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QUANTIFICATION OF MULTIPLE FLUOROPHORES AT THE SINGLE CELL AND SUBCELLULAR LEVEL

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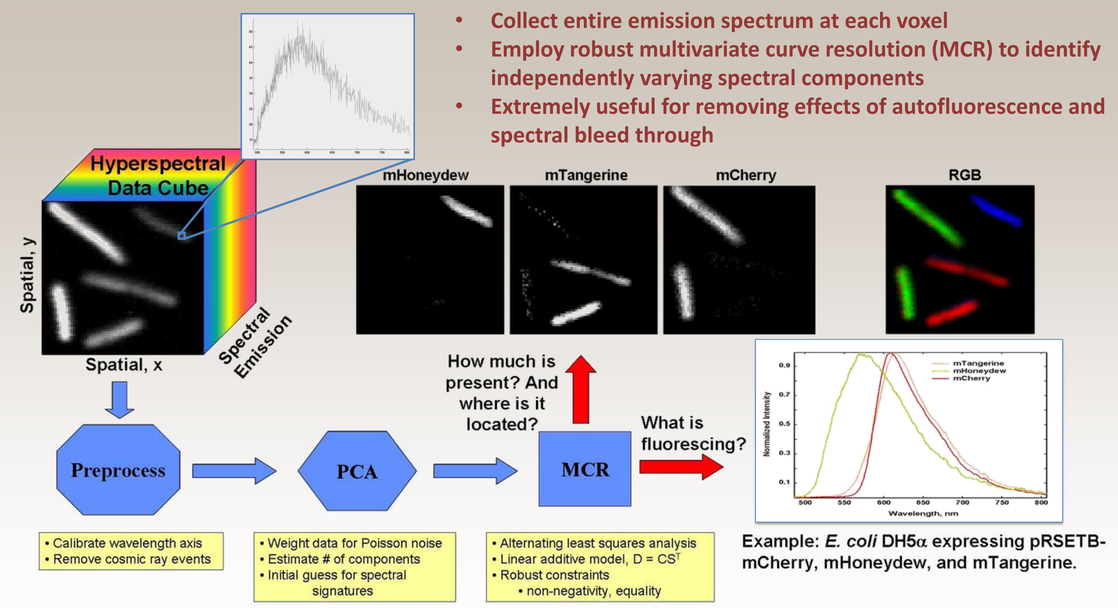
Introduction

Spectral imaging and spectral unmixing methods have emerged in recent years as important tools for quantitative cellular biology. The additional spectral information enables a higher degrees of mutliplexing in both fluorescently labeled probes as well as a suite of endogenous biomolecular signatures. In this poster we detail the spectroscopic imaging and multivariate analysis approach, specifically for direct visualization and quantification of multiple receptors involved in fungal recognition – a feat which is not possible with current biological assays such as western blots. We show, the importance of using advanced spectral unmixing methods to isolate and remove the confounding effect of the overlapping host cell autofluorescence spectrum in order to accurately detect single receptor dynamics *in vitro* and *in vivo.* Additionally, in a separate application we show the spectral variability of non-targeted probes due to cellular compartments can be exploited to classifiy cells following viral infection.

Hyperspectral Imaging and Multivariate Analysis



- Collect entire emission spectrum at each voxel
- Employ robust multivariate curve resolution (MCR) to identify independently varying spectral components
- Extremely useful for removing effects of autofluorescence and spectral bleed through

Example: *E. coli* DH5α expressing pRSETB-mCherry, mHoneydew, and mTangerine.

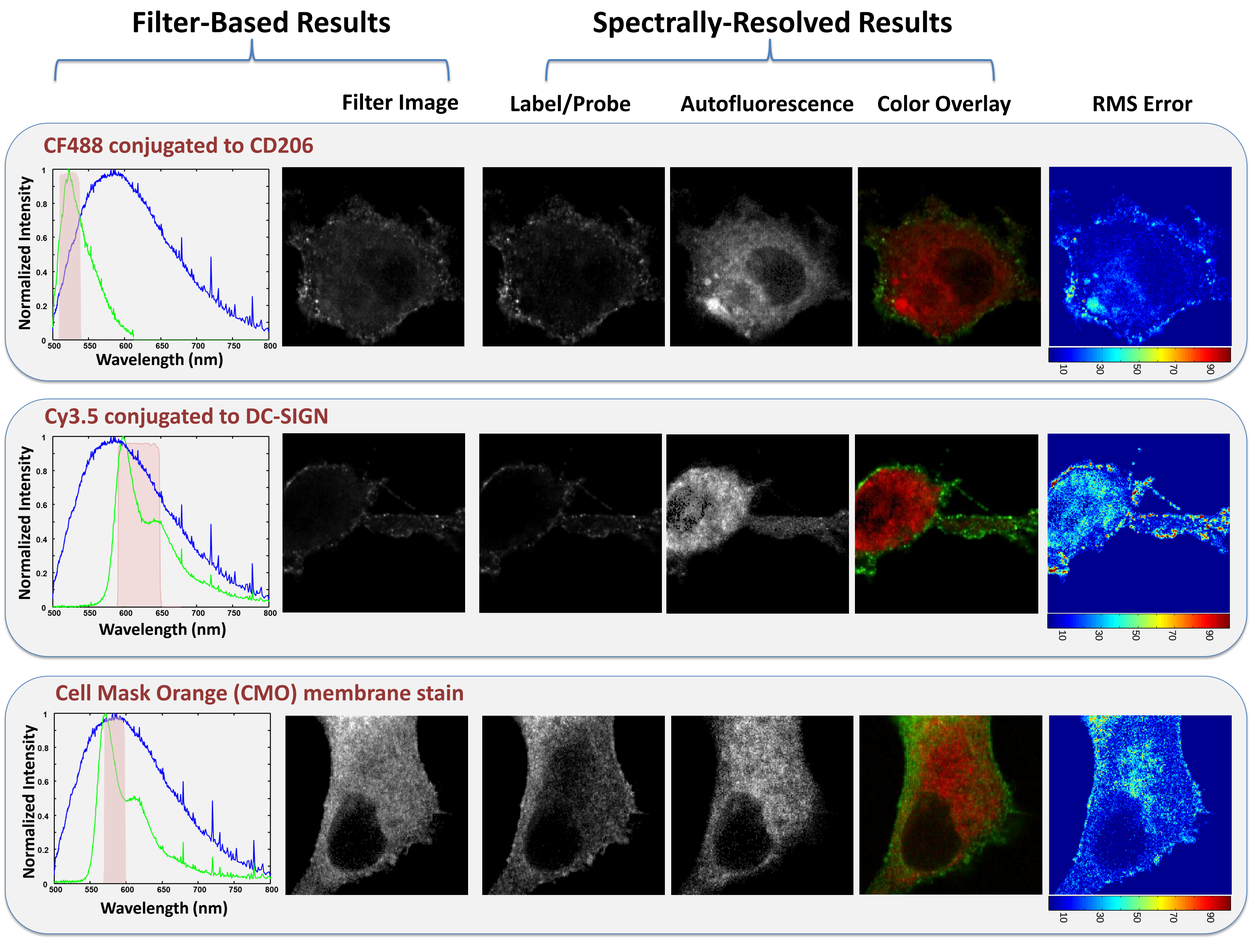
References: *Microscope- Sinclair, et. al., Applied Optics, 45, 6283-6291 (2006)*
MCR- Haaland, et al., Proc. SPIE, Vol. 4959, 55 (2003) ; Jones, et. al., J Chemom, 22:482-490 (2008); Jones, et. al., J Chemom, 117:149-158 (2012)
Autofluorescence removal- Davis, et. al., Microsc. Microanal. 16, 478–487 (2010)

Effect of Autofluorescence on Accuracy of Detection

Overall Goal: ***Determine spatial temporal dynamics of 4 fungal recognition receptors simultaneously during fungal infection.*** **A cell membrane stain is also required to localize the fungal attachment site. Initial investigations revealed cellular autofluorescence of similar intensities as the probes. Does this significantly affect results?**

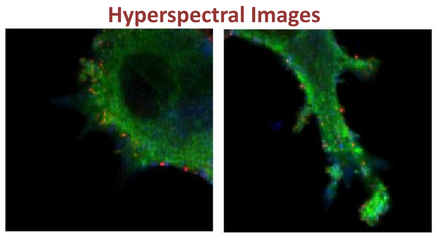
Current Study Design

- Human primary dendritic cells
- Fixed and labeled with fluorescently-conjugated primary antibodies and/or stained with membrane stain
- Imaging performed with 488 nm excitation 60x oil objective (NA=1.4)
- Spectral image data and standard filter curves for the dyes were used to calculate a filter image.
- Root mean-squared errors were calculated on a per pixel basis.



Images are 25μ x 25 μm. All grayscale images are scaled from min to 85% of max intensity. Color channels in red/green overlay images have been adjusted independently for visualization

Hyperspectral Images



Color map:
Cy3.5:DC-SIGN
Cell Mask Orange (CMO)
CF488:CD-206

- Autofluorescence is at the same level as the receptor probes and stains, therefore will pose a problem in quantitative analysis.
- Errors can also lead to higher # of receptors being identified.
- The greatest RMS errors are observed with Cy3.5, least with CF488.

Exploiting Spectral Variability for Classification

Overall Goal: ***Identify infected cells from a large population using only native signatures and simple, non-targeted probes.*** **We performed a feasibility study and show those results here to highlight the suitability of hyperspectral imaging for exploiting the spectral characteristics of non-targeted fluorescent probes resulting from the microenvironments of within a cell.**

Current Study Design

- Mouse macrophage cells (P388D1s)
- Infected with Sendai virus (16 HA units/ml)
- Stained with Acridine Orange (5 μM for 30 min)
- Imaging performed with 488 nm excitation and 60x oil objective (NA=1.4) at 18-22 hrs post infection.

MCR Component Spectra

* Indicates spectral component was important for classifying infected from uninfected cells.

Scatterplot

Mean intensities are plotted for each component on a per cell basis after applying a common threshold to each component to ensure that noisy pixels were not included in the mean calculation.

Resulting Color Overlay Images

Color map:
Acridine Orange (RNA)
Acridine Orange (DNA)
Shift of AO (RNA)

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