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Validation of Excitation-Scan Hyperspectral Multi-faceted Mirror Array Prototype System Advancements to Hyperspectral Imaging Applications

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ABSTRACT

Hyperspectral imaging technologies (HSI) have undergone rapid development since their beginning stages. While original applications were in remote sensing, other uses include agriculture, food safety and medicine. HSI has shown great utility in fluorescence microscopy for detecting signatures from many fluorescent molecules; however, acquisitions speeds have been slow due to light losses associated with spectral filtering. Therefore, we designed a novel light emitting diode (LED)-based rapid excitation scanning hyperspectral imaging platform allowing users to obtain simultaneous measurements of fluorescent labels without compromising acquisition speeds. Previously, we reported our results of the optical ray trace simulations and the geometrical capability of designing a multifaceted mirror imaging system as an initial approach to combine light at many wavelengths. The design utilized LEDs and a multifaceted mirror array to combine light sources into a liquid light guide. The computational model was constructed using Monte Carlo optical ray software (TracePro, Lambda Research Corp.). Recent prototype validation results show that when compared to a commercial emission scanning spectral confocal microscope (Zeiss-LSM-980), the novel LED-based excitation scanning HSI prototype successfully detected and separated six fluorescent labels from a custom 6-label African green monkey kidney epithelial cells. We report on the prototype's ability to overcome limitations of acquisition speeds, sensitivity, and specificity present in conventional systems. Future work will evaluate prototype's light losses to determine latent design modifications needed to demonstrate the system's feasibility as a promising solution for overcoming HSI acquisition speeds. This work was supported by NSF award MRI1725937.

Keywords: Systems Design, LED, Tissue imaging, HSI, Spectroscopy, Microscopy, Transmission, Spectral, Fluorescence, Molecules, Cells

1. INTRODUCTION

Since its original development by NASA for remote sensing1, HSI technologies have made an impact as an effective tool in a wide range of fields[2,3,4] for detecting certain targets and materials[3,5]. Specifically, in the medical field, HSI has shown great promise in tissue discrimination due to its ability to separate signatures from various fluorescent molecules[6–10]. A key aim in HSI medical applications is detection of cancerous cells and effectively identifying characteristic components of cells and tissues[11,12]. The standard approach is to utilize fluorescence emission-scanning HSI, where the fluorescence emission is filtered over a broad wavelength range while exciting at a single band[9]. However, emission scanning approaches present limitations such as reduced sensitivity due to light attenuation from spectral filtering thus, result in increased acquisition times and/or limited signal-to-noise ratios. Increased acquisition times limit the ability to resolve features of temporally-changing/dynamic cell signaling events. We have previously shown that excitation scanning HSI offers promising solution for overcoming limitations presented by emission scanning due to its ability to filter the fluoresce excitation instead of the fluorescence emission and thus providing 30-100X increased sensitivity when compared to current emission scanning systems[12,14,15]. The goal of this work is to validate a novel LED-based excitation scanning HSI prototype which we have previously reported on, using computational modeling[15,16]. Here, we report validation results of the excitation scanning HSI prototype when implemented so as to detect the entire fluorescence emission in a single channel, while simultaneously providing spectral discrimination by quickly scanning through a range of excitation wavelength bands. The prototype utilizes LEDs and a multifaceted mirror array to combine single-band light sources into a liquid light guide (LLG). We present results of comparing the LED-based excitation

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scanning HSI platform to a commercial emission scanning spectral confocal microscope (Zeiss-LSM-980). Hyperspectral imaging technologies (HSI) have undergone rapid development since their beginning stages. While original applications were in remote sensing, other uses include agriculture, food safety and medicine. HSI has shown great utility in fluorescence microscopy for detecting signatures from many fluorescent molecules; however, acquisitions speeds have been slow due to light losses associated with spectral filtering. Therefore, we designed a novel light emitting diode (LED)-based rapid excitation scanning hyperspectral imaging platform allowing users to obtain simultaneous measurements of fluorescent labels without compromising acquisition speeds. Previously, we reported our results of the optical ray trace simulations and the geometrical capability of designing a multifaceted mirror imaging system as an initial approach to combine light at many wavelengths. The design utilized LEDs and a multifaceted mirror array to combine light sources into a liquid light guide. The computational model was constructed using Monte Carlo optical ray software (TracePro, Lambda Research Corp.). Recent prototype validation results show that when compared to a commercial emission scanning spectral confocal microscope (Zeiss-LSM-980), the novel LED-based excitation scanning HSI prototype successfully detected and separated six fluorescent labels from a custom 6-label African green monkey kidney epithelial cells. We report on the prototype's ability to overcome limitations of acquisition speeds, sensitivity, and specificity present in conventional systems. Future work will evaluate prototype's light losses to determine latent design modifications needed to demonstrate the system's feasibility as a promising solution for overcoming HSI acquisition speeds.

2. MATERIALS AND METHODS

2.1 Cell preperation

African green monkey kidney epithelial cells were utilized to prepare custom slides with 6 fluorescent labels by Abberior GmbH. To evaluate the performance of the LED-based excitation-scanning HSI system for spectral separation capabilities, labels were selected to cover a wide spectral range (Table.1). An additional unlabeled slide was prepared to measure cellular autofluorescence. All cells were fixed and stained by Abberior in accordance with Wurm et al.17 and cell specimens were embedded in Abberior Mount Solid Antifade (item number: MM-2013-2X15ML)

Table.1 *Slide number and staining conditions*

Slide #	Stain
1	Fixed and unstained cell slide
2	F-actin – Abberior STAR GREEN phalloidin
3	Mitochondria – Abberior STAR520SXP goat anti-rabbit IgG
4	Double stranded DNA – Abberior LIVE 560 DNA
5	Vimentin – Abberior STAR ORANGE goat anti-chicken IgY
6	*Golgi* apparatus – Abberior STAR RED goat anti-guinea pig IgG
7&8	Multi-labeled slides with labels of slides 1 – 6

2.2 Hyperspectral imaging excitation scanning microscope set-up

Excitation light from the custom spectral source was coupled into an inverted epifluorescence microscope (TE2000-U, Nikon Instruments, Melville, NY) using a 3 mm LLG. Images were acquired using a 60X water immersion objective (Plan Apo VC 60x/1.2 WI, Nikon Instruments, Melville, NY), and a high sensitivity sCMOS camera (Prime 95B, Teledyne Photometrics, Tucson, AZ).

2.3 Image acquisition

Images were acquired for each sample (6-label slide, single-label control slides, and unlabeled autofluorescence control slide) on each spectral microscope systems (LED-based system, & Zeiss LSM 980). Imaging parameters were set accordingly based on the system used (Table.2)[18].

Table.2 *Image acquisition parameters and microscope configuration*

Microscope	Light Source	Objective Lens Parameter(s)	Detector
Inverted epifluorescence widefield microscope (TE2000-U, Nikon Instruments, Melville, NY)	LED-based Platform LED wavelengths: 365, 375, 385, 405, 415, 420, 430, 450, 470, 490, 515, 520 Power: 100%	Plan Apo VC 60x/1.2 WI (Nikon Instruments)	Prime 95B sCMOS camera (Teledyne Photometrics, Tucson, AZ) 4 averaging 1*s* per wavelength-band integration time 2x2 binning no gain or offset 40 MHz readout speed
Zeiss LSM 980 commercial emission-scanning spectral confocal microscope system (Carl Zeiss Microscopy, LLC, White Plains, NY)	Laser 405 nm at 10% power 488 nm at 2% power 561 nm at 0.2% power 639 nm at 2% power	C-Apochromat 40x/1.2W Autocorrect UV VIS IR (Zeiss Microscopy)	32 channel GaAsP-PMT 4 averaging 2 *min* 22 *sec* exposure/dwell time 0 offset, 0 binning Digital gain 1 Gain 650 V

2.4 Image analysis

To account for wavelength-dependent attenuation, excitation intensity was corrected to a flat spectral response as described in *Fink et. al.*[5]. In summary, a spectral correction coefficient was calculated for each wavelength band to account for wavelength dependent illumination intensity. Spectral correction was performed by sequentially illuminating each LED (at maximum power output) and measuring the optical power output available at the microscope stage. Spectral image stacks were then corrected by first identifying a blank (background) region in each image and extracting the mean spectrum from that region. The mean background spectrum was then subtracted from each spectral image stack and the stack was subsequently multiplied by the spectral correction factor to correct the image to a flat spectral response (Eq. (1)).

$$I_{corrected} = \left(I_{raw} - I_{dark} \cdot CC\right), \qquad (1)$$

where I_{raw} is the original spectral image, I_{dark} is the background spectrum, and CC is the spectral correction coefficient. Spectral image data from the Zeiss LSM 980 was not corrected, as the spectral detector is factory calibrated to provide a flat spectral response. All corrections were performed using a custom MATLAB script (MathWorks, Inc., Natick, MA.).

Corrected spectral images were then processed to build a spectral library (Figure 1.) from single-label control samples, as described previously[1,5,6]. In brief, regions of high signal strength were selected within spectral images from each single-label control sample, as well as the unlabeled autofluorescence control. The mean spectrum from each region was extracted and saved within a library for spectral unmixing. Due to high cellular autofluorescence, for a subset of samples, a fraction of the autofluorescence spectrum was subtracted from the single-label spectrum before saving the single-label spectrum within the spectral library. The test image of the 6-label sample was then linearly unmixed using a non-negatively constrained linear unmixing algorithm. Unmixed images were linearly scaled, false colored, and overlayed for visualization.

Example spectral unmixing code is available at https://www.southalabama.edu/centers/bioimaging/resources.html

3. RESULTS AND DISCUSSION

3.1 Spectral library

Spectral images from each single label control sample were used to build a spectral library using the LED-based excitation scanning HSI system and corrected to a flat spectral response. Spectral images were also acquired from the Zeiss LSM 980 commercial emission-scanning spectral confocal microscope system that is factory calibrated to achieve a flat spectral response. Regions of interest (ROIs) were selected, and the mean spectrum of each ROI was measured. A separate spectral library was created for each spectral microscope system: LED-based excitation scanning platform, & Zeiss LSM 980 (Figure 1) and later imported into MATLAB for linear unmixing.

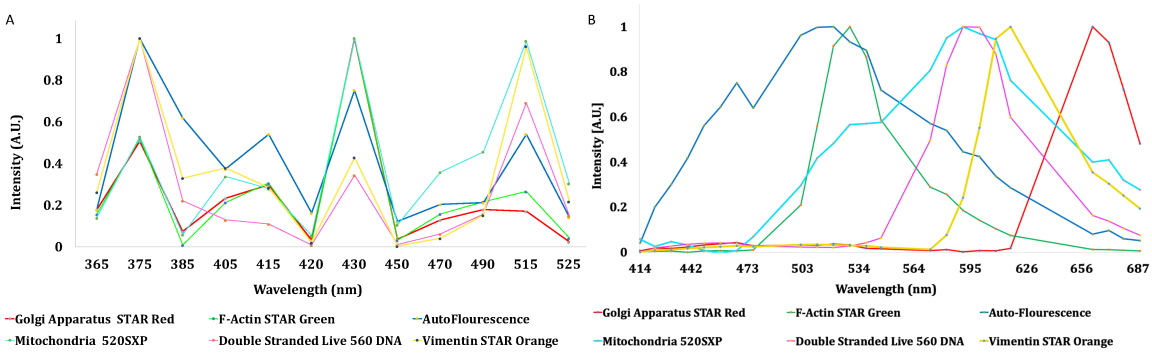

Figure 1. Spectral libraries were comprised for two spectral microscopy systems: A) excitation-scanning system using LEDs for spectral excitation and implemented on an inverted widefield microscope platform (novel LED-based excitation scanning HSI prototype), and B) commercial emission scanning spectral confocal microscope (Zeiss LSM 980).

3.2 Image analysis results

Spectral images of the mixed label sample that were acquired using each spectral microscope platform were linearly unmixed, false colored, and merged for visualization (Figure 2.). Most labels were easily identified on all three systems. When compared to the Zeiss LSM 980 (emission scanning), the LED-based excitation scanning HSI system better detected *golgi*, autofluorescence, mitochondria, double stranded DNA, and vimentin. However, f-actin was more clearly identified through the Zeiss LSM. The clear delineation of f-actin is primarily due to the optical sectioning capabilities provided by the Zeiss LSM 980, being a confocal microscope as opposed to the other system which is built on a widefield microscope platform. In future studies, it is possible that a higher labeling density of f-Actin may result in improved detection through the excitation-based scanning system.

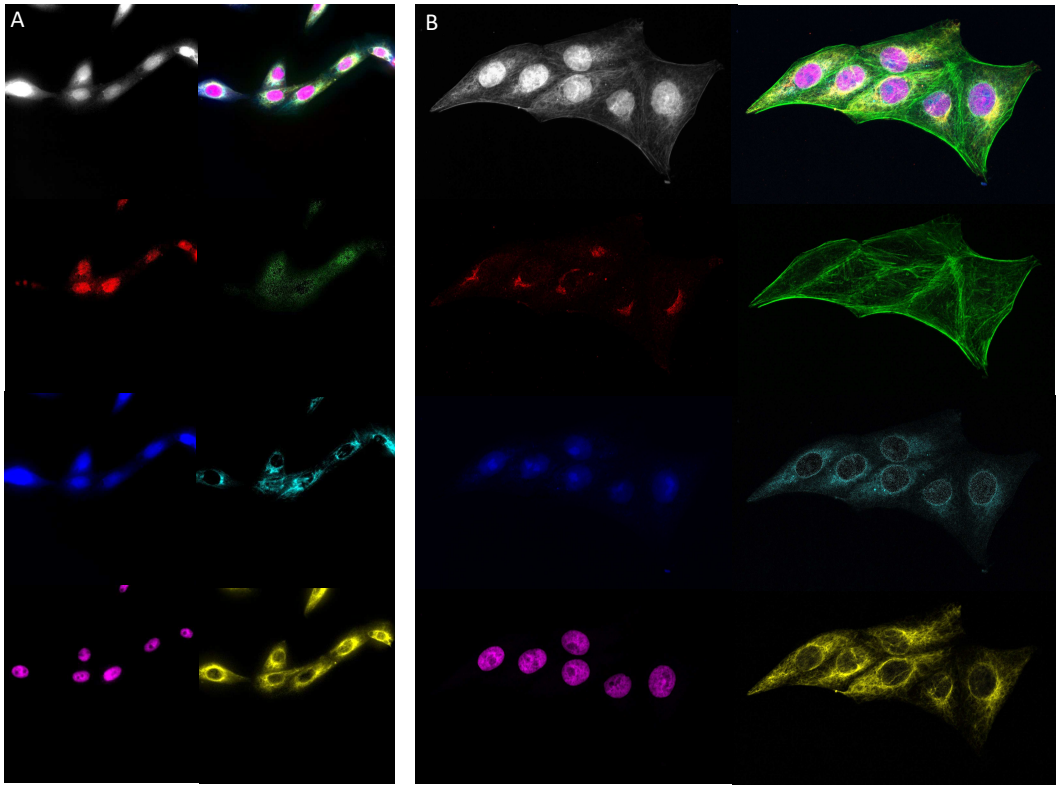

Figure 2. Comparison of image data acquired from *two* spectral imaging microscopy systems: A) *LED-based prototype – a* novel excitation-scanning spectral widefield system that uses an array of LEDs and multifurcated mirror for spectral illumination*, and* B) a commercial emission-scanning spectral confocal microscope (Zeiss LSM 980). For each image panel, the upper left image displays the sum of all wavelength bands for the spectral image stack while the upper right image displays the results of linear unmixing, as a merged and false-colored composite image. The individual components identified through linear unmixing are displayed in the bottom six images have been visualized with a color lookup table to match the colors shown in the false-colored and merged image, and are described from left-to-right and top-to-bottom: red – *golgi* apparatus labeled with STAR Red, green – f-Actin labeled with STAR Green, blue – cellular autofluorescence, cyan – mitochondria labeled with 520SXP, magenta – double-stranded DNA labeled with Live 560, and yellow – vimentin labeled with STAR Orange.

4. CONCLUSION AND FUTURE WORK

Hyperspectral imaging is a powerful tool for fluorescence microscopy applications for separating signatures from many fluorescent molecules. In this work, we have applied the first step in a prototype system validation for the high-speed excitation scanning system – an initial benchtop testing experiment to assess spectral discrimination capabilities using rapid hyperspectral imaging excitation scanning approach. The prototype consisting of LEDs, a multifaceted mirror array and a LLG showed promising results when compared to a commercial emission-scanning spectral confocal microscope (Zeiss LSM 980). By implementing LEDs, our system has decreased wavelength mechanical switching times from 250 milliseconds per wavelength to 10 microseconds. Future work will focus on improving imaging acquisition times as these were longer than desired for the LED-based excitation scanning HSI prototype due to optical transmission losses present in the prototype.

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