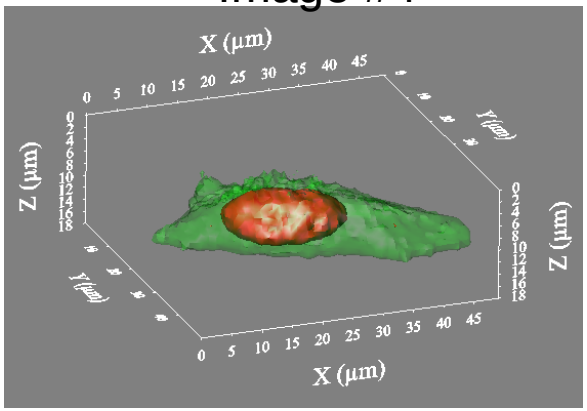


Image #4



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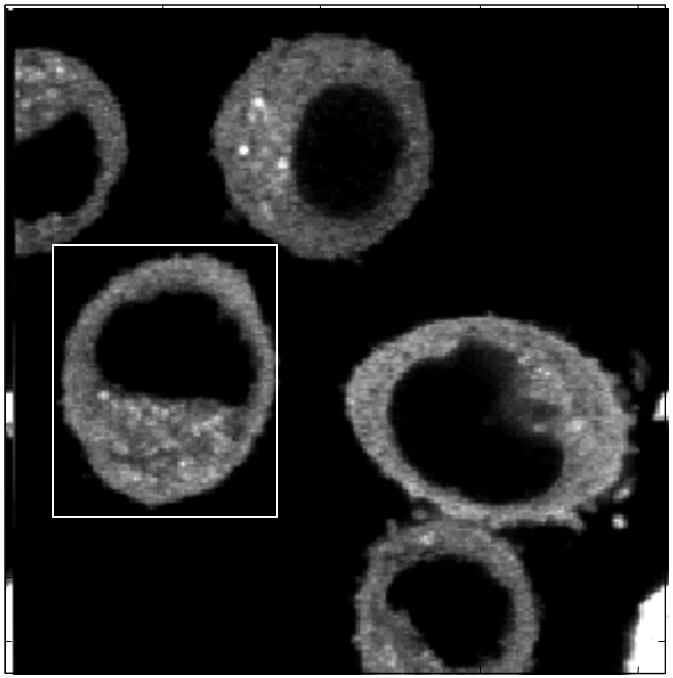
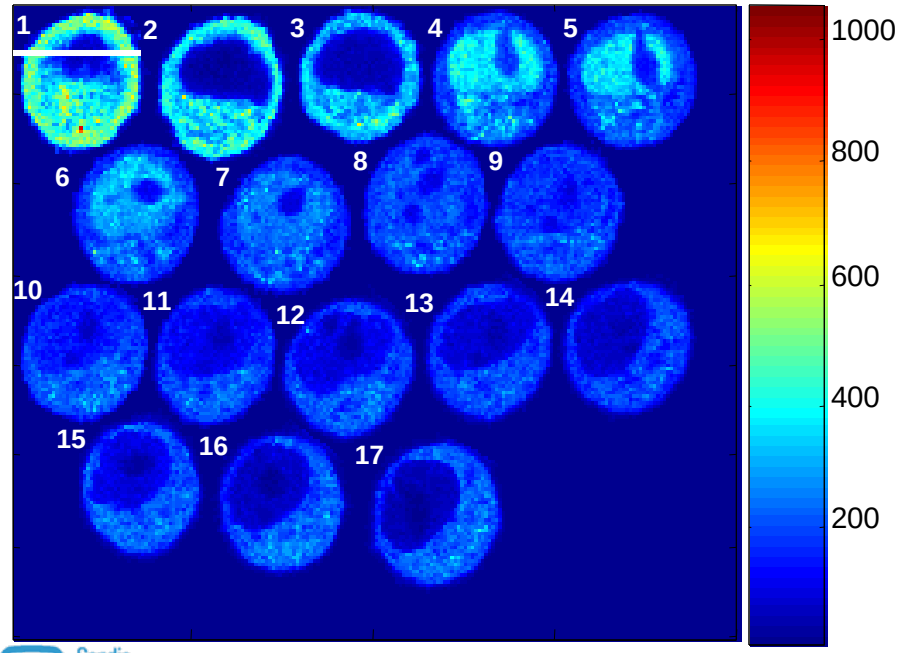
Time-Resolved Measurements: HeLa Oscillation Experiment GFP – RelA Reaction to TNFα

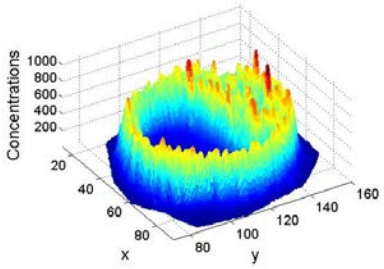
Brasier supplied cell lines

Same cell over the entire course of the experiment.

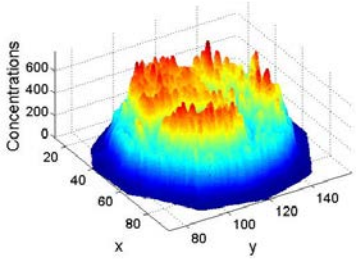


Relative GFP Concentrations





20 minutes



60 minutes

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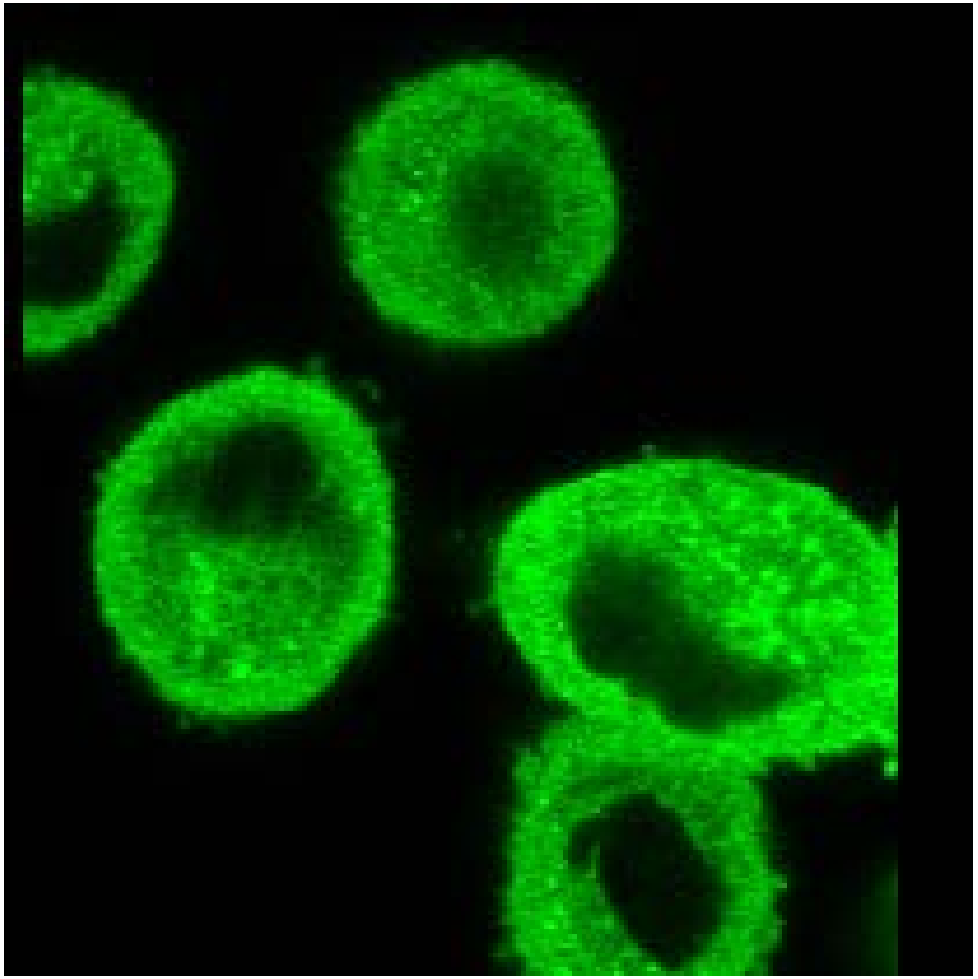
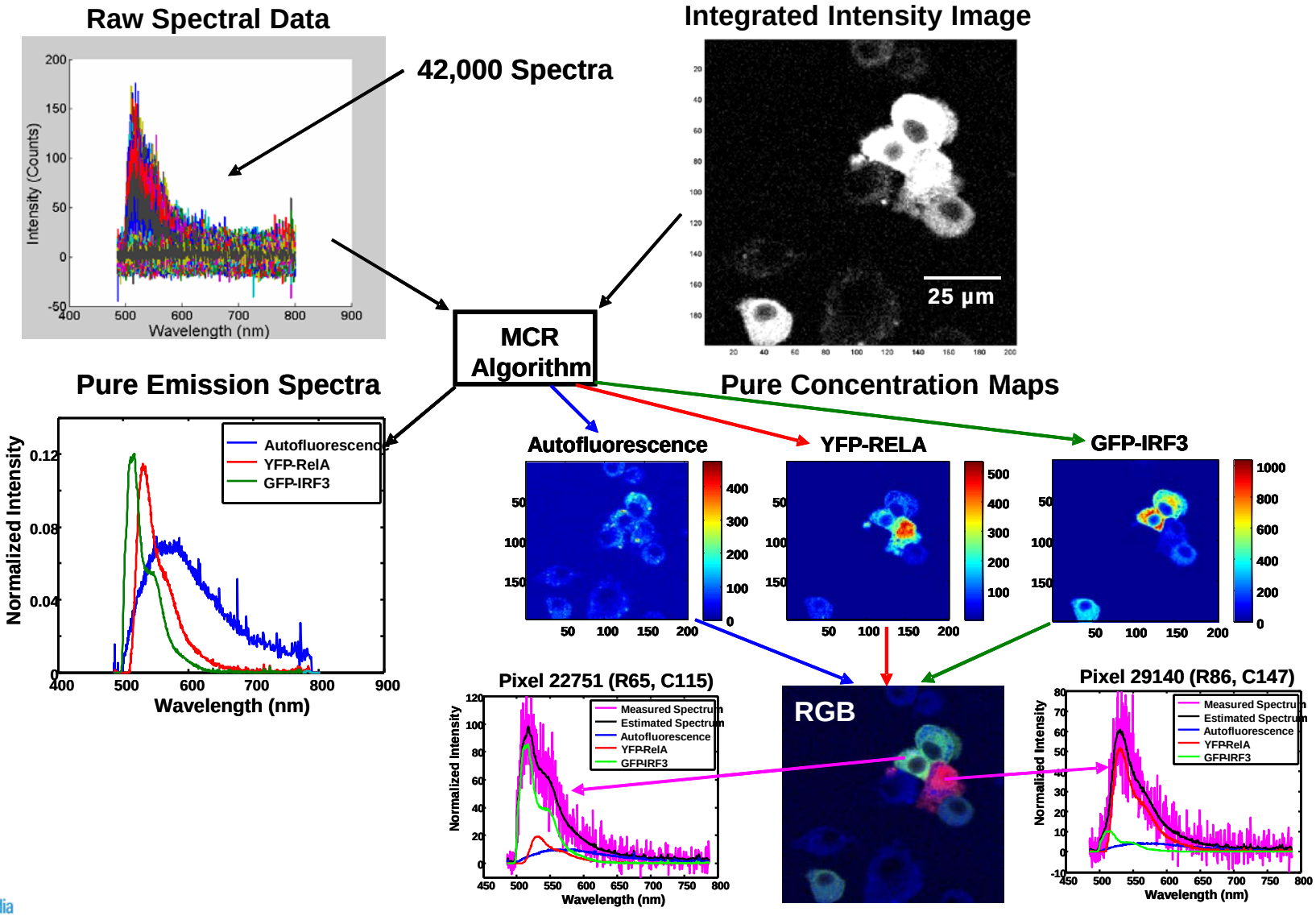
~ 20 min. intervals

HeLa Cell Movie

- HeLa cell data taken at approximately 20 minute intervals for ~6 hours
- GFP attached to RelA
- Cycling of GFP into and out of the nucleus when activated by TNFα



Multiple Fluorescent Proteins in RAW 264.7 Macrophage Cells



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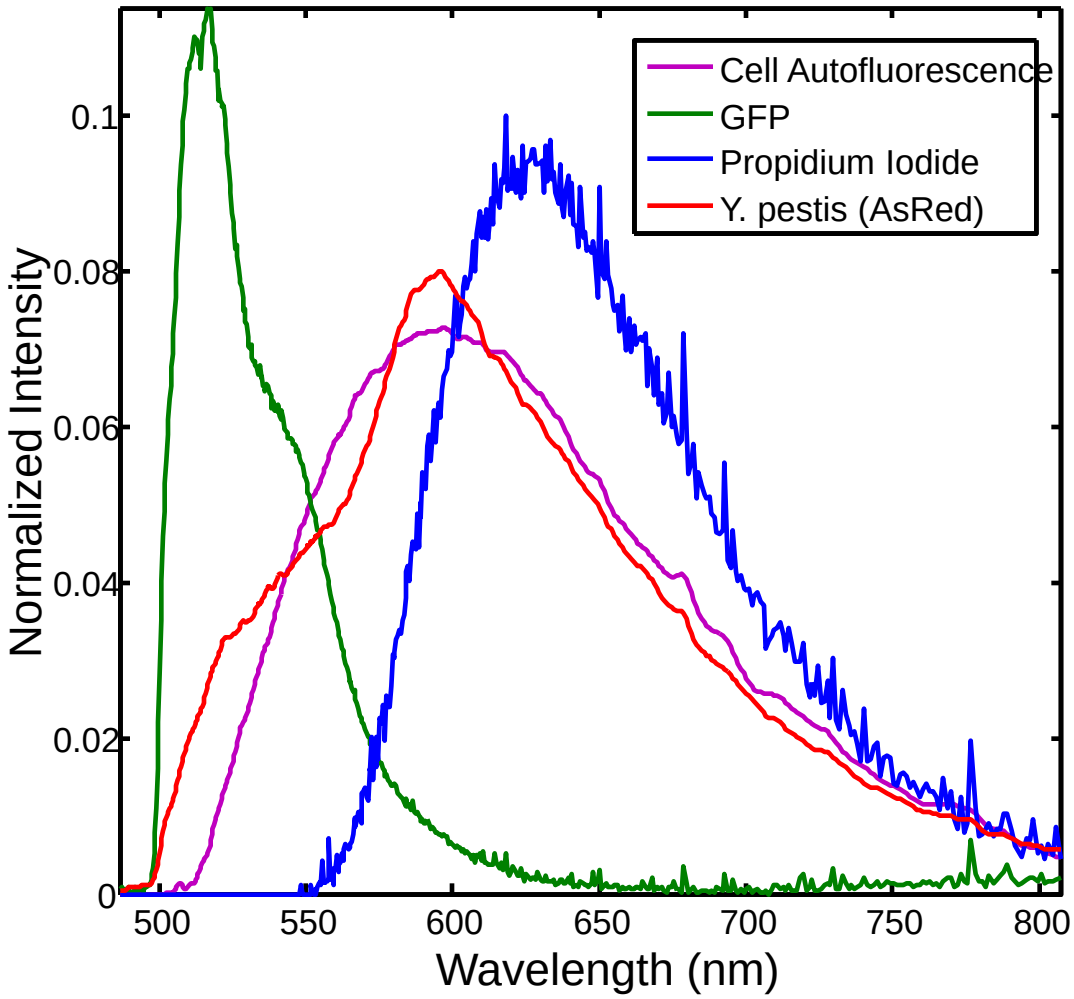
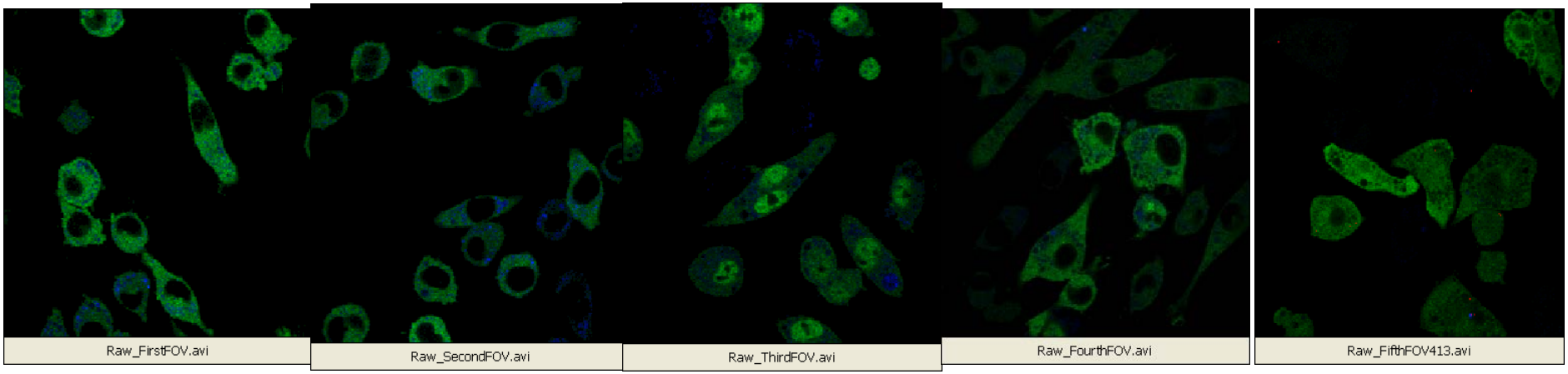
Live Macrophage Cells with GFP-RelA and AsRed-Labeled Y. Pestis (MCR Results)



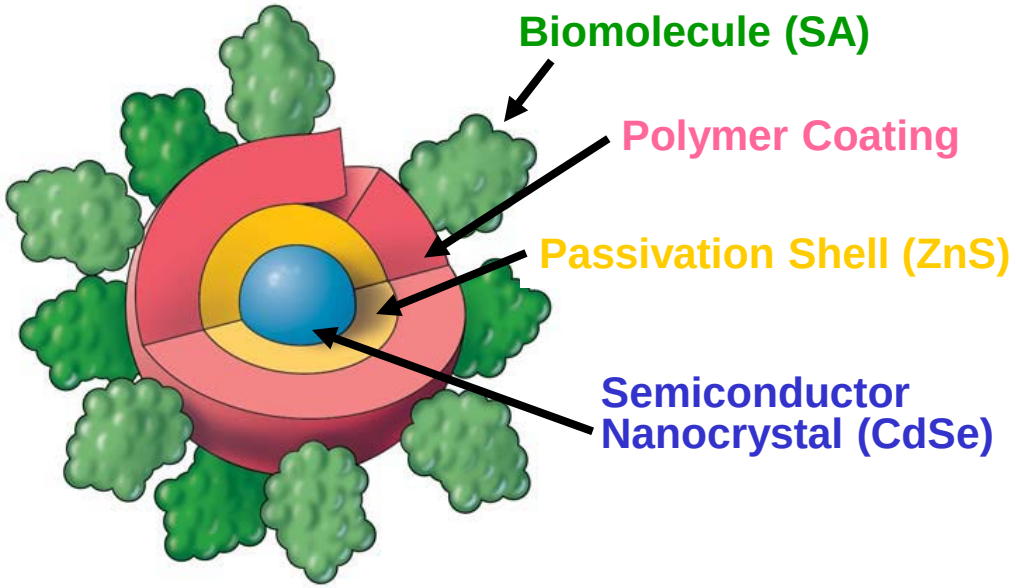
Live Macrophage Cells (RGB Movies)

1st FOV | 2nd FOV | 3rd FOV | 4th FOV | 5th FOV

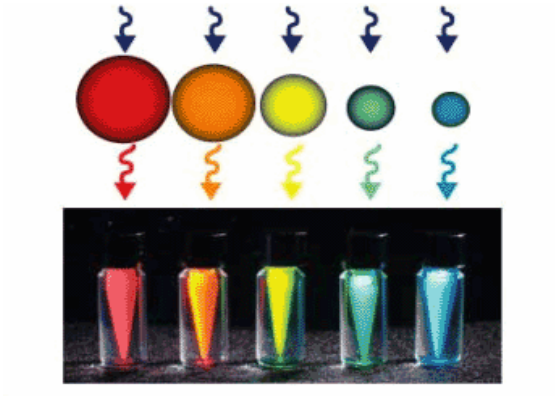


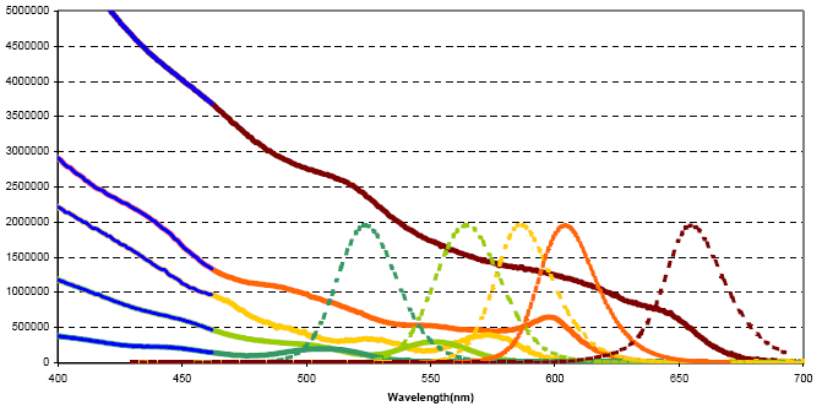
The pure-component spectra from the previous slide are the same colors for the RGB images (only GFP, AsRed and propidium iodide shown).

Quantum Dots



- Broad excitation spectrum
- Narrow emission band
- Brightness
- Photostability
- Flexible bioconjugation
- Electron dense





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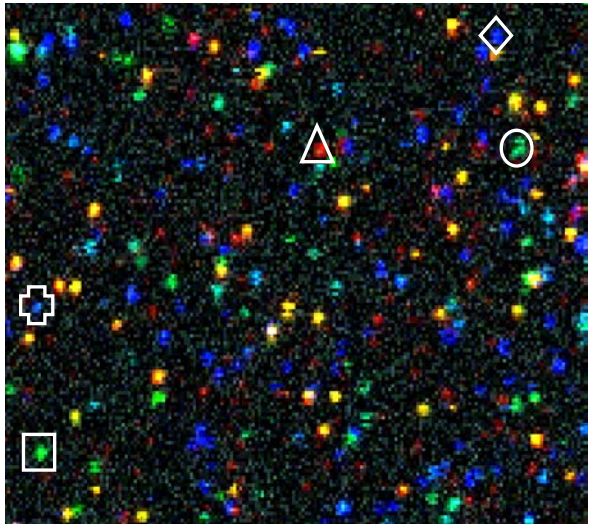
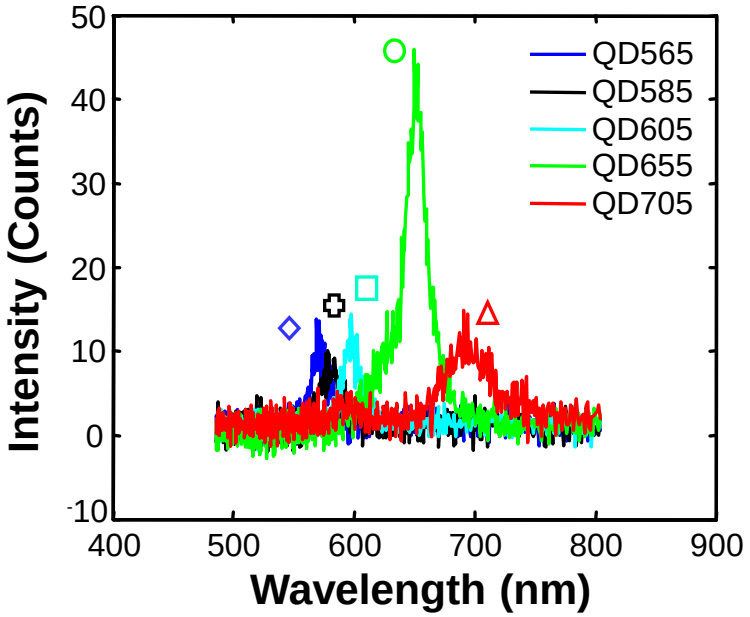
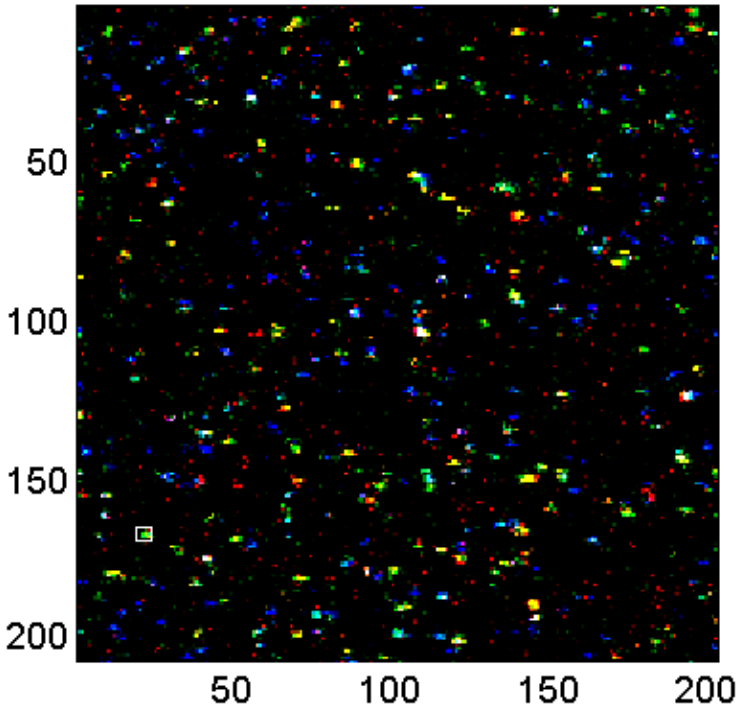
Hyperspectral Imaging of Quantum Dots

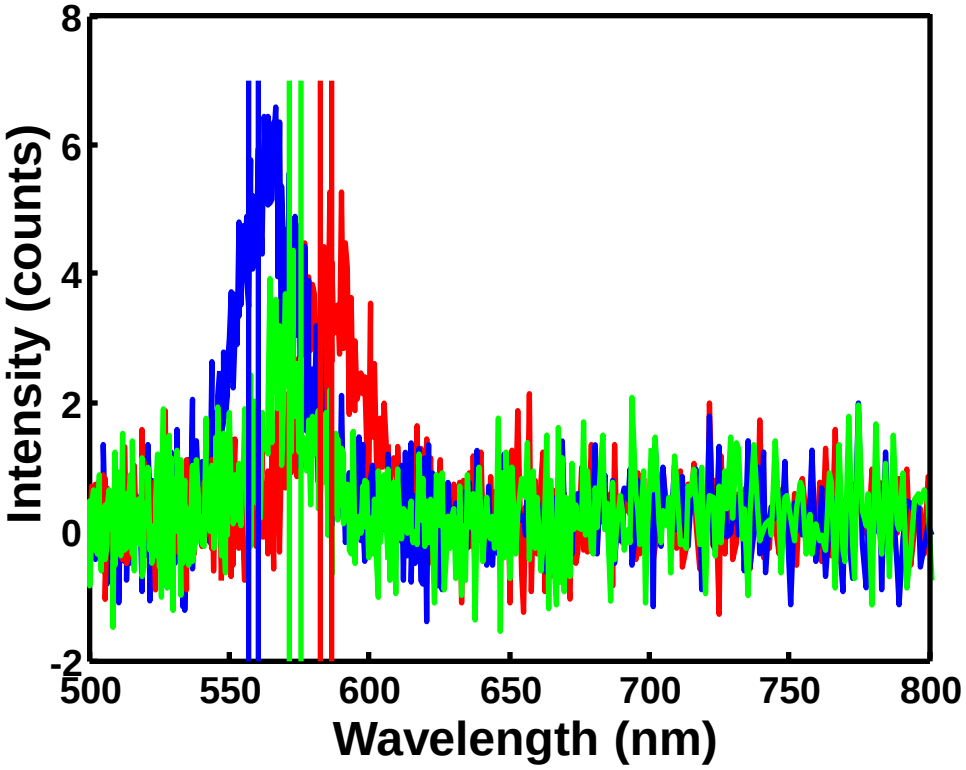
Mixture of Individual QDs (5 types)





565 nm QD Emission Spectra and Image from Simulated Filter-based Microscope





Spectra from individual pixels from different 565 nm QDs

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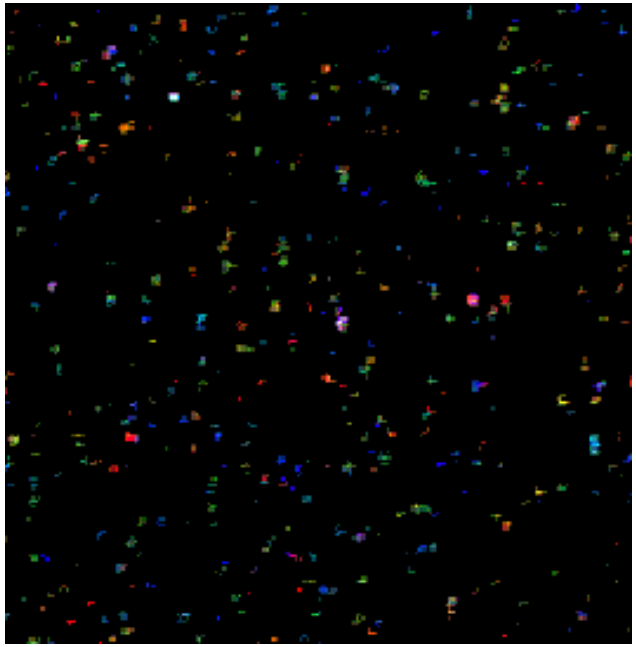
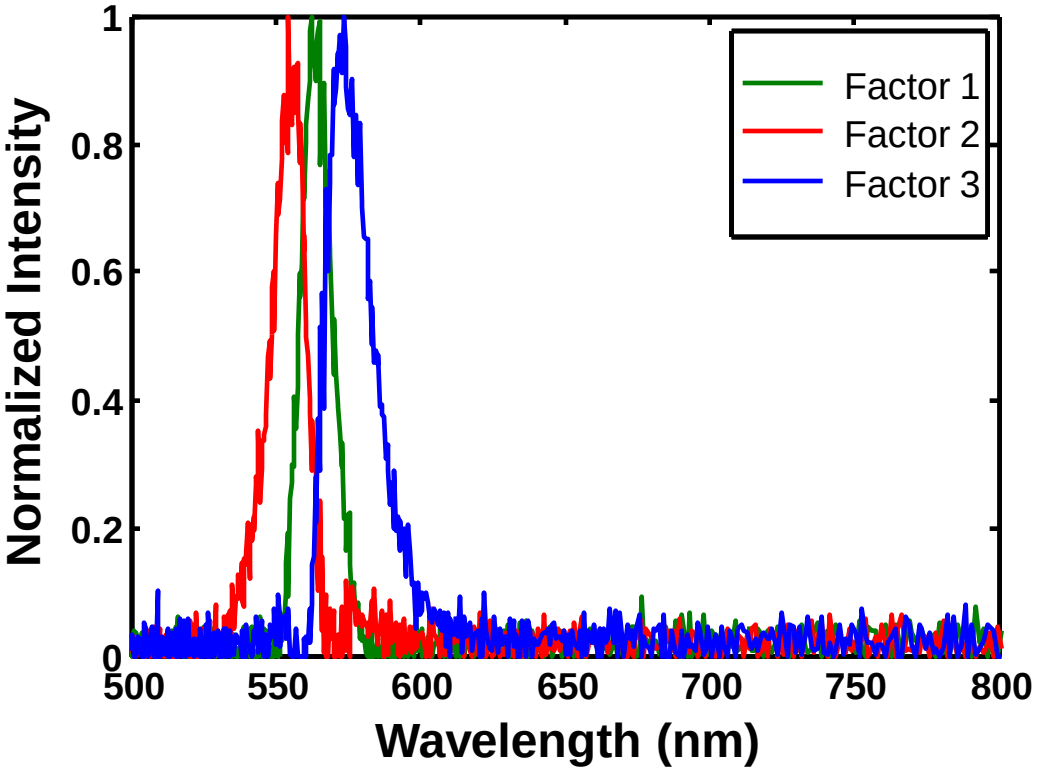
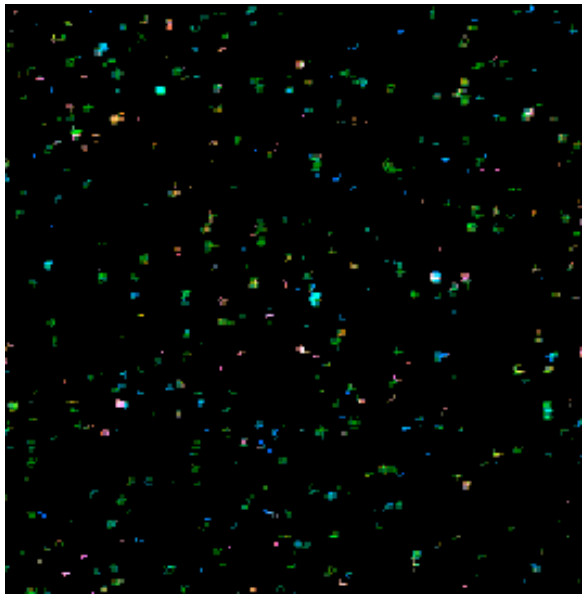
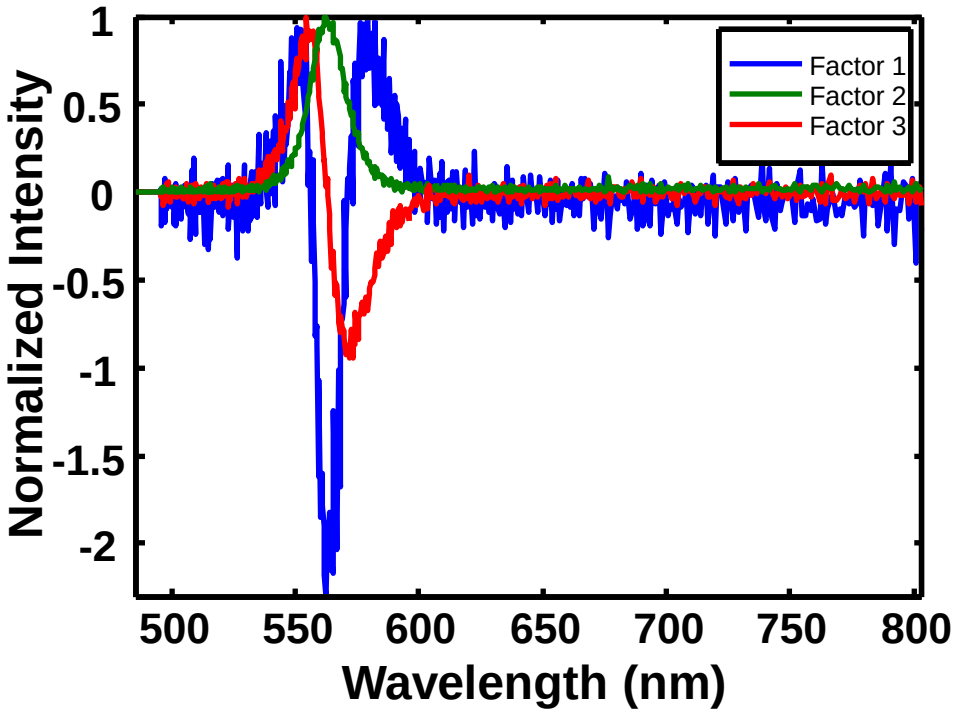
Pure 565 nm QD Emission Spectra and RGB Image from MCR Analysis





Alternative Pure 565 nm QD Emission Spectra and RGB Image from MCR Analysis

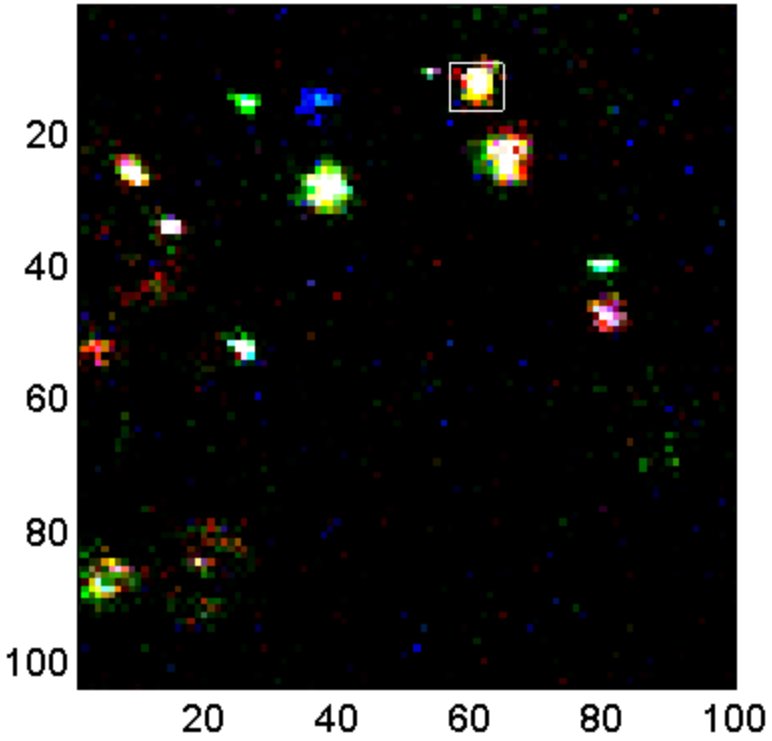


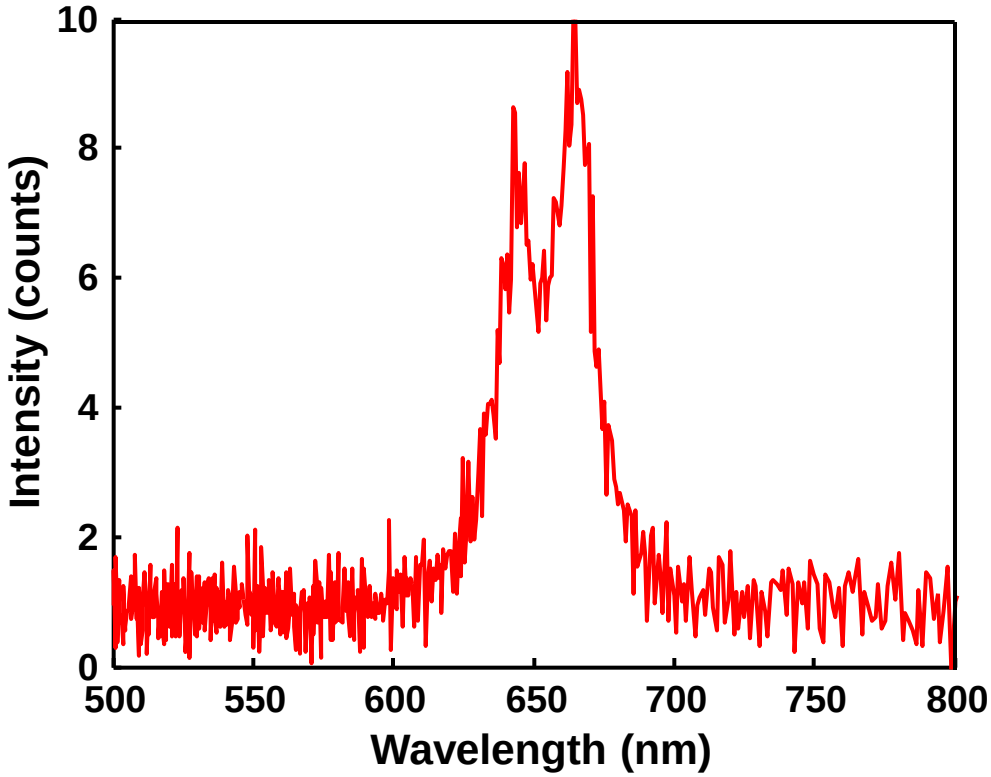


Time-Resolved Imaging of 655 nm QDs in Live Cells

- Commercial 655 nm QDs used to label IgE protein
- Investigate cell signaling of FcεRI (IgE Receptor) in live rat basophilic leukemia (RBL) cells in response to activation by divalent dinitrophenol
- Monitor the time-resolved motion of QD-labeled IgE on the membranes of live RBL cells with the hyperspectral microscope (currently 0.25 frames/sec)

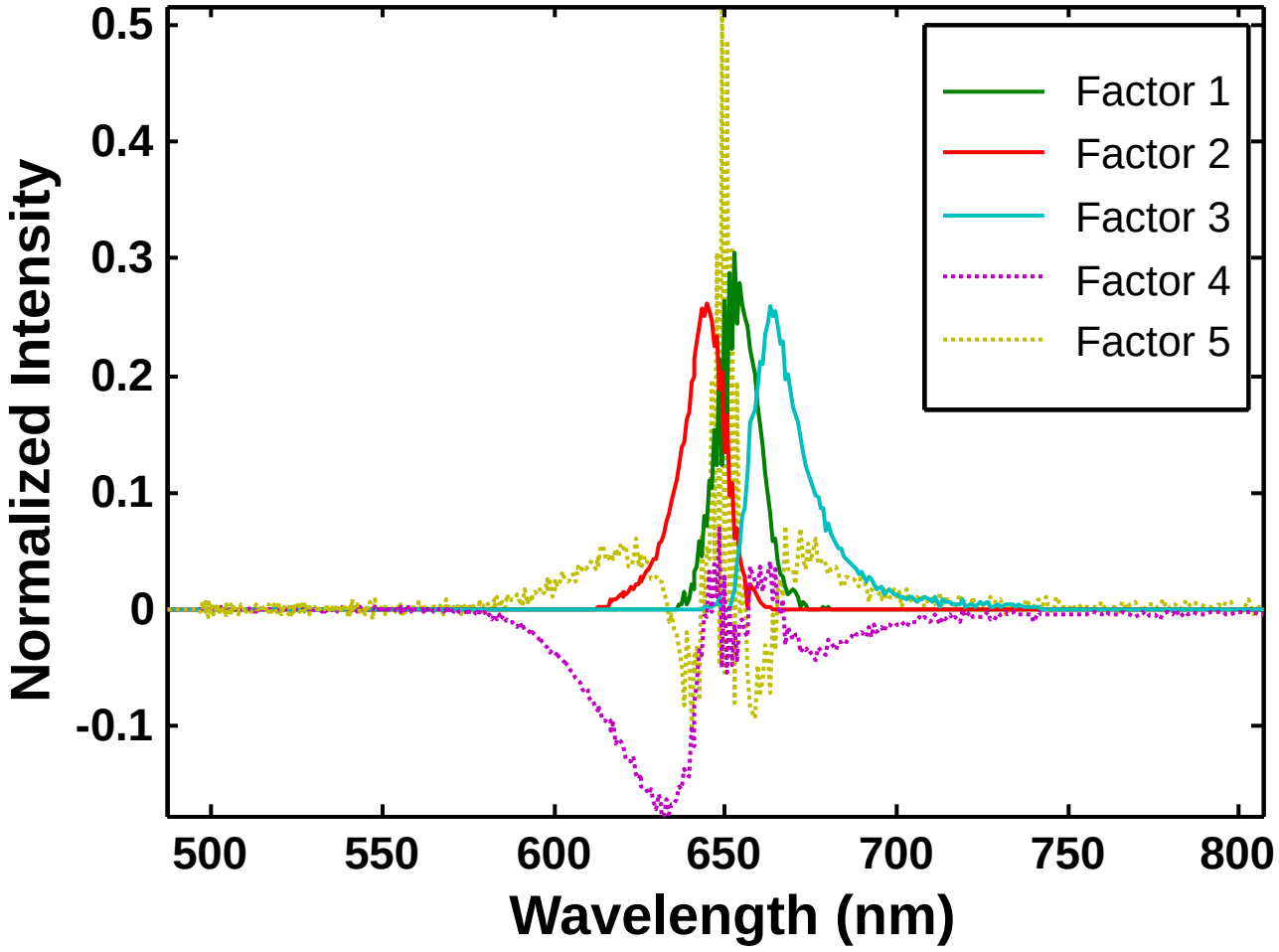
Single 655 nm QD Emission Spectrum from IgE-Labeled RBL Cells



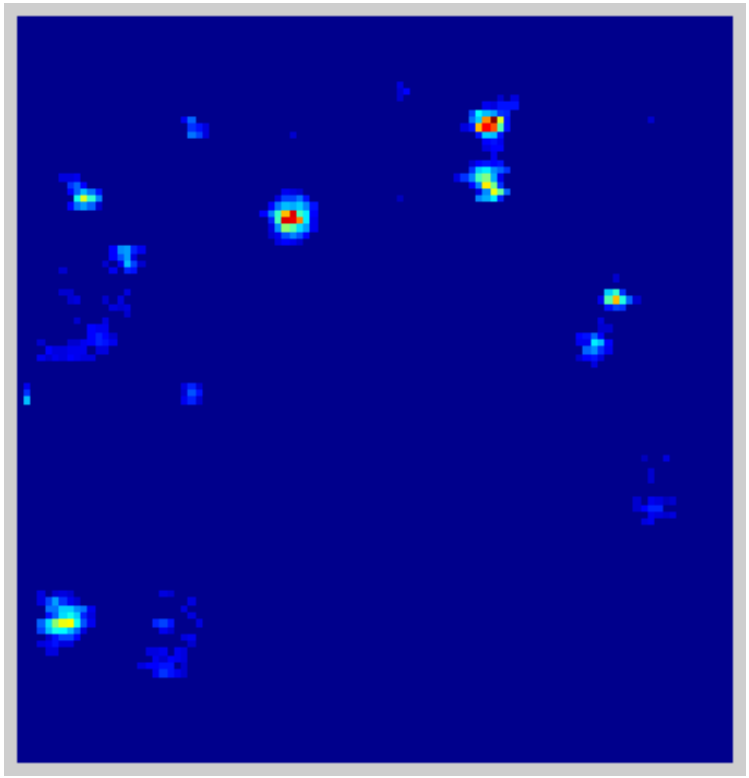


Sandia National Laboratories

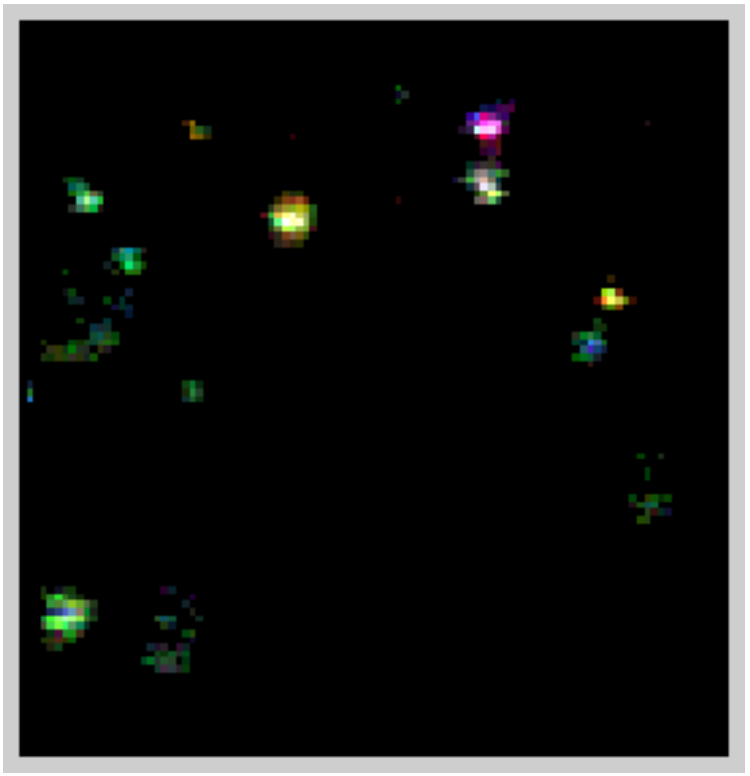
Pure 655 nm QD Emission Spectra from MCR Analysis of IgE-Labeled RBL Cells



Movies of 655 nm QDs on Live RBL cells (Labeled IgE Proteins)



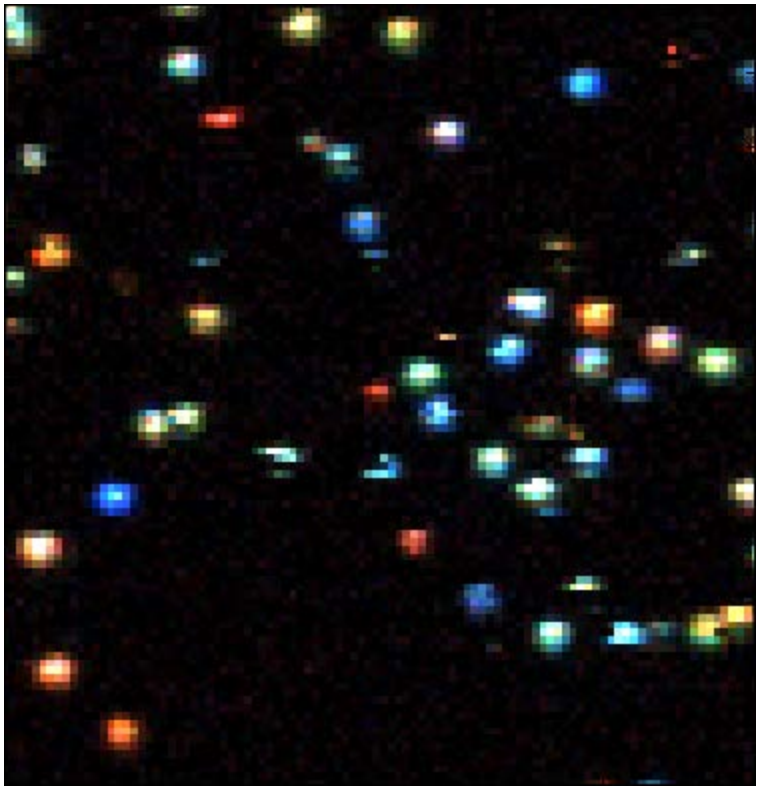
Total Intensity Image



RGB Image

24 x real time

Movie of 655 nm QDs on Glass Slides



RGB Image

24 x real time

Conclusions

- Sandia has unique hyperspectral microscopes for biotech applications
  - 2D hyperspectral microarray scanner
  - 3D hyperspectral confocal fluorescence microscope
- We have proprietary algorithms and software for rapid, accurate, reliable and quantitative analysis of huge data sets (confocal microscope images to remote sensing).
- New technologies have many biotechnology applications
  - Simultaneous monitoring of multiple fluorophores in live cells with optical sectioning capabilities
  - Photosynthetic pigments in cyanobacteria and corn leaves
  - Host-pathogen interactions
  - Monitoring of molecular interactions (FRET)
- Hyperspectral microscopy with MCR analysis is a new enabling technology for studying biological systems