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Fluorescent Hyperspectral Imaging of Biological Samples: Evaluating the sensitivity of our quantitative measurement

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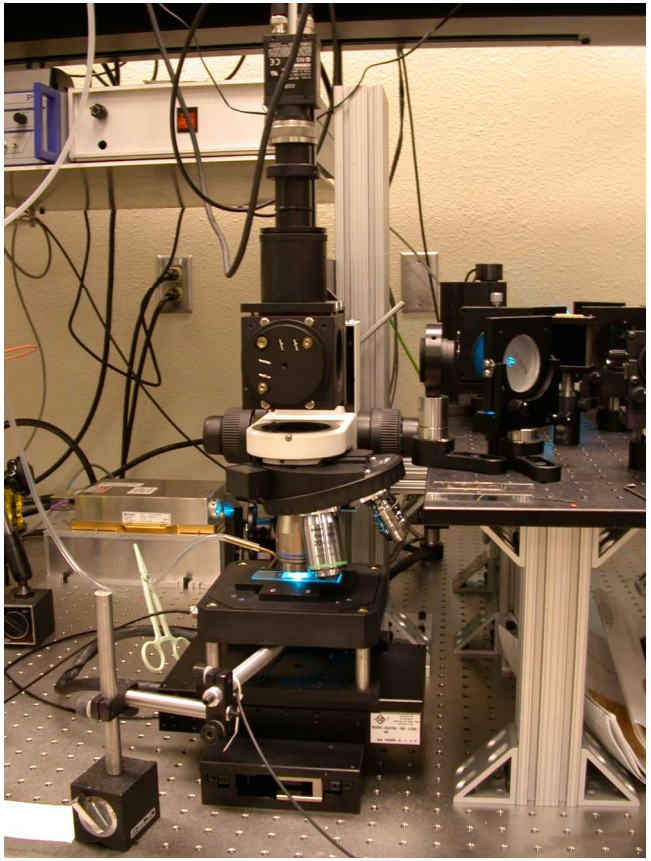
Outline

- Sandia's 3D Hyperspectral Fluorescence Imaging Efforts
  - 3D Confocal Hyperspectral Imager
  - Multivariate Curve Resolution (MCR)
- Evaluating the sensitivity of our quantitative measurement
  - Motivation
  - Approach
  - Factors that influence our sensitivity
- Application onto Simulation and Experimental Data
- Conclusions

3D Hyperspectral Confocal Fluorescence Microscope

- Fully confocal design
  - **high spatial resolution**
  - **optical sectioning**
- High optical throughput
  - **prism spectrometer**
  - **electron multiplying CCD**
- Performance Specifications:
  - **488 nm laser excitation**
  - **10x, 20x, 60x, 100x objectives**
  - **Lateral Resolution = 0.25 μm**
  - **Axial Resolution = 0.60 μm**
  - **Spectral range 490-800 nm**
  - **Spectral resolution = 1-3 nm**
  - **Acquisition rate = 8300 spectra/s**



M. B. Sinclair, D. M. Haaland, J. A. Timlin, and H. D. T. Jones, "Hyperspectral confocal microscope," Applied Optics, 45, 6283-6291 (2006).

Advantages of Hyperspectral Imaging & Multivariate Curve Resolution

Discover & quantify all emitting species in a sample simultaneously

- No *a priori* knowledge required
- Increased sensitivity
- Extended dynamic range
- Improved accuracy, reliability, & quantitation
- Increase throughput – multiple overlapping dyes

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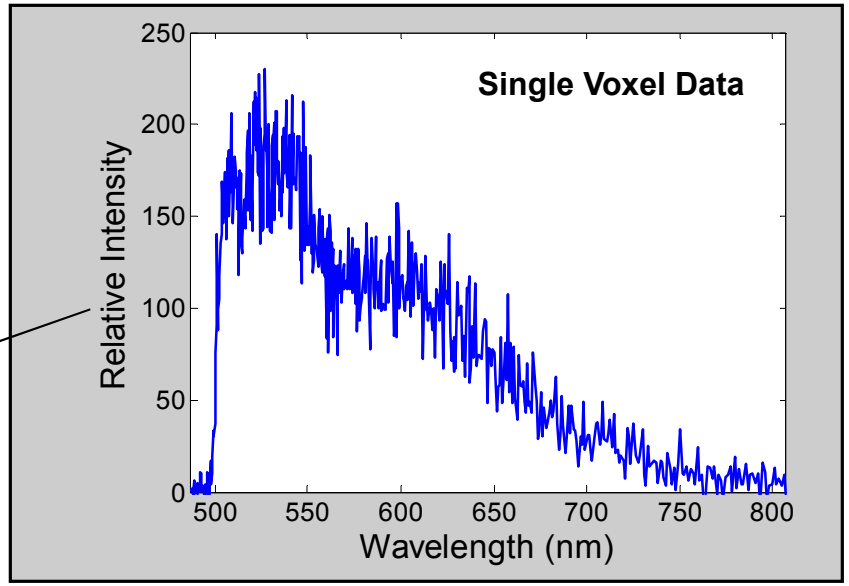
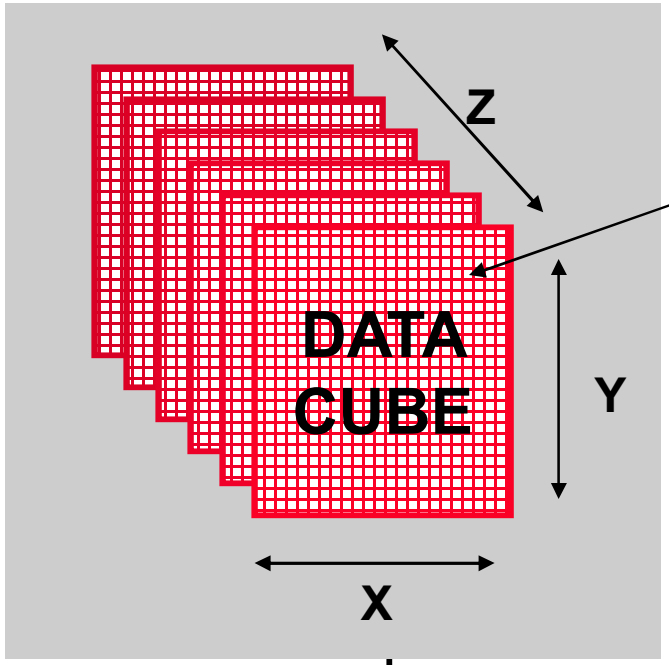
Multivariate Curve Resolution (MCR)

- **Assumptions**
  - **Linear additive model:** $D = CS^T + E$
  - **# of components is known or can be estimated**
- **Solve** $D_w = CS^T$ **with constrained alternating (rigorous) least squares methods**
  - $\hat{C} = D_w S^{T+}$, $\hat{S}^T = \hat{C}^+ D_w$, **solution**
- **Non-negativity Constrained Pure Components (S) and Concentrations (C)**





WHAT?

WHERE?
HOW MUCH?

Spectral Components

Concentration Maps

Preprocess

Initial Estimates

MCR

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Motivation and Approach

- Motivation
  - Allows for better interpretation of the results
  - Provides a mechanism to intelligently threshold the concentrations when reconstructing images from the MCR results
  - Understand the limits of our quantitative image measurements
- Approach
  - Understand the noise characteristics of our 3D Fluorescence Hyperspectral Imager and our imaging measurements
  - Develop methodology to determine the sensitivity of our measurement and conduct simulations of hyperspectral image data
  - Migrate this methodology to real data

Noise Sources that Influence the Sensitivity of our Measurement

Poisson Distributed Noise and Read Noise

Noise Variance = $(F_G \times \sigma_P^2) + \sigma_R^2$

F_G = gain factor, noise inflation due to EMCCD

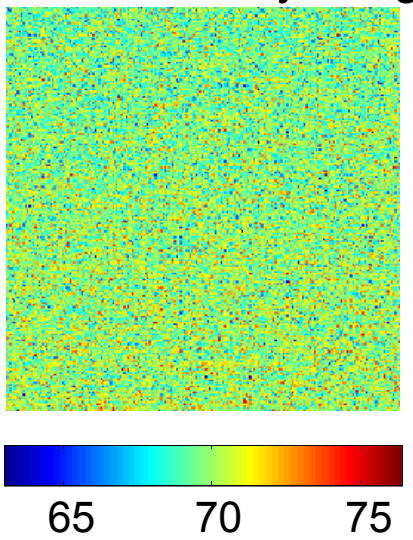
σ^2_P = Poisson Noise Variance
σ^2_R = Read noise variance

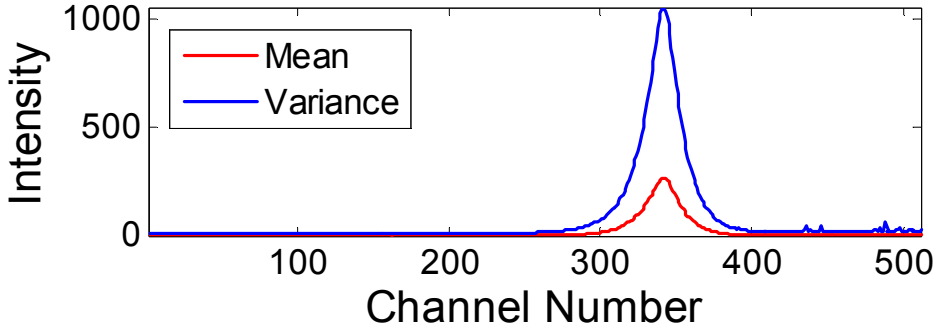
- **After knowing our major noise sources, what influences the sensitivity of our measurement?**
  - **Number of fluorophores**
  - **Amount of Spectral overlap**
  - **Amount of Spatial overlap**
  - **Relative intensity of the spectrally overlapped fluorophores**

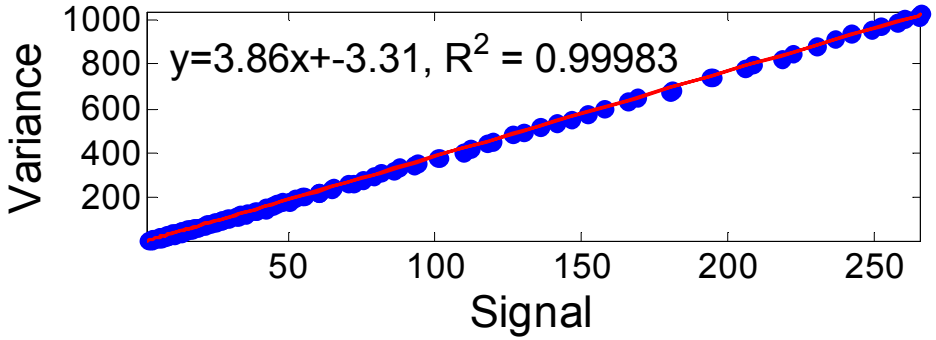
Calculating the Gain Factor

- **Determining the gain factor**
  - **Collect a spatially uniform emission image**
    - **Red LED source placed under the objective**

Mean Intensity Image







Gain Factor = 3.86

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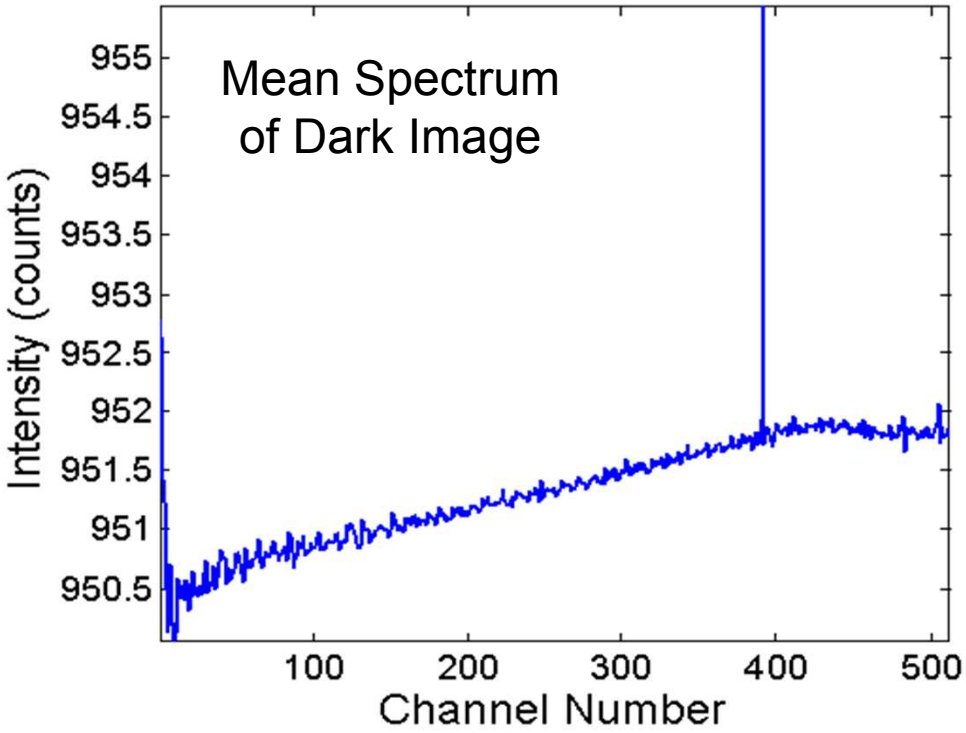
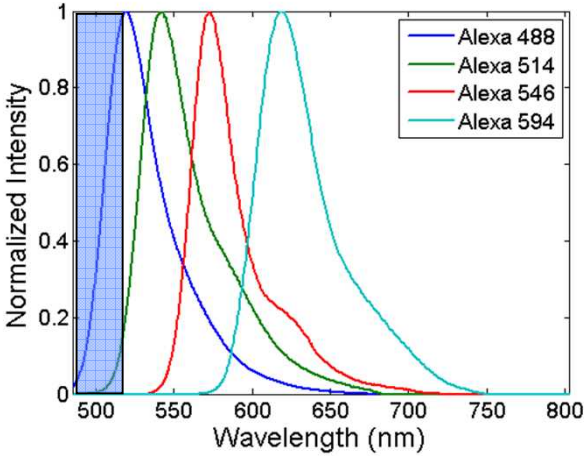
Generating Read Noise Image

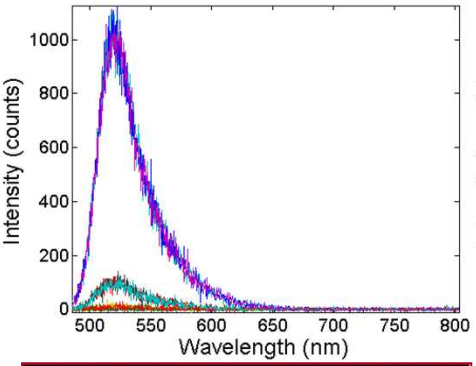
- Collect a dark image of the same size as your sample image
- Preprocessing
  - Remove cosmic spikes
  - Remove structured noise component
    - Pulls out in the first EV of PCA
    - Reconstruct data without first EV

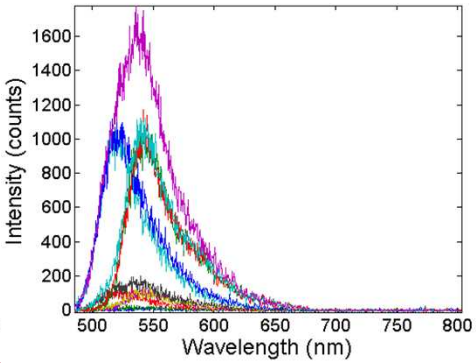


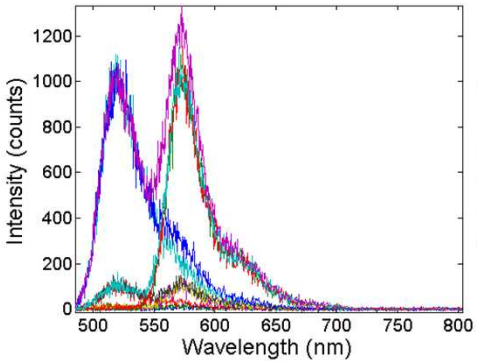
Simulation Data

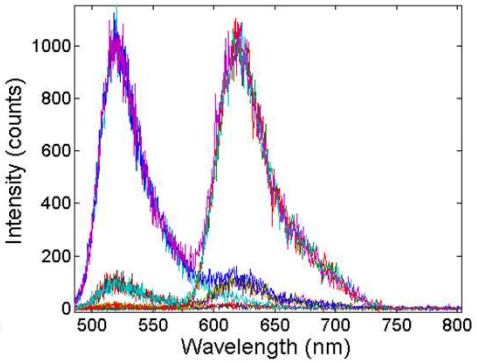
- Goal: Develop a simulation spectral image data set to understand how the following factors influence the sensitivity of the quantitative measurement each pixel in the image
  - Amount of Spectral overlap
  - Amount of Spatial overlap
  - Relative intensity of the spectrally overlapped fluorophores
- Compare with hyperspectral imaging simulation results with filter-based microscope simulation results

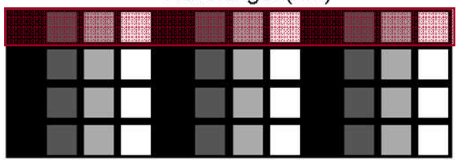








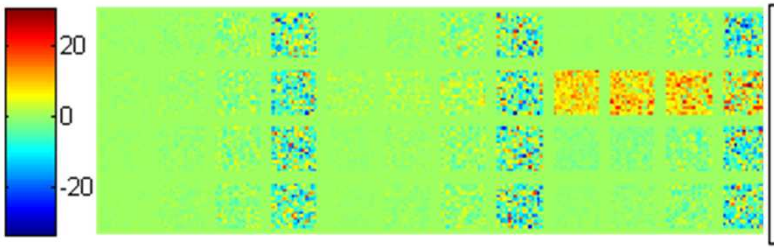


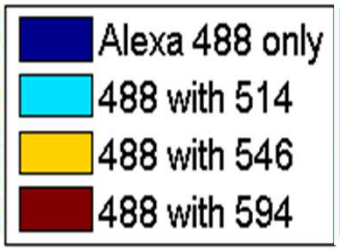


Alexa 488 Alexa 514 Alexa 546 Alexa 594

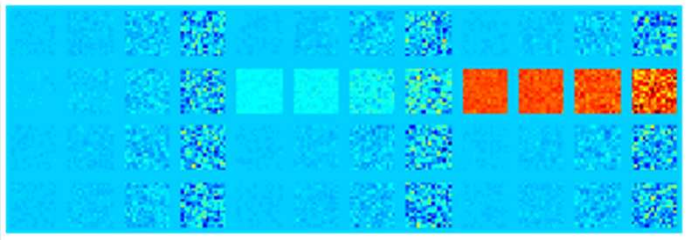
Simulation Results

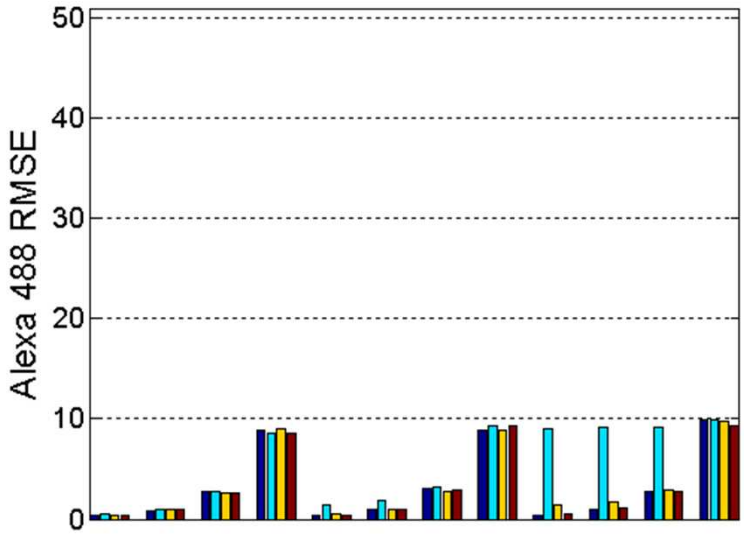
HSI Simulation
Alexa 488 Errors

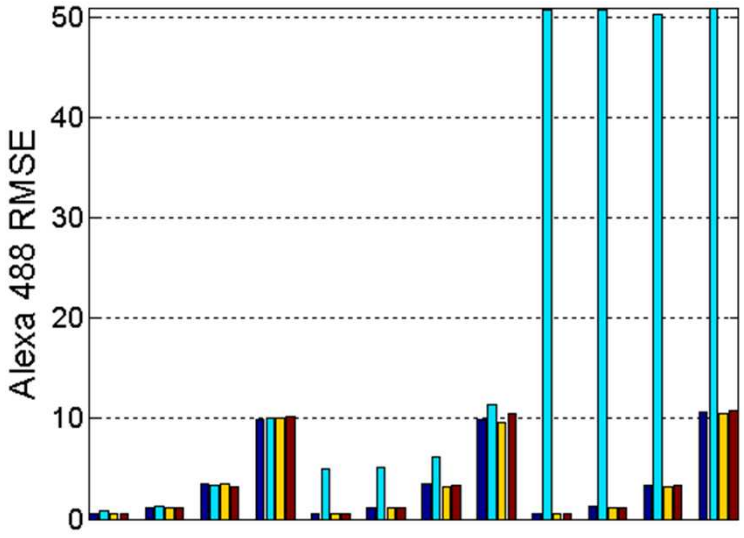




Filter-based Simulation
Alexa 488 Errors



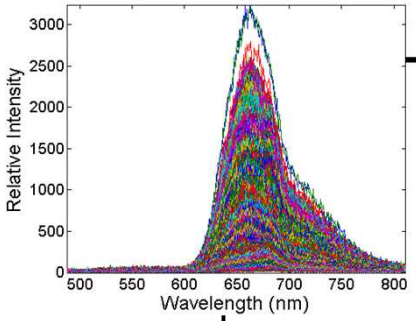




Hyperspectral imaging is limited by instrumental noise

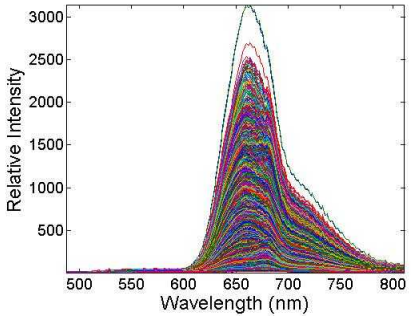
Filter-based imaging is limited by spectral cross-talk

Estimating the Confidence Limits for our MCR results



MCR

S_T, C_T → Reconstruct Data



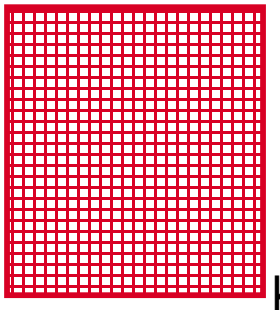
\+ Noise (Poisson, Read)

CLS, S_T

C_N

$(C_{N_{ijk}} - C_{T_{ik}})^2$

RMSE x 2 → **95% Confidence**

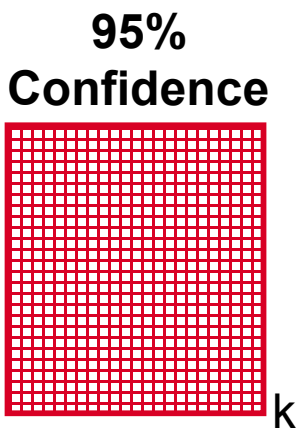


k

i^{th} pixel
j^{th} iteration
k^{th} component

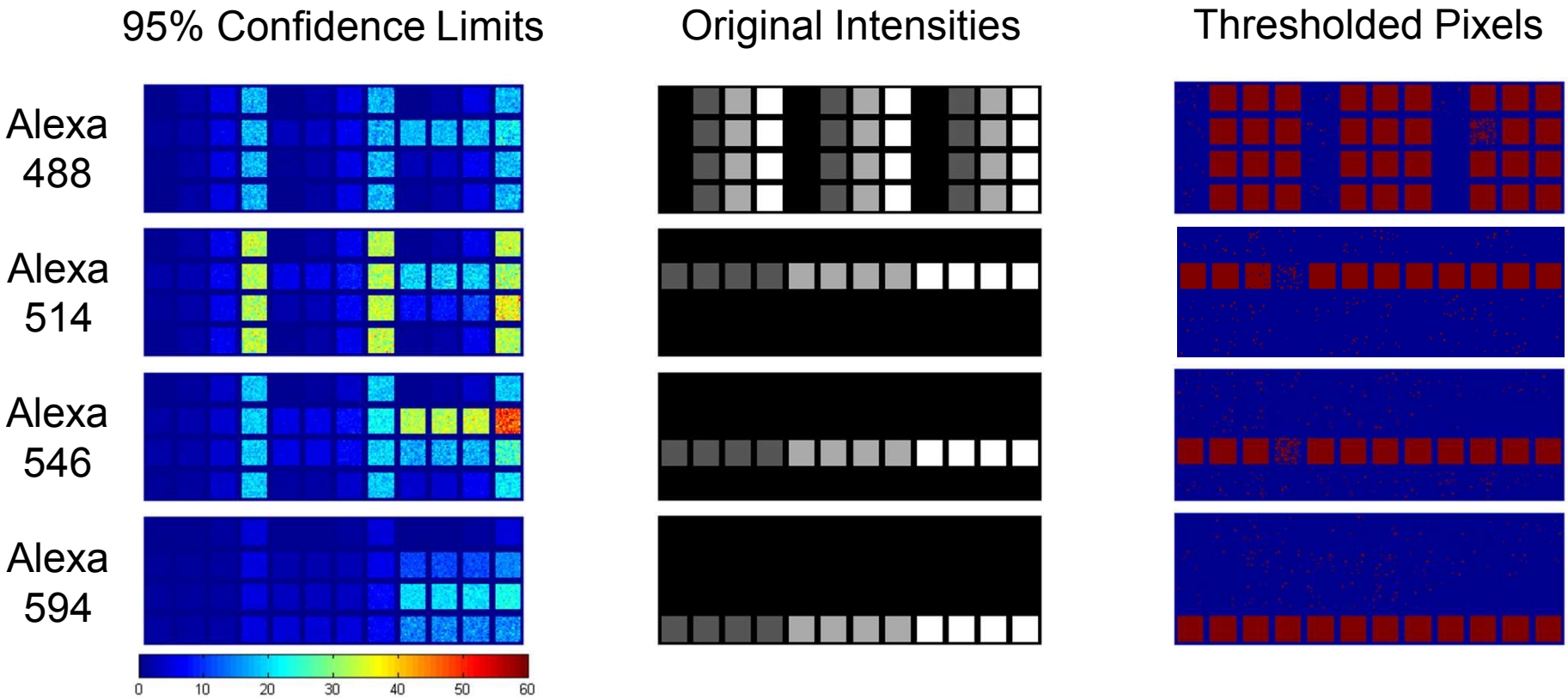
20 iterations were used to understand the concentration variance to estimate the RMSE.

Image Thresholding



- Per Component
  - Find the pixels whose concentrations fall below the 95% confidence limit and reset those concentrations to zero
- Reconstruct Image

Noise Thresholding - Simulation Example

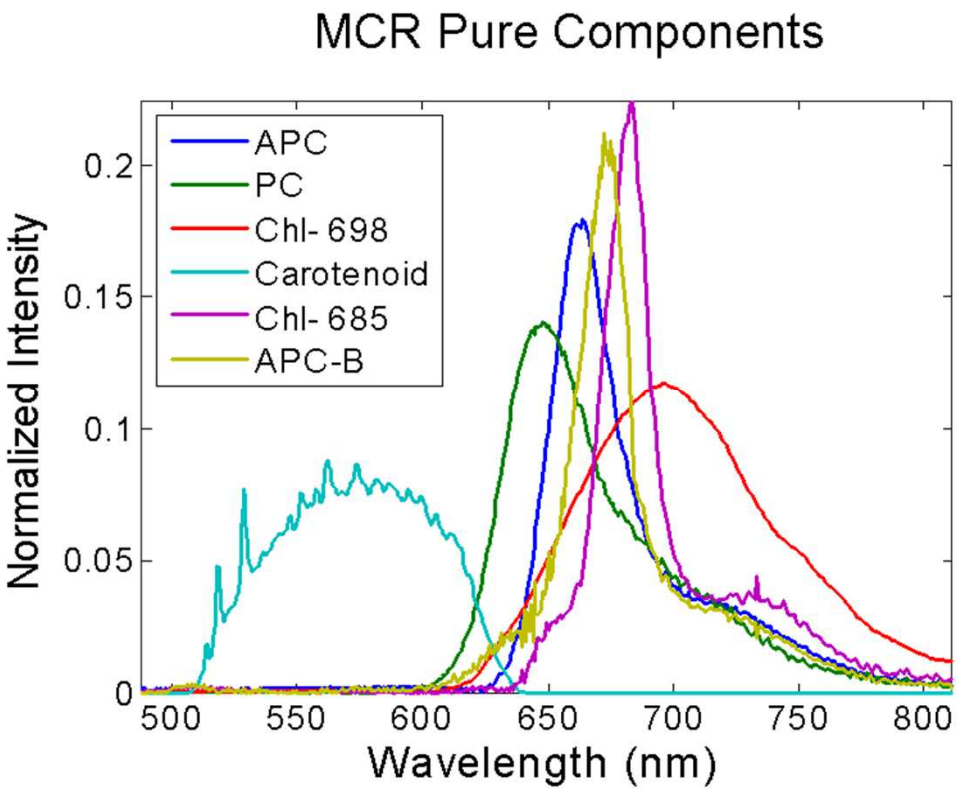


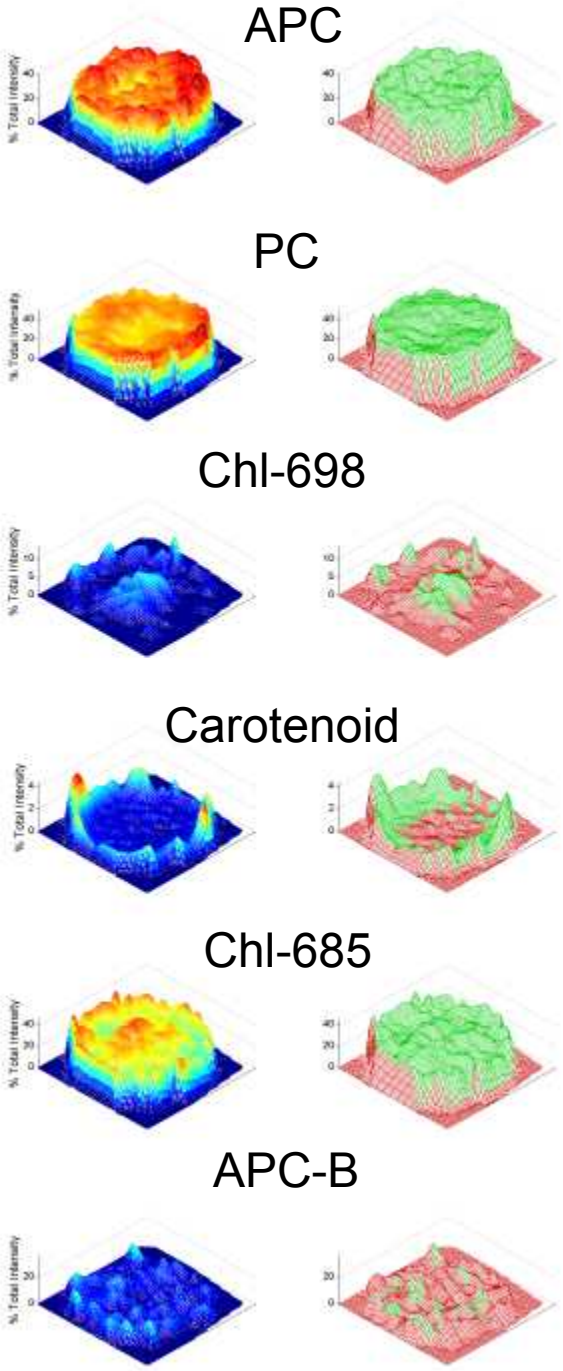
In this example, our detection and measurement sensitivity is severely impacted when there is spectral and spatial overlap combined with a 100 fold relative intensity change between the fluorophores of interest.

Synechocystis Example

- Use hyperspectral imaging to identify and map photosynthetic pigments in 3 dimensions
  - Native fluorescence of *Synechocystis* sp. PCC 6803
    - Cells are less than 2 μm in diameter
  - This system has been difficult to study with commercial instrumentation due to high amount of spectral congestion (fluorescent components all lie within 40 nm of each other)
- Wild-type and mutant strains were used to identify spectral components and map their locations in 3 dimensions
  - Seven strains (2 wild type, 5 mutant)
  - Mutants lack specific genes which affect properties such as chlorophyll synthesis

MCR Results with 95% Confidence Limits





W. F.J. Vermaas, J. A. Timlin, H. D.T. Jones, M. B. Sinclair, L. T. Nieman, S. Hamad, D. K. Melgaard, and D. M. Haaland, “In vivo Hyperspectral Confocal Fluorescence Imaging to Determine Pigment Localization and Distribution in Cyanobacterial Cells,” accepted to PNAS.

Conclusions

- With the proper understanding of your noise sources:
  - Realistic simulation data sets can be created to guide the experimental design of your biological imaging experiments.
    - Types of fluorophores, relative concentrations, targeted locations, etc.
  - Once the experiments are conducted and images analyzed, confidence limits can be calculated and intelligent thresholds can be applied to the images.
    - Allows for better interpretation of the results
- Hyperspectral imaging coupled with MCR is an excellent and necessary tool for imaging many biological systems.
  - Multiple overlapping fluorophores
  - Improved accuracy, reliability, & quantification
  - Extracts underlying relationships from complex datasets

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    - Wim Vermaas, Dmitrii Vavilin, Sawsan Hamad, Danny Yao, Allison van de Meene, and Robert W. Roberson

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