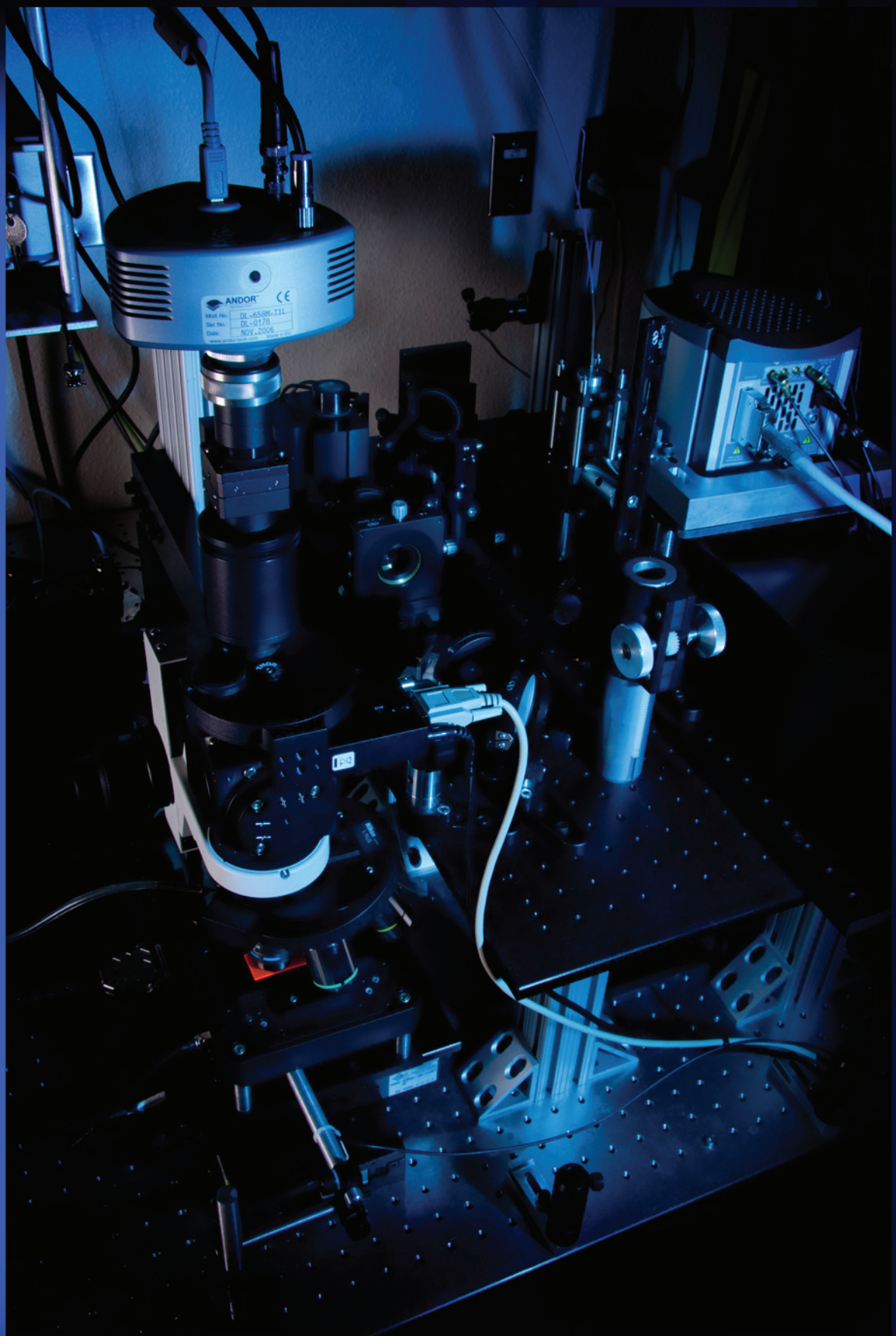
Hyperspectral Confocal Fluorescence Microscope System

SAND2009-1343P

R&D 100 ENTRY



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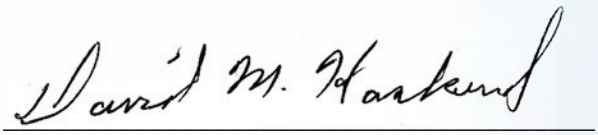
AFFIRMATION: I affirm that all information submitted as a part of, or supplemental to, this entry is a fair and accurate representation of this product.



David M. Haaland

JOINT ENTRY

N/A

PRODUCT NAME

Hyperspectral Confocal Fluorescence Microscope System

BRIEF DESCRIPTION

The product is a hyperspectral confocal fluorescence microscope with associated multivariate analysis software used to discover and quantify all the individual fluorescing species in three dimensions (3D).

Cover: Sandia National Laboratories, a hyperspectral confocal microscope. Photo by Lloyd Wilson.

Hyperspectral Confocal Fluorescence Microscope System

PRODUCT FIRST MARKETED OR AVAILABLE FOR ORDER

August 8, 2008.

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PRODUCT PRICE

As yet, no commercial units have been produced. Initial commercial units are estimated to cost between $300,000 and $500,000. The Cooperative Research and Development Agreement (CRADA) between Sandia National Laboratories and Monsanto Corp. has resulted in an improved design and construction of another hyperspectral confocal fluorescence microscope at Monsanto Corporation.

PATENTS OR PATENTS PENDING

Seven US patents issued.

1. Michael R. Keenan and Paul G. Kotula, "Apparatus and System for Multivariate Spectral Analysis," US Patent 6,584,413 issued June 24, 2003.
2. Michael R. Keenan and Paul G. Kotula, "Method of Multivariate Spectral Analysis," US Patent 6,675,106 issued January 6, 2004.
3. David M. Haaland and David K. Melgaard, "Generalized Augmented Classical Least Squares Methods," US Patents 6,687,620, 6,842,702, and 6,922,645 issued February 3, 2004, January 11, 2005, and July 26, 2005, respectively.
4. Michael R. Keenan, "Efficient Out-of-Core Algorithm for Analysis of Very Large Multivariate," US Patent 7,283,684 issued October 16, 2007.
5. Michael R. Keenan, "Improved Algorithm for Analysis of High Dimension Spectral Images," US Patent 7,400,772 issued July 15, 2008.
6. Michael R. Keenan and Mark H. Van Benthem, "Fast Combinatorial Algorithm for the Solution of Linearly Constrained Least Squares Problems," US Patent 7,451,173 issued November 11, 2008.
7. Michael R. Keenan, "Method of Exploiting Bias in Factor Analysis Using Constrained Alternating Least Squares Algorithms," US Patent 7,472,153 issued December 30, 2008.

Three US patent applications filed.

8. Michael B. Sinclair, "Hyperspectral Confocal Microscope," US patent application 11/334,840, filing date January 19, 2006.
9. Michael R. Keenan, "Methods for Spectral Image Analysis by Exploiting Spatial Simplicity," US patent application 11/233,223, filing date September 22, 2005.
10. Christopher L. Stork and Michael R. Keenan, "Method to Analyze Remotely Sensed Spectral Data," US patent application 11/410,445, filing date April 25, 2006.

Hyperspectral Confocal Fluorescence Microscope System

PRODUCT'S PRIMARY FUNCTION

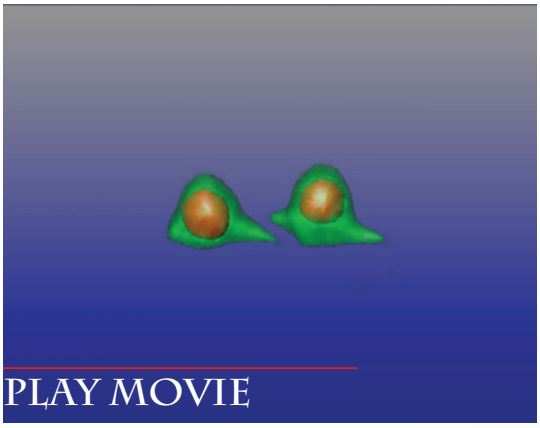
Researchers at Sandia National Laboratories (Sandia) have designed and constructed a new hyperspectral confocal fluorescence microscope. Hyperspectral microscopes image hundreds of spectral wavelengths when obtaining spectral images.

...the new microscope system allows us to rapidly "discover" all emitting fluorescence species in the image and to determine their relative concentrations throughout the image without any *a priori* information.

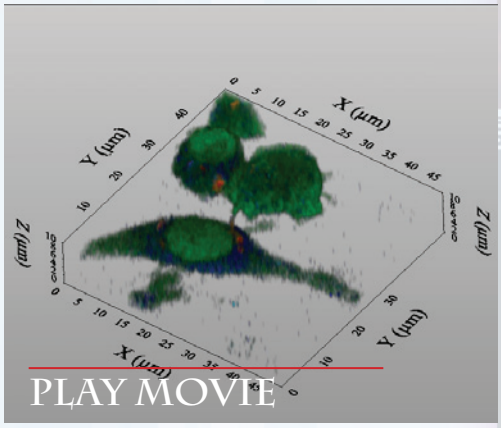
This patent-pending technology for the hyperspectral microscope has been combined with Sandia's unique and proprietary multivariate algorithms and software to form a complete system for the extraction of quantitative image information from the hyperspectral images at diffraction-limited spatial resolutions (250 nanometers (nm) in x and y and 600 nm in z). The hyperspectral microscope uses 488 nm laser excitation and collects 512 spectral emission wavelengths at each voxel (3D pixel) in the image over the spectral range from 500 to 800 nm at a spectral resolution of 1-3 nm, and at an imaging rate of 8300 spectra/sec (with extension to 64,000 spectra/sec in the future). These data acquisition speeds exceed the acquisition of other available hyperspectral imaging microscopes available in the research community.

The high acquisition speed is accomplished using an Electron Multiplying Charge Coupled Device (EMCCD) from Andor™ Technology with a special readout feature that allows continuous partial frame readout. The new readout mode specified by Sandia has made possible these extremely high hyperspectral imaging speeds. Our multivariate curve resolution (MCR) software employs new algorithmic approaches to accomplish dramatically faster computation of the rigorous, constrained alternating least squares MCR analysis. Thus, the new microscope system allows us to rapidly "discover" all emitting fluorescence species in the image and to determine their relative concentrations throughout the image without any

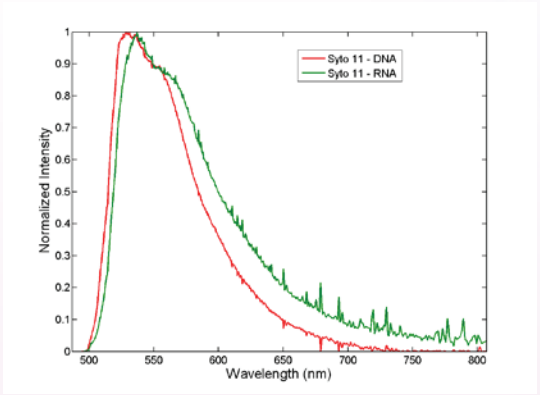
a priori information. Included in the hyperspectral imaging system are software programs for controlling the microscope and its data collection, as well as spectral image viewing software to view both the raw image data and the spectral and image results from the MCR analyses.



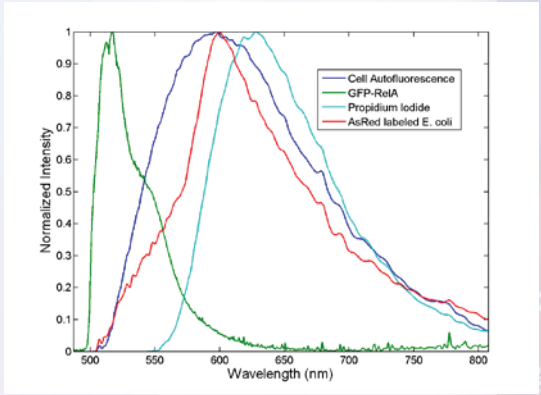
Rendered 3D image of live macrophage cells stained with Syto 11 dye. The colors in the image correspond to the colors in the adjacent plot of pure fluorescence components. The use of the RNA component (throughout the cytoplasm and nucleus) and the DNA component (nucleus only) allow the separation and imaging of the cytoplasm and nucleus with a single stain. These images were able to yield the nuclear to cytoplasm volume ratio which is important in determining the kinetics of the cell signaling process when the cells are exposed to pathogens.



3D image of live macrophage cells during exposure to E. coli. The colors in the image correspond to the colors in the adjacent plot of pure fluorescence components. There are several E. coli bacteria (red, AsRed fluorescent protein) that have already entered macrophage cells and one dead E. coli cell (cyan, i.e., propridium idodide viability stain) outside the cells. The GFP labeled RelA protein (green) has translocated into the cell nucleus for the two macrophage cells which have E. coli present in the cell. RelA is known to translate into the nucleus as part of the cell signaling process when under attack by pathogens. The autofluorescence is also observed at a low level in the cytoplasm (blue). The four fluorescent emission sources would not have been uniquely imaged and identified without the use of the Sandia hyperspectral confocal fluorescence microscope coupled with the MCR analysis.



Pure-component fluorescent components from MCR analysis of hyperspectral image of live macrophage cells stained with the nucleic acid stain Syto 11. The MCR analysis was able to "discover" and resolve two Syto 11 fluorescence components; one shifted relative to the other. These slightly shifted Syto 11 emission components are consistent with the stain being associated either with DNA (short wavelength component) or RNA (long wavelength component), respectively. Note the extremely high spectral overlap that would have been impossible to resolve and quantitatively image with a filter-based commercial microscope.



Pure-component fluorescent components from MCR analysis of hyperspectral image of live macrophage cells with invading E. coli bacteria. The macrophage cells were genetically modified to express the RelA protein with green fluorescent protein (GFP) and the E. coli express the AsRed fluorescent protein. The macrophage cell also contained the broad autofluorescent component and propidium idodide that was added as a monitor for cell viability. Note the extremely high spectral overlap that would have been impossible to resolve and quantitatively image with a filter-based commercial microscope.

PRODUCT'S COMPETITORS

Competing commercial technology products:

- Zeiss LSM710 META
- LightForm PARISS®
- Nikon Eclipse C1si
- WITec alpha300 S Scanning Near-field Optical Microscope

We are aware of only one commercial supplier of a hyperspectral near-confocal fluorescence microscope. LightForm, Inc. supplies a hyperspectral attachment (PARISS®) for commercial microscopes, but because it is a line-scanning imaging system rather than a point-scanning system, it is not a true confocal system. Therefore, the Lightform attachment does not achieve the high spatial resolution in all three spatial dimensions that is possible with the Sandia hyperspectral microscope.

Zeiss (LSM710 META) and Nikon (Eclipse C1si) supply multispectral confocal fluorescence microscopes with spectral information collected in only 32 wavelengths (i.e., multispectral rather than hyperspectral). WITec sells a hyperspectral scanning near-field optical microscope (alpha300 S Scanning Near-field Optical Microscope) system used for hyperspectral confocal fluorescence imaging with higher spatial resolution but slower imaging capabilities than the Sandia microscope. Prairie Technologies, Inc. is developing a new hyperspectral confocal microscope, but its product is not yet available.

...there is no comparable hyperspectral confocal fluorescence microscope system that both performs hyperspectral imaging and can adequately analyze the spectral images when the pure fluorescence species are unknown in the exact form present in the sample image.

Although all of these companies have software solutions to perform the spectral unmixing (i.e., determining the relative concentrations of the various fluorescence components in the collected spectral images), they rely on separately measured pure fluorescence spectra or library spectra to perform the unmixing. This approach can be problematic, because oftentimes the pure

emission spectra cannot be obtained separately — they may not exist in the sample without the presence of other overlapping emission sources. Thus, unlike the Sandia algorithms and software, they do not have methods to discover all the pure emission sources in the spectral image solely from the collected image data. Consequently, errors will result in the quantitative composition maps if unexpected emission components are present, or if environmental effects in the sample change the spectral position or spectral shape of the emission bands. Analyses of our hyperspectral images have demonstrated that fluorescence spectra of the pure emission sources are often greatly influenced by the local environment of the sample.

Zeiss does have a blind unmixing that does not require the knowledge of the pure emission spectra, but the Zeiss approach involves a slow search method that often results in incorrect solutions. Our MCR algorithm approach allows blind unmixing of the hyperspectral images to "discover" and quantify all the independently varying fluorescence species in the image. Unexpected or unknown fluorescence species in the imaged samples can be accurately detected and quantified. In addition, any changes in the shape or position of the fluorescence spectra of the emitting species are readily identified and will not result in quantitative errors in the composition of the concentration maps of the fluorescent components. In fact, with the Sandia microscope and analysis approach, changes in the pure emission spectra can be used to indirectly monitor the local environment of the fluorescent molecules in biological samples.

The Sandia system has the ability to perform hyperspectral imaging in a purely discovery mode for all those samples where the set of emission components is either not known or where the emission component spectra are dependent on the local environment of the sample.

Independent commercial software that performs MCR on spectral data exists, but the commercial MCR software is either too slow to be useful for large hyperspectral images, or it uses short-cut methods that are not guaranteed to converge and often yields incorrect solutions. Therefore, there is no comparable hyperspectral confocal fluorescence microscope system that both performs hyperspectral imaging and

can adequately analyze the spectral images when the pure fluorescence species are unknown in the exact form present in the sample image.

The Sandia system has the ability to perform hyperspectral imaging in a purely discovery mode for all those samples where the set of emission components is either not known or where the emission component spectra are dependent on the local environment of the sample. Our system can obtain relative concentration maps of each of the emission components in the samples without fear of spectral cross talk from overlapping spectral components. This gives our system a quantitative advantage, and allows our microscope to accurately observe emission species that other microscopes either miss entirely or measure inaccurately.

Although other researchers have recently developed a few hyperspectral confocal fluorescence microscopes, none has the multivariate image analysis capabilities that make the Sandia system unique.

We often find that with our hyperspectral microscope and MCR analysis, we are able to see features and components in living samples that no one has ever seen before. Although other researchers have recently developed a few hyperspectral confocal fluorescence microscopes, none has the multivariate image analysis capabilities that make the Sandia system unique.

COMPARISON MATRIX

Product	Sandia Microscope	Zeiss LSM710 META	Nikon Eclipse C1si	WiTec	Light Form PARISS	Competitive advantage of Sandia microscope
Number of spectral channels	512	3 to 34	32	1600	Variable, depends on detector and microscope selected	Advantage over multispectral systems
Speed of Image collection	intermediate	fast	fast	slow	Variable, depends on detector and microscope selected	Fastest for true hyperspectral mode
Confocal?	yes	yes	Yes	yes	Only in one dimension	No advantage
Spectral resolution	1 to 3 nm	3 nn at best resolution	2.5-10 nm	1 nm	Variable	Highest spectral resolution with high-speed hyperspectral system
Spectral range	490 to 800 nm	Variable	400-750 nm	500 to 800 nm	365 to 920 nm	No advantage
Fast MCR software	yes	no	no	no	no	Only system with fast, accurate MCR analysis

HOW PRODUCT IMPROVES UPON COMPETITION

The Sandia hyperspectral microscope system collects more spectral wavelengths and/or collects the images more rapidly than the competing technology. The analysis software is dramatically more flexible and allows rigorous, least squares analysis of the spectral images without any *a priori* information being required. This capability means that all the independently varying emission sources in the sample image can be discovered and quantified with negligible cross talk between spectrally or spatially overlapped fluorophores. As a hyperspectral microscope system that includes Sandia's proprietary MCR analysis software, the Sandia system is unique in its capabilities. These unique capabilities not only allow the Sandia microscope system to discover all the independently varying emission species in any sample that is imaged but also allows the image concentration maps of each fluorophore to be quantified with unprecedented accuracy.

When operated in discovery mode, this microscope system can uncover new fluorescent species in samples that may not have been known to exist. It also allows an expansion of the structural stains and molecular fluorophores that biologists can introduce into biological samples simultaneously since this new microscope and analysis system can accurately multiplex and recover the individual composition maps of each fluorophores, even those that are highly overlapped spectrally and/or spatially.

We have even been able to discover and identify fluorescence species whose emission spectra are separated by only 2 nm. For example, Syto 13 nucleic acid dye was found to have a 2 nm shift in peak wavelength maximum depending on whether the dye was attached to Deoxyribonucleic Acid (DNA) or Ribonucleic Acid (RNA), making possible the imaging of DNA and RNA locations in live cells for the first time. Many other similarly new and exciting discoveries have been made with this microscope given its powerful new capabilities.

Hyperspectral Confocal Fluorescence Microscope System

PRODUCT'S PRINCIPAL APPLICATIONS

Our current microscope system can image samples that have fluorescence species present that are excited by the 488 nm laser excitation. The system is optimized for small heterogeneous samples such as living cells and tissue samples containing multiple endogenous and/or exogenous fluorescence species. We have primarily used the microscope to measure biological samples such as living plant, animal and bacterial cells, thin animal and plant tissue samples, and biofilms on water purification membranes. All of these applications of the microscope system allow us to obtain greater information content from a given sample by multiplexing with many fluorophores present in the sample or providing unprecedented quantitative imaging or both. The microscope is excellent for imaging plant and cyanobacteria cells that contain multiple fluorescent photosynthetic pigments. The improvement in the information content and detail with the new microscope relative to commercial optical filter-based microscopes is readily apparent from the images in figure 1. The image on the right-hand side was obtained by the commercial microscope and required the introduction of a fluorescent dye in order to see the epidermis structure of the corn leaf. The image of a similar corn leaf sample with the hyperspectral microscope was able to resolve five emission components, three of which are represented in the RGB (red, green, blue) image on the

We have primarily used the microscope to measure biological samples such as living plant, animal and bacterial cells, thin animal and plant tissue samples, and biofilms on water purification membranes.

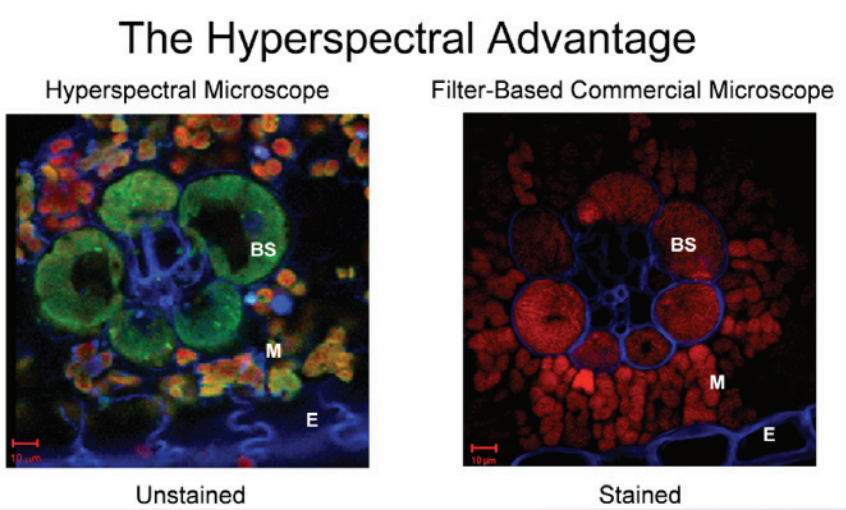


Figure 1. *Cross section of a corn leaf. BS, bundle sheath; M, mesophyll; E epidermis.* ***Left image:*** *2-dimensional RGB image obtained from the MCR analysis of a hyperspectral image of a thin corn leaf section using the Sandia microscope. The red, green and blue colors represent the relative concentrations of 3 of the 5 fluorescent pigments in the corn leaf. No external staining was used to obtain this image.* ***Right image:*** *The image of a similar corn leaf section obtained from a commercial optical-filter based confocal microscope. Note the blue epidermis features required the use of an external stain to visualize the epidermis. The comparison of these images demonstrates the power of the new Sandia microscope system.*

left-hand side of figure 1. Figure 2 shows the raw spectral data and integrated intensity image of the photosynthetic cyanobacteria, Synechocystis. It also shows the analysis process and results obtained from the MCR analysis that converted the raw spectral image data into pure emission spectra and quantitative relative concentration maps for the six photosynthetic pigments that were discovered by

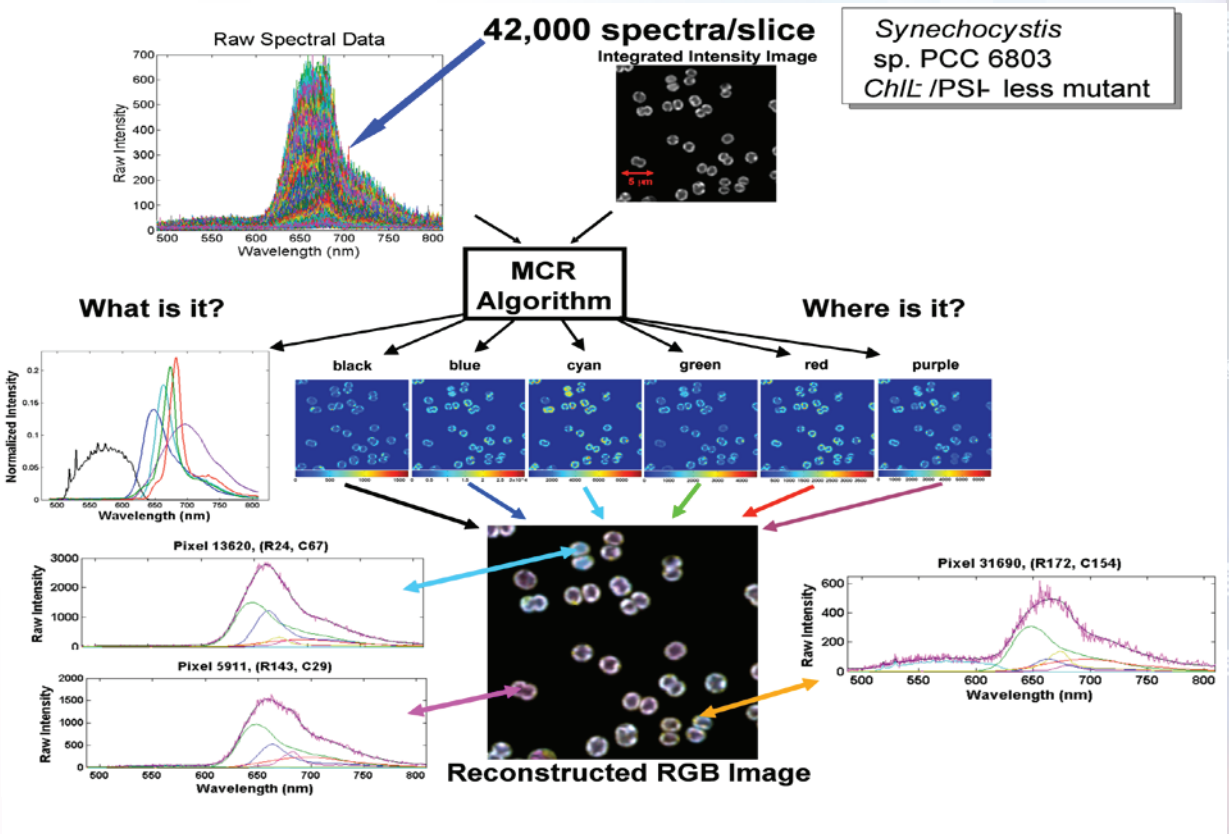


Figure 2. *Hyperspectral image results from a genetic mutant of Synechocystis sp. PCC 6803 cyanobacteria.* ***Top left:*** *Spectra obtained from a single slice of the live Synechocystis cyanobacteria. Top right: Integrated intensity image of Synechocystis obtained by integrating the spectral intensity data on the left.* ***Middle images:*** *MCR results obtained from the analysis of the hyperspectral image of the Synechocystis including the six emission spectra of the fluorescent components in the cyanobacteria* ***(middle left)*** *and the corresponding six individual composition maps* ***(middle right)*** *resulting from the MCR analysis. Bottom images: RGB image obtained from a composite of the six individual composition maps. The three spectral plots correspond to data obtained from three separate spatial pixels in the image. The individual spectral plots contain the raw spectrum from the selected pixel (magenta), the MCR fitted spectrum (black), and the amounts of the six individual pure spectra required to achieve the best MCR fitted spectrum. These plots demonstrate the heterogeneity of composition within and between individual Synechocystis cyanobacteria.*

our microscope and analysis methods (see Vermaas et al., "In vivo Hyperspectral Confocal Fluorescence Imaging to Determine Pigment Localization and Distribution in Cyanobacterial Cells," Proceedings of the National Academies of Sciences, 105, 4050-4055 (2008)).

The microscope has also been used to perform *in situ* monitoring of the synthesis of quantum dots in microfluidic platforms to better understand the kinetic reaction mechanisms and rate constants involved in their synthesis. Autofluorescence, exogenous fluorophores, stains, and quantum dots have all been used as emission sources during the imaging of samples of interest to Sandia and our university and industrial collaborators. By cleanly and quantitatively separating cell autofluorescence from the exogenous fluorophores, we have been able to obtain unprecedented image contrast and have eliminated any ambiguity in the proportion of the fluorescence that is caused by native fluorescence or the exogenously added fluorophores. We have been able to greatly increase information content in an image of a sample by adding many spectrally overlapping fluorophores to each sample. Each fluorophore can be used to identify structural components in a living cell, monitor cell viability, and follow the generation and location of multiple proteins with the use of multiple genetically modified fluorescent proteins each with a different color. We can even even monitor the metabolic state of the cell by separating and quantifying the reduced and oxidized flavin autofluorescent components in the cell.

OTHER APPLICATIONS

The new microscope system can image any sample that can be placed under the microscope objective and has fluorescence species that can be excited by the laser. Thus, the application space in hyperspectral fluorescence imaging is quite large. In addition, the MCR software is not restricted to the analysis of fluorescence image data. The MCR approach can be applied to any hyperspectral image data. We have applied it to hyperspectral infrared images, Raman images, energy dispersive spectral images obtained from transmission electron and scanning electron microscopes, and secondary scattering ion mass spectral images. We have even used the MCR software to analyze hyperspectral infrared and visible images obtained in remote sensing from airborne platforms.

Sandia and the University of New Mexico Cancer Research Facility used an earlier version of the microscope in a joint research project. This research focused on gene expression microarrays for studying genetic markers for Leukemia and treatment outcomes. We imaged microarray slides with the earlier hyperspectral microscope and helped analyze the microarray data.

Laboratory Directed Research and Development (LDRD) funding has driven a host of new applications for MCR. In the area of material durability diagnostics, one LDRD developed infrared spectroscopic techniques to quickly identify chemical constituents of materials and then make inferences about their aging characteristics and viability, work directly impacting evaluation of the aging of materials used in airplanes and nuclear reactors.

Taken as a whole, our work has elicited funding from the Department of Energy's Genomes to Life program, National Institute of Health funding for rat brain imaging, gene expression analysis, and microarray scanning, Environmental Protection Agency funding of a gene expression program, and a Cooperative Research and Development Agreement (CRADA) with Monsanto to develop improved seed-based products for biofuels.

Hyperspectral Confocal Fluorescence Microscope System

2009

SUMMARY

With this new microscope system, large numbers of fluorophores can be monitored simultaneously without cross talk to achieve higher throughput, greater quantitative accuracy, and increased reliability. The hyperspectral microscope has been especially useful for multiplexed 3D imaging of live cells at diffraction-limited spatial resolutions in a large variety of biological applications. This microscope system is able to collect hyperspectral images of 512 wavelengths at unprecedented acquisition speeds of 8300 spectra/sec. The associated MCR software uses new algorithmic approaches to perform rigorous constrained alternating least squares analyses at computational speeds and robustness that far outperform externally available software. In combination, the microscope and software provide a unique system that allows us to discover and quantify fluorescence species that other microscopes are not able to distinguish or quantify.

The associated MCR software uses new algorithmic approaches to perform rigorous constrained alternating least squares analyses at computational speeds and robustness that far outperform externally available software.

Hyperspectral Confocal Fluorescence Microscope System

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APPENDICIES ITEMS

Appendix A

Articles about the Hyperspectral Confocal Fluorescence Microscope System

- *Applied Spectroscopy*
- *In vivo hyperspectral confocal fluorescence imaging to determine pigment localization and distribution in cyanobacterial cells*
- *Hyperspectral microarray scanning: impact on the accuracy and reliability of gene expression data.*
- *Weighting hyperspectral image data for improved multivariate curve resolution results*

Appendix B

Letters of Support/Testimonials

- *Wim Vermaas, Professor, ASU*
- *Monsanto*

Appendix C

Seven US patents issued.

1. Michael R. Keenan and Paul G. Kotula, "Apparatus and System for Multivariate Spectral Analysis," US Patent 6,584,413 issued June 24, 2003.
2. Michael R. Keenan and Paul G. Kotula, "Method of Multivariate Spectral Analysis," US Patent 6,675,106 issued January 6, 2004.
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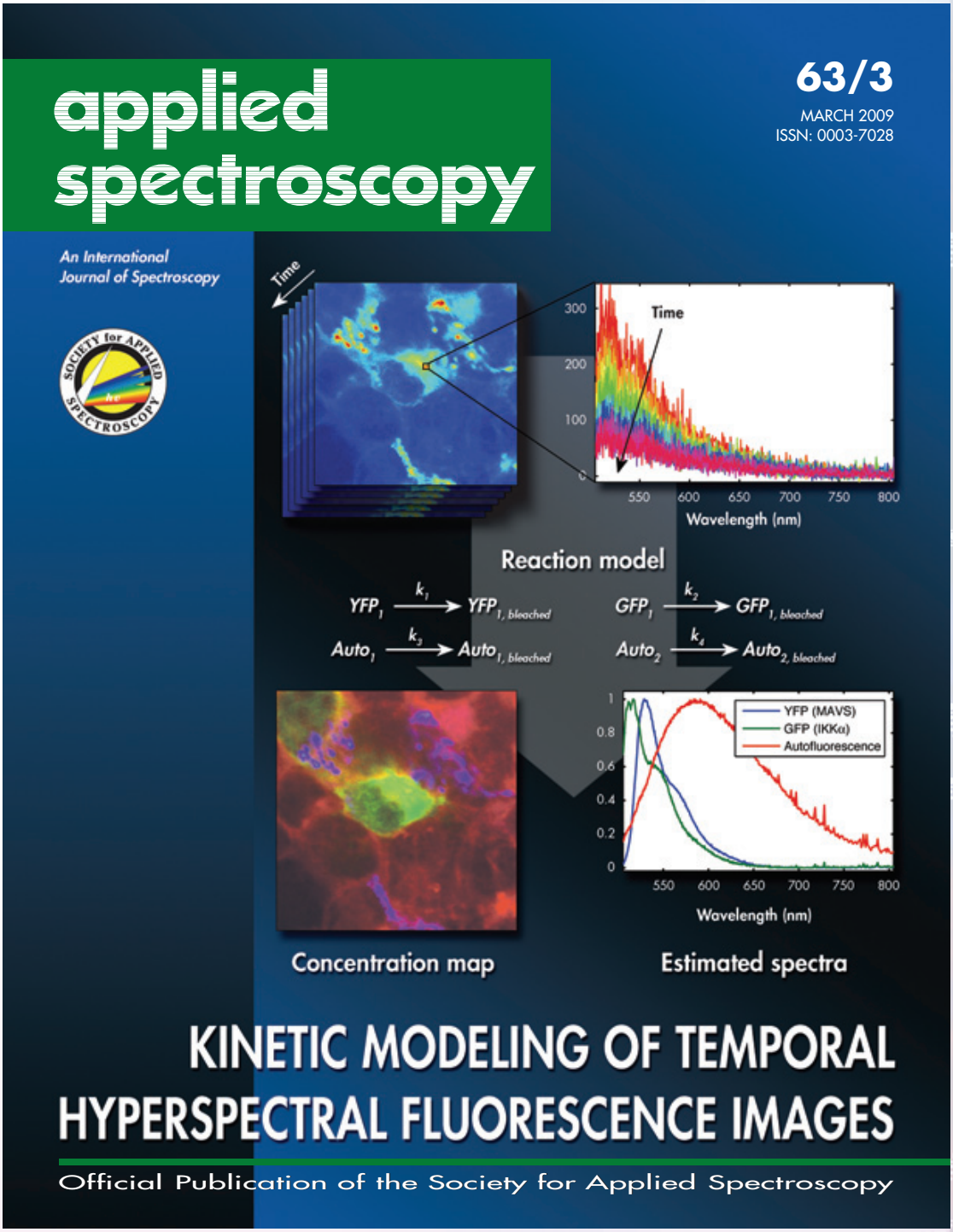
5. Michael R. Keenan, "Improved Algorithm for Analysis of High Dimension Spectral Images," US Patent 7,400,772 issued July 15, 2008.
6. Michael R. Keenan and Mark H. Van Benthem, "Fast Combinatorial Algorithm for the Solution of Linearly Constrained Least Squares Problems," US patent 7,451,173 B1 issued November 11, 2008.
7. Michael R. Keenan, "Method of Exploiting Bias in Factor Analysis Using Constrained Alternating Least Squares Algorithms," US patent 7,472,153 issued December 30, 2008.

APPENDIX ITEM A ARTICLES

2009

Applied Spectroscopy



Novel methods for fitting kinetic models to temporally resolved hyperspectral images of fluorescently labeled cells can be used to mathematically resolve pure-component spatial concentration maps, pure-component spectra, and pure-component reaction profiles. At each pixel in a temporally resolved hyperspectral image ***(upper left corner)****, a set of fluorescence spectra are recorded as a function of time* ***(upper right corner)****. Kinetic fitting is performed by postulating a reaction model consisting of a combination of several first-order decays* ***(center)****. Identification of a parsimonious and statistically sufficient model yields spatial concentration maps of each fluorophore* ***(lower left corner)*** *and their respective pure component spectra* ***(lower right corner)****.*

APPENDIX ITEM A ARTICLES

In vivo hyperspectral confocal fluorescence imaging to determine pigment localization and distribution in cyanobacterial cells

PNAS PNAS PNAS PNAS

In vivo hyperspectral confocal fluorescence imaging to determine pigment localization and distribution in cyanobacterial cells

Wim F. J. Vermaas*†, Jerilyn A. Timlin‡, Howland D. T. Jones‡, Michael B. Sinclair‡, Linda T. Nieman‡§, Sawsan W. Hamad*, David K. Melgaard‡, and David M. Haaland‡

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Edited by Elisabeth Gantt, University of Maryland, College Park, MD, and approved January 25, 2008 (received for review August 27, 2007)

Hyperspectral confocal fluorescence imaging provides the opportunity to obtain individual fluorescence emission spectra in small (≈0.03-μm^3) volumes. Using multivariate curve resolution, individual fluorescence components can be resolved, and their intensities can be calculated. Here we localize, *in vivo*, photosynthesis-related pigments (chlorophylls, phycobilins, and carotenoids) in wild-type and mutant cells of the cyanobacterium *Synechocystis* sp. PCC 6803. Cells were excited at 488 nm, exciting primarily phycobilins and carotenoids. Fluorescence from phycocyanin, allophycocyanin, allophycocyanin-B/terminal emitter, and chlorophyll *a* was resolved. Moreover, resonance-enhanced Raman signals and very weak fluorescence from carotenoids were observed. Phycobilin emission was most intense along the periphery of the cell whereas chlorophyll fluorescence was distributed more evenly throughout the cell, suggesting that fluorescing phycobilisomes are more prevalent along the outer thylakoids. Carotenoids were prevalent in the cell wall and also were present in thylakoids. Two chlorophyll fluorescence components were resolved: the short-wavelength component originates primarily from photosystem II and is most intense near the periphery of the cell; and the long-wavelength component that is attributed to photosystem I because it disappears in mutants lacking this photosystem is of higher relative intensity toward the inner rings of the thylakoids. Together, the results suggest compositional heterogeneity between thylakoid rings, with the inner thylakoids enriched in photosystem I. In cells depleted in chlorophyll, the amount of both chlorophyll emission components was decreased, confirming the accuracy of the spectral assignments. These results show that hyperspectral fluorescence imaging can provide unique information regarding pigment organization and localization in the cell.

cyanobacteria | photosynthetic pigments | multivariate curve resolution

Cyanobacteria convert light energy to chemical energy by means of photosynthesis, using water as a source of electrons for CO_2 reduction and O_2 production. A key part of the photosynthesis process is light absorption (harvesting) by pigments, followed by excitation transfer to reaction center chlorophyll (Chl) *a* of photosystems (PS) II and I (1). These processes take place in thylakoid membranes that in cyanobacteria generally form an extensive internal membrane complex of several layers along the periphery of the cytoplasm, with thylakoids found less frequently toward the center of the cell (2).

The pigments associated with the photosynthetic apparatus are bound to thylakoid proteins, modifying their spectral properties and providing a spatial distribution that aids in the efficiency of light harvesting and energy transfer to reaction center Chls. Pigments bound to integral membrane proteins in reaction center complexes in thylakoids of cyanobacteria include Chl *a* [≈40 per PS II (3) and ≈100 per PS I (4)] and carotenoids; the latter act in photoprotection and ^{3}Chl quenching but do not effectively transfer energy to Chl in cyanobacterial PS II (5). Carotenoids are also present in the outer cell membrane and cytoplasmic membrane of cyanobacteria, whereas Chl is not (6, 7). Additional light-harvesting capability, primarily for PS II, is provided by phycobilisomes, which are pigment-binding complexes in the cytoplasm that associate with thylakoids to enable energy transfer to Chl (8, 9). Phycocyanin (PC), allophycocyanin (APC), and allophycocyanin-B (APC-B) are the main phycobilisome pigments in *Synechocystis* sp. PCC 6803 (10).

Chl and phycobilisome pigments fluoresce at room temperature with spectral maxima in the 640- to 700-nm range. PC emits fluorescence with an ≈650-nm maximum, APC at 665 nm, and APC-B at 675 nm, and the main emission wavelength of Chl is at 685 nm (11). Phycobilisomes are highly fluorescent in isolated form, but the fluorescence yield is decreased in intact systems because *in vivo* the excitation energy is transferred efficiently from PC to APC to APC-B or to long-wavelength APC associated with the ApcE protein (terminal emitter) (12) and eventually to Chl in the thylakoid membranes.

Much is known regarding cyanobacterial cell architecture and thylakoid organization (2, 13–15), the structure of individual pigment-binding complexes (16, 17), the distribution of photosynthetic complexes in fixed thylakoid membranes (18), and the ability of phycobilisomes to dynamically associate with photosynthetic complexes in the membrane (19–21). However, photosynthetic pigments and their interactions have not yet been visualized distinctly *in vivo* because of their spectral overlap. Spectral congestion (fluorescence emission maxima that are different by <20–30 nm) is common in photosynthetic systems that depend on spectral overlap for efficient energy transfer and presents a major problem in data analysis/interpretation. Confocal laser scanning microscopy coupled with spectral imaging techniques has the potential to visualize photosynthetic pigments even amidst spectral overlap. However, because of low spectral resolution of current commercial instrumentation and the absence of methods available for robust analysis of spectrally and spatially overlapped spectral images, application of this technique has been limited to systems with relatively few fluorescent pigments that are spectrally distinct and spatially isolated (22). The recent development of a high-resolution hyperspectral confocal fluorescence imaging microscope (23) and correspond-

Author contributions: W.F.J.V., J.A.T., H.D.T.J., M.B.S., and D.M.H. designed research; W.F.J.V., J.A.T., H.D.T.J., M.B.S., L.T.N., S.W.H., and D.M.H. performed research; W.F.J.V., J.A.T., H.D.T.J., M.B.S., S.W.H., D.K.M., and D.M.H. contributed new reagents/analytic tools; W.F.J.V., J.A.T., H.D.T.J., M.B.S., L.T.N., S.W.H., D.K.M., and D.M.H. analyzed data; and W.F.J.V., J.A.T., H.D.T.J., and D.M.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0708090105/DC1.

Hyperspectral microarray scanning: impact on the accuracy and reliability of gene expression data

BMC Genomics

BioMed Central

Research article

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Hyperspectral microarray scanning: impact on the accuracy and reliability of gene expression data

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Published: 11 May 2005

BMC Genomics 2005, **6**:72 doi:10.1186/1471-2164-6-72

Received: 15 February 2005

Accepted: 11 May 2005

This article is available from: http://www.biomedcentral.com/1471-2164/6/72

Abstract

Background: Commercial microarray scanners and software cannot distinguish between spectrally overlapping emission sources, and hence cannot accurately identify or correct for emissions not originating from the labeled cDNA. We employed our hyperspectral microarray scanner coupled with multivariate data analysis algorithms that independently identify and quantitate emissions from all sources to investigate three artifacts that reduce the accuracy and reliability of microarray data: skew toward the green channel, dye separation, and variable background emissions.

Results: Here we demonstrate that several common microarray artifacts resulted from the presence of emission sources other than the labeled cDNA that can dramatically alter the accuracy and reliability of the array data. The microarrays utilized in this study were representative of a wide cross-section of the microarrays currently employed in genomic research. These findings reinforce the need for careful attention to detail to recognize and subsequently eliminate or quantify the presence of extraneous emissions in microarray images.

Conclusion: Hyperspectral scanning together with multivariate analysis offers a unique and detailed understanding of the sources of microarray emissions after hybridization. This opportunity to simultaneously identify and quantitate contaminant and background emissions in microarrays markedly improves the reliability and accuracy of the data and permits a level of quality control of microarray emissions previously unachievable. Using these tools, we can not only quantify the extent and contribution of extraneous emission sources to the signal, but also determine the consequences of failing to account for them and gain the insight necessary to adjust preparation protocols to prevent such problems from occurring.

Background

Since their introduction in 1995 [1], DNA-based microarrays (also known as genechips) have driven an explosion in functional genomic analyses. All varieties of microarrays have in common the ability to perform binary

APPENDIX ITEM A ARTICLES

Weighting hyperspectral image data for improved multivariate curve resolution results

Special Issue

CHEMOMETRICS

Received: 18 January 2008, Revised: 19 May 2008, Accepted: 21 May 2008, Published online in Wiley InterScience: 29 July 2008

(www.interscience.wiley.com) DOI: 10.1002/cem.1170

Weighting hyperspectral image data for improved multivariate curve resolution results

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The combination of hyperspectral confocal fluorescence microscopy and multivariate curve resolution (MCR) provides an ideal system for improved quantitative imaging when multiple fluorophores are present. However, the presence of multiple noise sources limits the ability of MCR to accurately extract pure-component spectra when there is high spectral and/or spatial overlap between multiple fluorophores. Previously, MCR results were improved by weighting the spectral images for Poisson-distributed noise, but additional noise sources are often present. We have identified and quantified all the major noise sources in hyperspectral fluorescence images. Two primary noise sources were found: Poisson-distributed noise and detector-read noise. We present methods to quantify detector-read noise variance and to empirically determine the electron multiplying CCD (EMCCD) gain factor required to compute the Poisson noise variance. We have found that properly weighting spectral image data to account for both noise sources improved MCR accuracy. In this paper, we demonstrate three weighting schemes applied to a real hyperspectral corn leaf image and to simulated data based upon this same image. MCR applied to both real and simulated hyperspectral images weighted to compensate for the two major noise sources greatly improved the extracted pure emission spectra and their concentrations relative to MCR with either unweighted or Poisson-only weighted data. Thus, properly identifying and accounting for the major noise sources in hyperspectral images can serve to improve the MCR results. These methods are very general and can be applied to the multivariate analysis of spectral images whenever CCD or EMCCD detectors are used.

Keywords: hyperspectral imaging; spectral imaging; noise analysis; data weighting; multivariate analysis; multivariate curve resolution; MCR

1. INTRODUCTION

Multivariate curve resolution (MCR), when applied to hyperspectral images, is a powerful technique for investigating a variety of biological and related samples [1–7]. MCR analysis techniques have also been successfully applied to non-image spectral data including vibrational spectroscopy data [8]. MCR can provide relative quantitative analyses of spectral image data without the need for standards, and it can discover all the emitting species present in an image, even those about which we have no *a priori* information. As an example, we have used our new hyperspectral fluorescence confocal microscope and MCR to discover and identify all of the emitting photosynthetic pigments in live cyanobacteria cells (*Synechocystis* sp. PCC 6803) and to determine the spatial distribution of these pigments in these small cells (~2 μm diameter) [9]. In this example, we were able to resolve multiple fluorescent components with highly overlapped emission spectra (the emission peaks were all within ~50 nm of each other). Without this combined hyperspectral microscope and MCR analysis system, it would not have been possible to obtain the true spatial distribution of each pigment within these small photosynthetic cells. We have also found MCR to be a valuable tool in quantifying multiple known fluorophores and confirming the presence of an unknown contaminant fluorophore when using our hyperspectral microarray scanner to interrogate DNA microarray slides [7]. These examples demonstrate the importance and benefits of combining hyperspectral imaging and MCR analysis for exploring unknown biological samples or confirming the relative spatial distributions of samples with known fluorophores present.

As previously touted, MCR is a powerful analysis tool; however, it can only be as good as the data input to the algorithm. Attention needs to be given to the preprocessing and preparation of the data prior to performing the MCR analysis to remove unnecessary spectral features that may be present in the data (cosmic spike removal, dark image subtraction and baseline correction). Another important reason for data preprocessing is to properly account for the heteroscedastic nature of all the major noise sources present in the spectral images. The goal of weighting is to redistribute the noise uniformly across the spectral and spatial dimensions, since the MCR algorithm is optimized for uniform and uncorrelated noise. With proper handling of heteroscedastic noise in spectral images, MCR results are often improved and smaller spectral components can be more readily discovered [10]. In addition, appropriately weighting the data for the noise characteristics of each spectral image can

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APPENDIX ITEM B LETTER OF SUPPORT/TESTIMONIAL

Wim Vermaas, Professor, ASU

ASU SCHOOL OF Life Sciences
ARIZONA STATE UNIVERSITY

February 9, 2009

Dr. David M. Haaland
Sandia National Laboratories
MS0895
PO Box 5800
Albuquerque, NM 87185-0895

RE: R&D 100 Awards

Dear Dave:

I am very happy to hear that you are submitting the hyperspectral confocal fluorescence microscope that Mike Sinclair and you developed for consideration for an R&D 100 Award. Such an award would be so well deserved!

As you know, we have used the microscope for our joint project on imaging of cyanobacterial cells, which has resulted in a first publication last year (Vermaas et al. (2008) Proc Natl Acad Sci USA 105, 4050-4055). Cyanobacteria, which are about 1.5 µm in diameter and thereby just a little larger than the intrinsic resolution of optical microscopy, contain a large number of pigments with fluorescence properties that spectrally are only 10-40 nm different from each other and that occur at very close proximity in the cells. As a consequence, conventional confocal fluorescence microscopy is unable to resolve the different components. However, resolution of the components within the cell is very important as this informs us regarding the position of these fluorescent pigments inside the cells; and knowing the position of these components in turn informs us regarding where corresponding protein complexes are located in the cell. Your hyperspectral confocal fluorescence microscope and its software is unique in that the instrument is able to discern these components that have very similar fluorescence emission characteristics and are located in similar parts of the cell. Based on the data collected on your microscope, we have been able to directly monitor the position and quantity of spectrally overlapping chromophores in cells in vivo. Moreover, we have been able to verify the obtained results and assignments by using mutants that lack specific chromophores. The observed heterogeneity in the content of different parts of thylakoid membranes in the cell was novel, and would not have been detected if not for your hyperspectral imager.

Your hyperspectral fluorescence imager is truly unique and represents a breakthrough in what can be gleaned from fluorescence microscopy. From my own experience I know that commercial microscopes do not even come close in terms of their resolution and performance. You have an outstanding machine that can provide very detailed insights in small, living cells, without artifacts. This is something the field has long been waiting for!

With best regards,

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APPENDIX ITEM B LETTER OF SUPPORT/TESTIMONIAL

Maria Cristina Ubach, Ph.D, Monsanto Company

MONSANTO imagine

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Letter of Support for Sandia National Laboratories' Hyperspectral Confocal Fluorescence Microscope System

In May 2006, Monsanto Company established a cooperative research and development agreement (CRADA) with Sandia National Laboratories to bring in-house advanced analytical technologies for Agricultural applications. The hyperspectral confocal fluorescence microscope was selected as one of the most impactful new technologies that Sandia has developed recently for immediate application to plant cell research. The ability to measure the entire emission spectrum for each tri-dimensional voxel, coupled with high spatial resolution, high sensitivity, and confocality, makes this new confocal microscope a powerful tool for the study of biological systems and quantitation of biological processes. This product enabled us to visualize cellular structures and components not detected by commercially available confocal microscopes, and it enabled the development of new approaches and protocols impossible to carry out successfully due to technical limitations of traditional confocal microscopes. I foresee this product to be the first of a long series of hyperspectral imaging systems setting the standard for the world of fluorescence-based research.

Maria Cristina Ubach, Ph.D.
Plant Biotechnology and Plant Cell Biology
Monsanto Associate Fellow

APPENDIX ITEM C PATENTS

Michael R. Keenan and Paul G. Kotula, "Apparatus and System for Multivariate Spectral Analysis," US Patent 6,584,413 issued June 24, 2003.

US006584413B1

(12) **United States Patent**
Keenan et al.

(10) **Patent No.: US 6,584,413 B1**
(45) **Date of Patent: Jun. 24, 2003**

(54) **APPARATUS AND SYSTEM FOR MULTIVARIATE SPECTRAL ANALYSIS**

(75) Inventors: **Michael R. Keenan**, Albuquerque, NM (US); **Paul G. Kotula**, Albuquerque, NM (US)

(73) Assignee: **Sandia Corporation**, Albuquerque, NM (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 225 days.

(21) Appl. No.: **09/872,740**

(22) Filed: **Jun. 1, 2001**

(51) **Int. Cl.**[7] **G06F 19/00**

(52) **U.S. Cl.** **702/28**; 702/194; 702/196

(58) **Field of Search** 702/27, 28, 189, 702/190, 194, 196, 197; 250/306–317, 336.1–395; 356/300–334

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5,379,352	A	1/1995	Sirat et al.	382/41
5,583,951	A	12/1996	Sirat et al.	382/232
5,596,195	A	1/1997	Obori et al.	250/310
5,610,836	A	3/1997	Alsmeyer et al.	702/27
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Co–pending US patent application claims "*Method of Multivariate Spectral Analysis*", M. R. Keenan, et al, commonly assigned to Sandia Corporation, Albuquerque, New Mexico, Docket No. SD–6728.

B. Cross, "*Scanning X–Ray Fluorescence Microscopy and Principal Component Analysis*", Proc. 50th Annual Meeting of the Electron Microscopy Society of America Held jointly with the 27th Annual Meeting of the Microbeam Analysis Society and the 19th Annual Meeting of the Microscopical Society of Canada/Societe de microscopie du Canada(1992) pp. 1752–1753.

D. M. Hawkins and D. J. Olive, "*Improved feasible solution algorithms for high breakdown estimation*", Elsevier Computational Statistics & Data Analysis 30 (1999) pp. 1–11.

P. G. Kotula and M. R. Keenan, "*Automated unbiased information extraction of STEM–EDS spectrum images,*" Paper presented at 2nd Conf. Int. Union Microbeam Analysis societies, Kailua–Kona, Hawaii, Jul. 9–13, 2000 pp. 147–148.

P. G. Kotula and M. R. Keenan, "*Information Extraction: Statistical Analysis to get the most from Spectrum Images*" Microsc. Microanal. 6 (Suppl 2: Proceedings), Aug. 2000, pp. 1052–1053.

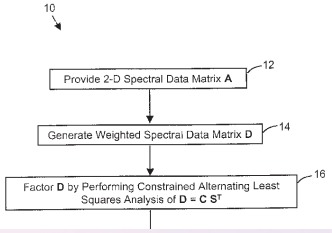
(List continued on next page.)

Primary Examiner—Craig Hallacher
Assistant Examiner—Blaise Mouttet
(74) *Attorney, Agent, or Firm*—Robert D. Watson

(57) **ABSTRACT**

An apparatus and system for determining the properties of a sample from measured spectral data collected from the sample by performing a method of multivariate spectral analysis. The method can include: generating a two-dimensional matrix A containing measured spectral data; providing a weighted spectral data matrix D by performing a weighting operation on matrix A; factoring D into the product of two matrices, C and S^T, by performing a constrained alternating least-squares analysis of $D=CS^T$, where C is a concentration intensity matrix and S is a spectral shapes matrix; unweighting C and S by applying the inverse of the weighting used previously; and determining the properties of the sample by inspecting C and S. This method can be used by a spectrum analyzer to process X-ray spectral data generated by a spectral analysis system that can include a Scanning Electron Microscope (SEM) with an Energy Dispersive Detector and Pulse Height Analyzer.

21 Claims, 21 Drawing Sheets



APPENDIX ITEM C PATENTS

Michael R. Keenan and Paul G. Kotula, "Method of Multivariate Spectral Analysis," US Patent 6,675,106 issued January 6, 2004.

US006675106B1

(12) **United States Patent**
Keenan et al.

(10) **Patent No.: US 6,675,106 B1**
(45) **Date of Patent: *Jan. 6, 2004**

(54) **METHOD OF MULTIVARIATE SPECTRAL ANALYSIS**

(75) Inventors: **Michael R. Keenan**, Albuquerque, NM (US); **Paul G. Kotula**, Albuquerque, NM (US)

(73) Assignee: **Sandia Corporation**, Albuquerque, NM (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 227 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/873,078**

(22) Filed: **Jun. 1, 2001**

(51) **Int. Cl.**[7] **G06F 19/00**; G06F 17/16

(52) **U.S. Cl.** **702/28**; 702/194; 702/196

(58) **Field of Search** 702/27, 28, 189, 702/194, 196, 197; G06F 19/00, 17/16

(56) **References Cited**

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5,583,951	A	12/1996	Sirat et al.	382/232
5,596,195	A	1/1997	Obori et al.	250/310
5,610,836	A	3/1997	Alsmeyer et al.	364/498
5,701,074	A	* 12/1997	Zhu	324/307
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Co–pending US patent application claims "*Apparatus and System for Multivariate Spectral Analysis*", M. R. Keenan, et al, commonly assigned to Sandia Corporation, Albuquerque, New Mexico.

B. Cross, "*Scanning X–Ray Fluorescence Microscopy and Principal Component Analysis*", Proc. 50th Annual Meeting of the Electron Microscopy Society of American Held jointly with the 27th Annual Meeting of the Microbeam Analysis Society and the 19th Annual Meeting of the Microscopical Society of Canada/Societe de microscopie du Canada(1992) pp. 1752–1753.

D. M. Hawkins and D. J. Olive, "*Improved feasible solution algorithms for high breakdown estimation*", Elsevier Computational Statistics & Data Analysis 30 (1999) pp. 1–11.

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P. G. Kotula and M. R. Keenan, "*Information Extraction: Statistical Analysis to get the most from Spectrum Images*" Microsc. Microanal. 6 (Suppl 2: Proceedings). Aug. 2000, pp. 1052–1053.

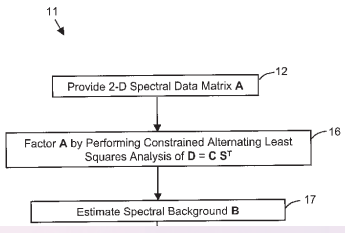
(List continued on next page.)

Primary Examiner—Stephen D. Meier
Assistant Examiner—Blaise Mouttet
(74) *Attorney, Agent, or Firm*—Robert D. Watson

(57) **ABSTRACT**

A method of determining the properties of a sample from measured spectral data collected from the sample by performing a multivariate spectral analysis. The method can include: generating a two-dimensional matrix A containing measured spectral data; providing a weighted spectral data matrix D by performing a weighting operation on matrix A; factoring D into the product of two matrices, C and S^T, by performing a constrained alternating least-squares analysis of $D=CS^T$, where C is a concentration intensity matrix and S is a spectral shapes matrix; unweighting C and S by applying the inverse of the weighting used previously; and determining the properties of the sample by inspecting C and S. This method can be used to analyze X-ray spectral data generated by operating a Scanning Electron Microscope (SEM) with an attached Energy Dispersive Spectrometer (EDS).

106 Claims, 21 Drawing Sheets



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David M. Haaland and David K. Melgaard, "Generalized Augmented Classical Least Squares Methods," US Patents 6,687,620, 6,842,702, and 6,922,645 issued February 3, 2004, January 11, 2005, and July 26, 2005, respectively.

US006687620B1

(12) **United States Patent**
Haaland et al.

(10) **Patent No.: US 6,687,620 B1**
(45) **Date of Patent: Feb. 3, 2004**

(54) **AUGMENTED CLASSICAL LEAST SQUARES MULTIVARIATE SPECTRAL ANALYSIS**

(75) Inventors: **David M. Haaland**, Albuquerque, NM (US); **David K. Melgaard**, Albuquerque, NM (US)

(73) Assignee: **Sandia Corporation**, Albuquerque, NM (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **10/209,841**

(22) Filed: **Jul. 31, 2002**

Related U.S. Application Data

(60) Provisional application No. 60/309,619, filed on Aug. 1, 2001, and provisional application No. 60/311,755, filed on Aug. 9, 2001.

(51) **Int. Cl.**[7] **G01N 33/48**; G06F 19/00

(52) **U.S. Cl.** **702/22**; 702/27; 702/30; 702/32

(58) **Field of Search** 702/22–26, 30, 702/181, 189–190, 196–199; 700/266–269; 73/23.36, 23.37, 23.41, 24.02; 250/339.08, 339.09, 338.5, 338.1, 339.07, 341.1, 339.11, 339.12, 339.13, 339.14

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(List continued on next page.)

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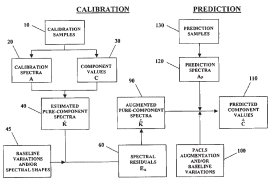
Assistant Examiner—John Le
(74) *Attorney, Agent, or Firm*—Kevin W. Bieg

(57) **ABSTRACT**

A method of multivariate spectral analysis, termed augmented classical least squares (ACLS), provides an improved CLS calibration model when unmodeled sources of spectral variation are contained in a calibration sample set. The ACLS methods use information derived from component or spectral residuals during the CLS calibration to provide an improved calibration-augmented CLS model. The ACLS methods are based on CLS so that they retain the qualitative benefits of CLS, yet they have the flexibility of PLS and other hybrid techniques in that they can define a prediction model even with unmodeled sources of spectral variation that are not explicitly included in the calibration model. The unmodeled sources of spectral variation may be unknown constituents, constituents with unknown concentrations, nonlinear responses, non-uniform and correlated errors, or other sources of spectral variation that are present in the calibration sample spectra. Also, since the various ACLS methods are based on CLS, they can incorporate the new prediction-augmented CLS (PACLS) method of updating the prediction model for new sources of spectral variation contained in the prediction sample set without having to return to the calibration process. The ACLS methods can also be applied to alternating least squares models. The ACLS methods can be applied to all types of multivariate data.

24 Claims, 25 Drawing Sheets

Spectral-Residual Augmented CLS



US006842702B2

(12) **United States Patent**
Haaland et al.

(10) **Patent No.: US 6,842,702 B2**
(45) **Date of Patent: Jan. 11, 2005**

(54) **AUGMENTED CLASSICAL LEAST SQUARES MULTIVARIATE SPECTRAL ANALYSIS**

(75) Inventors: **David M. Haaland**, Albuquerque, NM (US); **David K. Melgaard**, Albuquerque, NM (US)

(73) Assignee: **Sandia Corporation**, Albuquerque, NM (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **10/661,968**

(22) Filed: **Sep. 11, 2003**

(65) **Prior Publication Data**

US 2004/0064259 A1 Apr. 1, 2004

Related U.S. Application Data

(62) Division of application No. 10/209,841, filed on Jul. 31, 2002, now Pat. No. 6,687,620.

(60) Provisional application No. 60/311,755, filed on Aug. 9, 2001, and provisional application No. 60/309,619, filed on Aug. 1, 2001.

(51) **Int. Cl.**[7] **G06F 19/00**; G01N 33/48

(52) **U.S. Cl.** **702/18**; 250/573; 702/22; 702/27; 702/28

(58) **Field of Search** 702/22–26, 27, 702/28, 30, 181, 189–190, 196–199; 250/339.08, 339.09, 339.12, 559.1, 573

(56) **References Cited**

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* cited by examiner

Primary Examiner—John Barlow
Assistant Examiner—John Le
(74) *Attorney, Agent, or Firm*—Kevin W. Bieg

(57) **ABSTRACT**

A method of multivariate spectral analysis, termed augmented classical least squares (ACLS), provides an improved CLS calibration model when unmodeled sources of spectral variation are contained in a calibration sample set. The ACLS methods use information derived from component or spectral residuals during the CLS calibration to provide an improved calibration-augmented CLS model. The ACLS methods are based on CLS so that they retain the qualitative benefits of CLS, yet they have the flexibility of PLS and other hybrid techniques in that they can define a prediction model even with unmodeled sources of spectral variation that are not explicitly included in the calibration model. The unmodeled sources of spectral variation may be unknown constituents, constituents with unknown concentrations, nonlinear responses, non-uniform and correlated errors, or other sources of spectral variation that are present in the calibration sample spectra. Also, since the various ACLS methods are based on CLS, they can incorporate the new prediction-augmented CLS (PACLS) method of updating the prediction model for new sources of spectral variation contained in the prediction sample set without having to return to the calibration process. The ACLS methods can also be applied to alternating least squares models. The ACLS methods can be applied to all types of multivariate data.

48 Claims, 25 Drawing Sheets

$$\hat{K} = (\tilde{C}^T \tilde{C})^{-1} \tilde{C}^T$$

$$\begin{bmatrix} \\ \\ \\ \end{bmatrix} = \left\{ \begin{bmatrix} c_1\ c_1\ \ldots c \\ 1\ 2\ \ldots in \\ c_2\ c_2\ \ldots c \end{bmatrix} \begin{bmatrix} c_1\ c_2\ e \\ c_1\ c_2\ e \\ \vdots \\ c\ c\ e \end{bmatrix} \right\}^{-} \begin{bmatrix} c_1\ c_1\ \ldots C \\ 1\ 2\ \ldots in \\ c_2\ c_2\ \ldots c \end{bmatrix} \begin{bmatrix} \\ \\ \\ \end{bmatrix}$$

$$e = \hat{c} - c$$

US006922645B2

(12) **United States Patent**
Haaland et al.

(10) **Patent No.: US 6,922,645 B2**
(45) **Date of Patent: Jul. 26, 2005**

(54) **AUGMENTED CLASSICAL LEAST SQUARES MULTIVARIATE SPECTRAL ANALYSIS**

(75) Inventors: **David M. Haaland**, Albuquerque, NM (US); **David K. Melgaard**, Albuquerque, NM (US)

(73) Assignee: **Sandia Corporation**, Albuquerque, NM (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 25 days.

(21) Appl. No.: **10/963,195**

(22) Filed: **Oct. 12, 2004**

(65) **Prior Publication Data**

US 2005/0043902 A1 Feb. 24, 2005

Related U.S. Application Data

(62) Division of application No. 10/661,968, filed on Sep. 11, 2003, now Pat. No. 6,842,702, which is a division of application No. 10/209,841, filed on Jul. 31, 2002, now Pat. No. 6,687,620.

(60) Provisional application No. 60/309,619, filed on Aug. 1, 2001, and provisional application No. 60/311,755, filed on Aug. 9, 2001.

(51) **Int. Cl.**[7] **G01R 23/16**; G01N 23/48

(52) **U.S. Cl.** **702/76**; 702/18; 702/22; 702/27; 702/28

(58) **Field of Search** 702/18, 22–28, 702/30, 76, 85, 181, 189–190, 196–199; 250/339.09, 339.08, 339.12, 559.1, 573

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,341,257 B1 * 1/2002 Haaland 702/27
6,415,233 B1 * 7/2002 Haaland 702/22

* cited by examiner

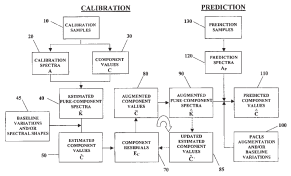
Primary Examiner—John Barlow
Assistant Examiner—John Le
(74) *Attorney, Agent, or Firm*—Kevin W. Bieg

(57) **ABSTRACT**

A method of multivariate spectral analysis, termed augmented classical least squares (ACLS), provides an improved CLS calibration model when unmodeled sources of spectral variation are contained in a calibration sample set. The ACLS methods use information derived from component or spectral residuals during the CLS calibration to provide an improved calibration-augmented CLS model. The ACLS methods are based on CLS so that they retain the qualitative benefits of CLS, yet they have the flexibility of PLS and other hybrid techniques in that they can define a prediction model even with unmodeled sources of spectral variation that are not explicitly included in the calibration model. The unmodeled sources of spectral variation may be unknown constituents, constituents with unknown concentrations, nonlinear responses, non-uniform and correlated errors, or other sources of spectral variation that are present in the calibration sample spectra. Also, since the various ACLS methods are based on CLS, they can incorporate the new prediction-augmented CLS (PACLS) method of updating the prediction model for new sources of spectral variation contained in the prediction sample set without having to return to the calibration process. The ACLS methods can also be applied to alternating least squares models. The ACLS methods can be applied to all types of multivariate data.

10 Claims, 25 Drawing Sheets

Concentration-Residual Augmented CLS



APPENDIX ITEM C PATENTS

David M. Haaland and David K. Melgaard, "Generalized Augmented Classical Least Squares Methods," US Patents 6,687,620, 6,842,702, and 6,922,645 issued February 3, 2004, January 11, 2005, and July 26, 2005, respectively.

US006922645B2

(12) **United States Patent**
Haaland et al.

(10) **Patent No.: US 6,922,645 B2**
(45) **Date of Patent: Jul. 26, 2005**

(54) **AUGMENTED CLASSICAL LEAST SQUARES MULTIVARIATE SPECTRAL ANALYSIS**

(75) Inventors: **David M. Haaland**, Albuquerque, NM (US); **David K. Melgaard**, Albuquerque, NM (US)

(73) Assignee: **Sandia Corporation**, Albuquerque, NM (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 25 days.

(21) Appl. No.: **10/963,195**

(22) Filed: **Oct. 12, 2004**

(65) **Prior Publication Data**

US 2005/0043902 A1 Feb. 24, 2005

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(52) **U.S. Cl.** **702/76**; 702/18; 702/22; 702/27; 702/28

(58) **Field of Search** 702/18, 22–28, 702/30, 76, 85, 181, 189–190, 196–199; 250/339.09, 339.08, 339.12, 559.1, 573

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,341,257 B1 * 1/2002 Haaland 702/27

6,415,233 B1 * 7/2002 Haaland 702/22

* cited by examiner

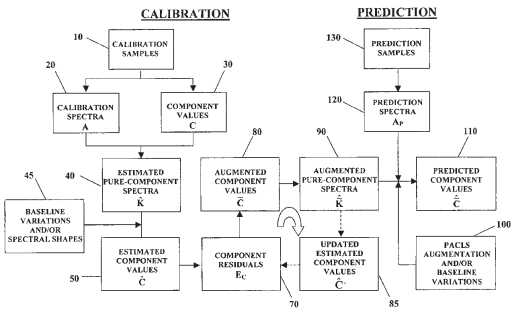
Primary Examiner—John Barlow
Assistant Examiner—John Le
(74) *Attorney, Agent, or Firm*—Kevin W. Bieg

(57) **ABSTRACT**

A method of multivariate spectral analysis, termed augmented classical least squares (ACLS), provides an improved CLS calibration model when unmodeled sources of spectral variation are contained in a calibration sample set. The ACLS methods use information derived from component or spectral residuals during the CLS calibration to provide an improved calibration-augmented CLS model. The ACLS methods are based on CLS so that they retain the qualitative benefits of CLS, yet they have the flexibility of PLS and other hybrid techniques in that they can define a prediction model even with unmodeled sources of spectral variation that are not explicitly included in the calibration model. The unmodeled sources of spectral variation may be unknown constituents, constituents with unknown concentrations, nonlinear responses, non-uniform and correlated errors, or other sources of spectral variation that are present in the calibration sample spectra. Also, since the various ACLS methods are based on CLS, they can incorporate the new prediction-augmented CLS (PACLS) method of updating the prediction model for new sources of spectral variation contained in the prediction sample set without having to return to the calibration process. The ACLS methods can also be applied to alternating least squares models. The ACLS methods can be applied to all types of multivariate data.

10 Claims, 25 Drawing Sheets

Concentration-Residual Augmented CLS



APPENDIX ITEM C PATENTS

Michael R. Keenan, "Efficient Out-of-Core Algorithm for Analysis of Very Large Multivariate," US Patent 7,283,684 issued October 16, 2007.

(12) **United States Patent**
Keenan

(10) **Patent No.: US 7,283,684 B1**
(45) **Date of Patent: Oct. 16, 2007**

(54) **SPECTRAL COMPRESSION ALGORITHMS FOR THE ANALYSIS OF VERY LARGE MULTIVARIATE IMAGES**

(75) Inventor: **Michael R. Keenan**, Albuquerque, NM (US)

(73) Assignee: **Sandia Corporation**, Albuquerque, NM (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 830 days.

(21) Appl. No.: **10/772,548**

(22) Filed: **Feb. 4, 2004**

Related U.S. Application Data

(60) Provisional application No. 60/472,447, filed on May 20, 2003.

(51) **Int. Cl.**
G06K 9/36 (2006.01)

(52) **U.S. Cl.** **382/276**; 382/235; 382/243; 382/277; 345/644

(58) **Field of Classification Search** 382/205, 382/235, 243, 277, 281; 345/644, 645
See application file for complete search history.

(56) **References Cited**

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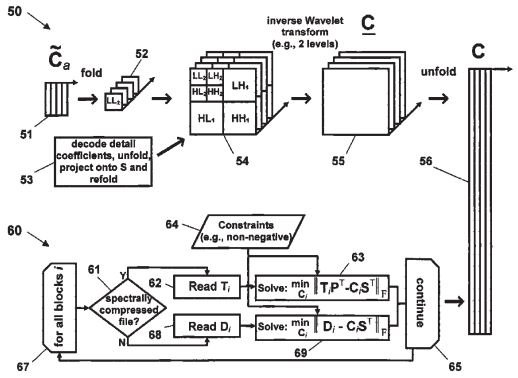
Primary Examiner—Yosef Kassa
(74) *Attorney, Agent, or Firm*—Kevin W. Bieg

(57) **ABSTRACT**

A method for spectrally compressing data sets enables the efficient analysis of very large multivariate images. The spectral compression algorithm uses a factored representation of the data that can be obtained from Principal Components Analysis or other factorization technique. Furthermore, a block algorithm can be used for performing common operations more efficiently. An image analysis can be performed on the factored representation of the data, using only the most significant factors. The spectral compression algorithm can be combined with a spatial compression algorithm to provide further computational efficiencies.

44 Claims, 16 Drawing Sheets



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Michael R. Keenan, "Improved Algorithm for Analysis of High Dimension Spectral Images," US Patent 7,400,772 issued July 15, 2008.

US007400772B1

(12) **United States Patent**
Keenan

(10) **Patent No.: US 7,400,772 B1**
(45) **Date of Patent: Jul. 15, 2008**

(54) **SPATIAL COMPRESSION ALGORITHM FOR THE ANALYSIS OF VERY LARGE MULTIVARIATE IMAGES**

(75) Inventor: **Michael R. Keenan**, Albuquerque, NM (US)

(73) Assignee: **Sandia Corporation**, Albuquerque, NM (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 833 days.

(21) Appl. No.: **10/772,805**

(22) Filed: **Feb. 4, 2004**

Related U.S. Application Data

(60) Provisional application No. 60/472,447, filed on May 20, 2003.

(51) **Int. Cl.**
G06K 9/36 (2006.01)
G06K 9/46 (2006.01)
H04B 1/66 (2006.01)

(52) **U.S. Cl.** **382/232**; 375/240

(58) **Field of Classification Search** 382/232–253; 356/300–305; 375/240, 240.01–240.24
See application file for complete search history.

(56) **References Cited**

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"Algorithms for constrained linear unmixing with application to the hyperspectral analysis of fluorophore mixtures," M.R. Keenan, J.A. Timlin, M.H. Van Benthem, and D.M. Haaland , Proc. SPIE 4816, 193-202 (2002).*

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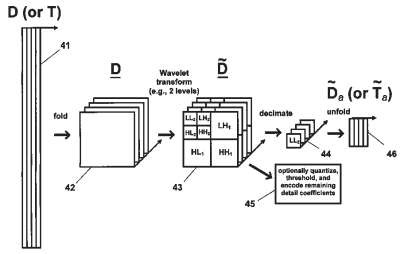
Primary Examiner—Bhavesh Mehta
Assistant Examiner—Manav Seth
(74) *Attorney, Agent, or Firm*—Kevin W. Bieg

(57) **ABSTRACT**

A method for spatially compressing data sets enables the efficient analysis of very large multivariate images. The spatial compression algorithms use a wavelet transformation to map an image into a compressed image containing a smaller number of pixels that retain the original image's information content. Image analysis can then be performed on a compressed data matrix consisting of a reduced number of significant wavelet coefficients. Furthermore, a block algorithm can be used for performing common operations more efficiently. The spatial compression algorithms can be combined with spectral compression algorithms to provide further computational efficiencies.

26 Claims, 16 Drawing Sheets



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