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HYPERSPECTRAL IMAGING SYSTEM FOR QUANTITATIVE IDENTIFICATION AND DISCRIMINATION OF FLUORESCENT LABELS IN THE PRESENCE OF AUTOFLUORESCENCE

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ABSTRACT

Multivariate data analysis applied to hyperspectral images offers the unique opportunity to dramatically increase the amount of information gained from a single biological sample. Numerous fluorescent tags can be used to perform multiple studies in parallel from a single hyperspectral image scan. Highly spatially and spectrally overlapping fluorphores can be separated even amidst a large autofluorescence background with the use of multivariate curve resolution methods. The results of two biological samples with multiple fluorescent labels are shown and compared to a traditional filter-based multispectral system. These examples illustrate the combined power of the hyperspectral microscope hardware and the multivariate image analysis software for biomedical imaging. This technique has the potential to be applied to a broad array of biological applications where fluorescent tags are a central and ubiquitous tool, and to biomedical areas that focus on the discovery and identification of weak, broad spectrum native fluorescence.

1. INTRODUCTION AND BACKGROUND

Multispectral imaging is a filter based technique in which images of a sample are obtained from several wavelength regions or channels. This is a spectral binning method where all emission photons falling within a given wavelength band are integrated into a single channel intensity. This is an excellent technique if fluorescence emissions do not overlap to give spectral crosstalk, and have high quantum yield to overcome losses associated with the use of filters. Typical exogenous fluorescent tags have FWHM spectral widths of 50 nm, effectively limiting their number to 4 or 5 in a single sample. Studies based on endogenous fluorescence are more limited as their spectral widths are easily greater than 100 nm. In practice with biomedical imaging samples like cells and tissue, multiple fluorescent tags with overlapping spectra are often imaged in the presence of underlying autofluorescence. As a result, the measured intensity of a fluorescent species of interest will be falsely high due to spectral crosstalk from spectrally near fluorophores.

This problem can be overcome by using spectral shape as a species identifier, instead of a single intensity binned over a wavelength range. Many more spectral channels must be collected to adequately sample a fluorescence spectrum to define the spectral shape. Commercial microscopes have been recently developed with up to 32 channels using photomultiplier tube arrays [1, 2]. In single detector instruments, gratings, prisms, or tunable filters are used to acquire many spectral channels [3, 4]. However, these instruments require repeated scanning, and are therefore prone to photobleach the sample.

An imaging system that could acquire numerous spectral channels simultaneously, so that component fluorophores could be identified based on their spectral shape, and could acquire images quickly to minimize the effects of photobleaching would be ideal. Two hyperspectral imaging microscopes that satisfy these requirements have been designed and constructed at Sandia National Laboratories. Hyperspectral imagers, originally developed for remote sensing, typically collect hundreds of spectral channels in a signal image scan. One imager at Sandia operates as a two-dimensional (2D) imager and has 455 spectral channels spanning 550-850 nm with a spectral resolution of 3 nm and a spatial resolution ranging from 3-30 μm depending on the objective used. This 2D system is a pushbroom line configuration; ideal for applications where large area scans are desired and optical sectioning is not necessary. The second imager provides up to three-dimensions (3D) of hyperspectral data and is fully confocal with ~0.25 μm lateral spatial resolution, ~0.6 μm axial resolution, and a spectral range and resolution of 500-800 nm and 3nm, respectively. The light collected from the sample is spectrally dispersed onto the CCD using a grating for the 2D system and a prism for the 3D system. In addition, the 3D system has a novel CCD readout mode that allows fast scanning. For example, the confocal system can acquire a 25 μm by 25 μm field in 5 seconds sampling every 0.12 μm. Detailed descriptions for each system are in references [5, 6].

The datacube generated by the 2D hyperspectral scanner consists of two spatial dimensions (x,y) and a third spectral dimension. Multiple x-y images can be acquired at

a particular focus in the sample via sample scanning to image areas up to size of an entire microscope slide. The 3D confocal hyperspectral system generates a similar datacube but with an added axial (z) spatial dimension. A combination of sample scanning and mirror scanning permits acquisition of x-y-spectral images at various focal depths within the sample. The excellent temporal resolution offered by either system also facilitates the ability to follow processes over time.

In order to extract the maximum amount of information from the complex hyperspectral data cubes, both imagers are coupled with custom multivariate data analysis tools to create two fast and efficient systems for imaging in multicolor. Custom software performs principal component analysis (PCA) to determine the number of fluorescing species within a hyperspectral image. Multivariate curve resolution (MCR) is then applied to extract the pure component spectra and their relative concentrations [7-9]. This requires no a priori knowledge of the fluorescing species (though a priori information can easily be incorporated if details are known about a sample). Fluorescence spectra that are highly spatially and spectrally overlapped can be easily separated. In fact, contaminants are often discovered within a sample that has not previously been known [10]. Even with high contamination, weak signals from fluorophores below the noise floor can be extracted as long as there are some differences in spectral shape and/or spatial variation [11]. This sensitivity permits the excitation of multiple, overlapping fluorohores with a single excitation laser.

In this communication, the unparalleled discovery, sensitivity, quantitation, and multiplexing capabilities of the 2D and 3D hyperspectral imaging systems will be demonstrated for several biomedical applications.

2. RESULTS AND DISCUSSION

Examples are given in which spectrally overlapping fluorescent dyes are isolated in the presence of autofluorescence contamination in a biological sample. These examples illustrate the combined power of the hyperspectral microscope hardware and the multivariate image analysis software for biomedical imaging. The first example will be of an *in-situ* hybridized rat brain section labeled with four different fluorescent markers. Large scans approximately 0.04 mm^2 were obtained with the 2D hyperspectral system and subsequently analyzed using multivariate analysis. Using the 3D confocal hyperspectral imager, yeast cells labeled with two highly spectrally overlapped fluorescent tags - green fluorescent protein (GFP) and yellow fluorescent protein (YFP) were examined.

Although only two examples will be presented in this communication, many samples of biological interest (such as rat basophilic leukemia (RBL) cells, live cyanobacteria, DNA microarrays, and lipid bilayers) have been interrogated with our hyperspectral imaging systems and in all cases the hyperspectral imaging provided a unique look at the samples unavailable with any other technique.

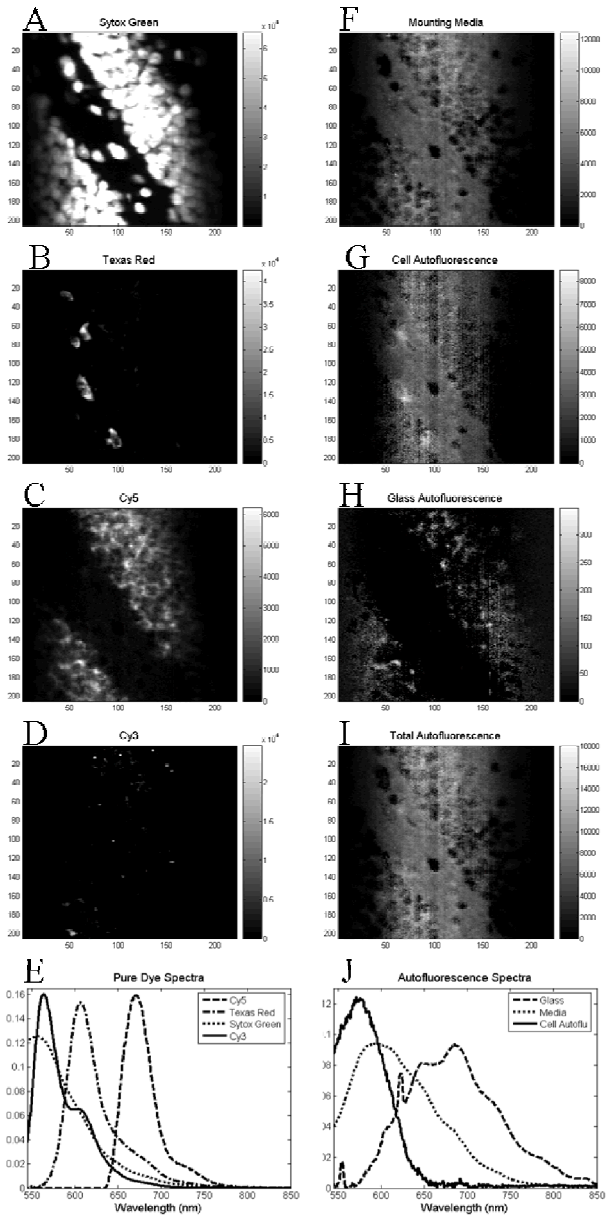

Figure 1. Rat brain tissue labeled with four different fluorescent labels. A-D. Extracted concentration images of dye labels. The Sytox green and Cy3 dyes are separated despite having almost complete spectral overlap. Image dimensions are 222 x 205 μm. E. Pure dye spectra. F-H. Concentration images of the component autofluorescence. H. Total autofluorescence concentration image. J. Spectra of the independent autofluorescence components.

2.1. Rat brain tissue

2D hyperspectral images were taken with 532 nm excitation and a 0.83 micron pixel size of the dentate gyrus region of a 25 micron thick slice of rat brain tissue that is labeled with Sytox green, Cy3, Cy5, and Texas red, to highlight the cell nuclei, *Arc* RNA, α-calcium/calmodulin kinase II RNA (CamII kinase), and Gad65/67 RNA, respectively. The purpose of this study was to explore behaviorally relevant neural circuits with temporal and 3D spatial resolution using specific behavior markers. As the details of this study are not the focus of this communication, the reader is referred to reference [12] for more specific study information.

Figure 1 shows the results of multivariate analysis of this hyperspectral image. The left column shows the dye concentration maps which contain the location of each dye within the rat brain. Sytox green is clearly seen in cell nuclei, as expected since it is used as a nuclear counterstain. Texas red labels Gad65/67 which is present in the cytoplasm of only a subset of the neurons known as interneurons. The Cy5 labels CamII kinase which is present primarily in the cellular cytoplasm. Cy3 is present where *Arc,* an immediate early gene, is expressed within small foci inside cell nuclei [13]. The pure spectrum, shown in Figure 1E, and relative concentration of each tag are identified despite their large spectral overlap and a large autofluorescence signal from several independent sources. It is important to note that the contribution from Sytox green and Cy3 are separated even though they have almost complete spectral overlap.

In addition to the RNA and nuclei labels, three sources of autofluorescence were discovered: Raman scattering and autofluorescence from the glass of the microscope slide coverslip (Figure 1H), cell autofluorescence (Figure 1G), and a surprisingly large contribution from the mounting media used to maintain tissue integrity after mounting onto the slide (Figure 1F). In a multispectral instrument, the total autofluorescence (shown in Figure 1I), would confound analysis and overwhelm the signal from Cy3 and Cy5. In this example our multivariate analysis tools identify the autofluorescence and effectively remove its effect from the label images, thus allowing a more accurate view of the dyes. The separation of Sytox and Cy3 emissions would not be possible with a multispectral instrument.

2.2. Yeast cells

3D confocal hyperspectral images were taken with 488 nm excitation of *Saccharomyces cerevisiae* yeast cells genetically engineered to produce GFP or YFP when exposed to galactose. Briefly, the dyes were labeled with a GAL1 promoter that is located on multiple copies of a 2-micron pYES2 plasmid. Cells in the sample had either GFP labeling only or YFP labeling only. Figure 2A is the concentration image for GFP, showing that it is taken up by the cell vacuoles when exposed to galactose.

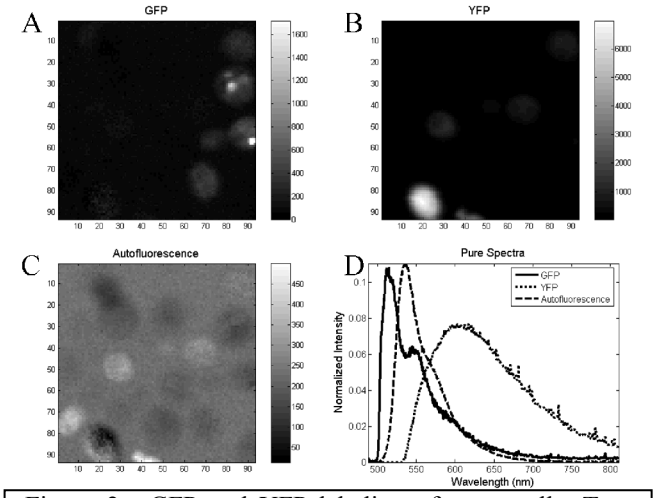

Figure 2. GFP and YFP labeling of yeast cells. Two separate groups of cells were combined in this sample. One labeled with GFP and another labeled with YFP. Cells are approximately 5 μm in diameter. A. GFP concentration image indicating GFP contained within cell vacuoles. B. YFP concentration image. C. Background autofluorescence. D. Plot of the pure spectra.

Figure 2B is the concentration image for YFP, where the dye is found primarily in the cell cytoplasm. There is a single brightly labeled cell and two fainter cells that are not in the confocal plane. Figure 2A and B show that the location of each dye is spatially distinct, facilitating the extraction of the pure dye spectra shown in Figure 2D. The low overall expression of GFP and YFP in the concentration images was because the cells were imaged before they had time to adequately react to galactose [14].

Figure 2C indicates that there is some underlying autofluorescence in the sample that can complicate accurate analysis of multispectral images. It is suspected that much of this autofluorescence is due to cells that are dead or dying. This leads to the potential possibility of using hyperspectral imaging for in situ cell viability monitoring during experimentation. The spectra for the three fluorescent components comprising the hyperspectral image is shown in Figure 2D. As seen with the rat brain study, the MCR analysis is capable of easily separating the highly spectrally overlapped GFP and YFP dyes that would otherwise be difficult to completely isolate from one another using a conventional multispectral system and filters.

3. CONCLUSIONS AND FUTURE WORK

The results presented in this communication demonstrate the unique and powerful capabilities of hyperspectral imaging. Used in combination with multivariate curve resolution analysis, multiple fluorophores, exogeneous and endogenous, can be monitored with high sensitivity within a

single sample. This permits highly multiplexed experiments to be performed, reducing sample to sample variation and the total number of samples that are needed for a single study. This has practical importance for expensive samples or animal studies where utilizing a large number of samples can be cost prohibitive.

Additionally, quantitative information can be gained on the relative contributions of the individual spectral components to the overall emission intensity when all sources of emission are identified. The concentration images presented in Figure 1 show that individual autofluorescence from the mounting media and cell autofluorescence would obscure the Cy5 fluorescence emission. Moreover, the high sensitivity of the system permits extraction of weak spectral components that would otherwise be lost in the noise or beneath another stronger spectral component as was seen with Sytox green and Cy3 in the rat brain tissue. This would have impact in biomedical areas where the discovery and identification of native fluorophores within tissue would greatly aid in the development and or refinement of diagnostic and screening instruments based on endogenous tissue fluorescence.

This high sensitivity to spectral shape also has the potential to be applied to biomedical applications to monitor the chemical environment within tissue during disease progression or treatment. Future studies have been planned that will couple time-resolved hyperspectral images with MCR to allow kinetic processes to be investigated.

In conclusion, the multivariate analysis and hyperspectral imaging systems developed at Sandia National Laboratories offers the unique opportunity to probe biological samples quantitatively and in a highly parallelized fashion using numerous spectrally overlapped fluorescent tags and or endogenous autofluorescence with high sensitivity.

4. ACKNOWLEDGEMENTS

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