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New 3-D Hyperspectral Confocal Microscope for *in vivo* Imaging of Cells

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Fluorescence imaging has become a critical tool for biologists in the examination of *in vivo* processes and structures in whole cells. Three-color confocal fluorescence imaging using filter-based microscopes can simultaneously image up to three spectral emission sources. However, problems with spectral cross-talk and autofluorescence can limit quantitative accuracy. Recently, multispectral fluorescence imaging (tens of wavelengths per pixel) has become available to improve quantitative accuracy and increase the numbers of fluorophores that can be monitored, but these tools lack the necessary multivariate analysis methods to fully characterize samples in the absence of reference spectra for all emitting species. In order to address these shortcomings with current imaging technologies, we have developed hyperspectral fluorescence imaging instruments which, when coupled with multivariate image analysis capabilities, can overcome many of the limitations of current commercial imaging methods.

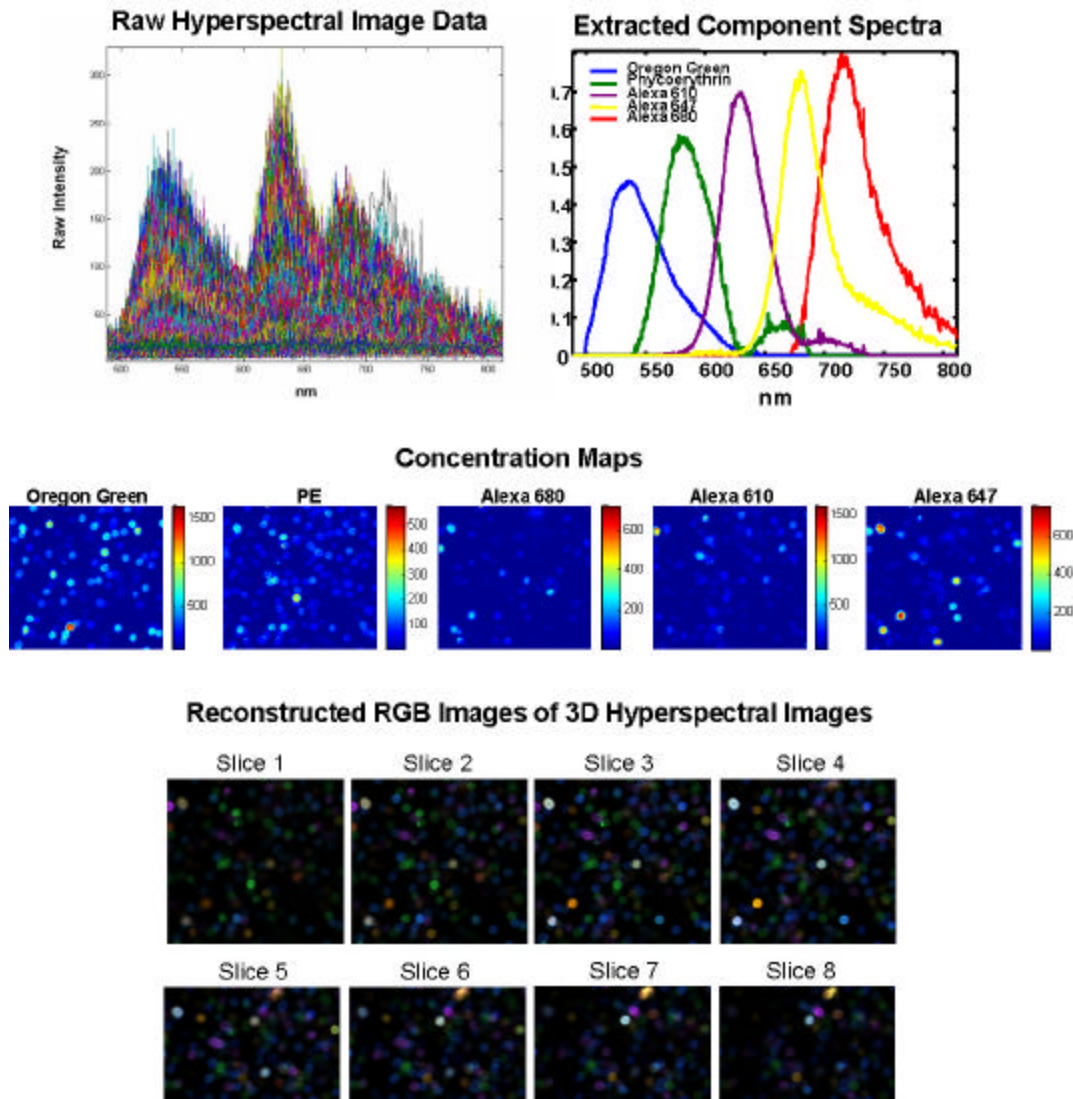
In hyperspectral imaging, an entire emission spectrum is collected at each image pixel (2-D) or voxel (3-D). Multivariate analysis software (employing Sandia's proprietary multivariate curve resolution [MCR] algorithm) is then used to process the spectra and identify and quantify all emission sources in a sample without *a priori* information about emitting species. Unlike the limitations of filter-based confocal fluorescence microscopes, large numbers of different fluorescent molecules can be simultaneously monitored with the hyperspectral microscope. Generally, there is little negative effect due to spectral cross-talk, interference from contaminant emission, or autofluorescence because independent concentration maps are produced for each emitting species. Thus, the hyperspectral imaging system is capable of higher quantitative accuracy relative to commercial microscopes and is capable of resolving many overlapping fluorophores simultaneously.

Our first hyperspectral imaging system was optimized as a 2-D microarray scanner and was the subject of an earlier DOE highlight. More recently we have designed and built a new state-of-the-art 3-D hyperspectral confocal fluorescence microscope. The new system operates with diffraction-limited spatial resolution in three dimensions (~250 nm in the x and y dimensions and ~600 nm in the z direction). It collects >8,300 full-emission spectra per second (512 wavelengths in the visible spectral region, 490 to 800 nm) with the use of a Sandia-designed imaging prism spectrometer. A state-of-the-art 2-D charge-coupled device (CCD) detector with electron multiplier gain gives the system high sensitivity for low-light applications.

The potential applications of this unique instrument are many. With the use of multiple donor-acceptor fluorescence dyes and the fluorescence resonance energy transfer (FRET)

method, our new microscope will allow us to simultaneously monitor the formation of multiple protein-protein complexes in living cells. The new system is also capable of performing single-point kinetics of processes on the 120 μsec time scale at diffraction-limited spatial resolutions. We can acquire 3-D images (~ 400 total pixels) of a single bacterium ($\sim 1.5 \mu\text{m}$ in diameter) in about 50 msec. This capability should permit the tracking of 3-D kinetic processes and subcellular interactions, even if the processes are tagged with severely overlapping fluorescent emissions or obscured by cellular autofluorescence. In addition, native fluorescent pigments can also be studied and we are now starting to apply our new imaging technology to the investigation of the photosynthetic processes in wild type and mutant cyanobacteria. These investigations will further our understanding of the photosynthetic process that is critical for the sequestration of carbon dioxide from the atmosphere. These studies would not be possible with non-hyperspectral fluorescence imaging instruments because the bacteria are small and the 4-5 pigments involved in photosynthesis are highly overlapped both spectrally and spatially.

The figure illustrates the power of the MCR software when applied to a 3-D hyperspectral confocal fluorescence image of fluorescent beads used to characterize the new spectrometer and associated multivariate analysis software. The MCR algorithm is able to resolve the five emitting species from these bioderivatized and multiply colored



fluorophore-coated silica beads. The figure shows the raw spectral data from a 2-D slice from the 3-D image and also shows the MCR results that yield the pure-component emission spectra from the five dyes present in the sample as well as the individual relative concentration maps of each fluorophore. Color composite images reconstructed for eight optical slices through the bead sample are also given in the figure. These images are created by assigning each of the fluorescent dye's concentration maps a color and creating an RGB image. Not only are we able to discover the pure emission spectra from this multicomponent sample, we are also able to derive quantitative concentration maps of the individual dyes without any *a priori* information about the dyes. Our new technology will directly impact the study of bacteria of interest to the Genomics:GTL program and it will also serve as an enabling technology for *in vivo* cell imaging in the broader scientific community.