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Hyperspectral imaging fluorescence excitation scanning (HIFEX) microscopy for live cell imaging

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ABSTRACT

In the past two decades, spectral imaging technologies have expanded the capacity of fluorescence microscopy for accurate detection of multiple labels, separation of labels from cellular and tissue autofluorescence, and analysis of autofluorescence signatures. These technologies have been implemented using a range of optical techniques, such as tunable filters, diffraction gratings, prisms, interferometry, and custom Bayer filters. Each of these techniques has associated strengths and weaknesses with regard to spectral resolution, spatial resolution, temporal resolution, and signal-to-noise characteristics. We have previously shown that spectral scanning of the fluorescence excitation spectrum can provide greatly increased signal strength compared to traditional emission-scanning approaches. Here, we present results from utilizing a Hyperspectral Imaging Fluorescence Excitation Scanning (HIFEX) microscope system for live cell imaging. Live cell signaling studies were performed using HEK 293 and rat pulmonary microvascular endothelial cells (PMVECs), transfected with either a cAMP FRET reporter or a Ca^{2+} reporter. Cells were further labeled to visualize subcellular structures (nuclei, membrane, mitochondria, etc.). Spectral images were acquired using a custom inverted microscope (TE2000, Nikon Instruments) equipped with a 300W Xe arc lamp and tunable excitation filter (VF-5, Sutter Instrument Co., equipped with VersaChrome filters, Semrock), and run through MicroManager. Timelapse spectral images were acquired from 350-550 nm, in 5 nm increments. Spectral image data were linearly unmixed using custom MATLAB scripts. Results indicate that the HIFEX microscope system can acquire live cell image data at acquisition speeds of 8 ms/wavelength band with minimal photobleaching, sufficient for studying moderate speed cAMP and Ca^{2+} events.

Keywords: Spectral, Spectroscopy, Signature, Fingerprint, Optical, Biopsy, Classification, EEM

1. INTRODUCTION

Spectral imaging technologies were originally developed by NASA for use in remote sensing applications[1,2]. Over the past half-century, spectral imaging technologies have found many additional applications, ranging from agricultural inspection[3] to biomedical imaging[4–7]. In the field of fluorescence microscopy, spectral imaging and hyperspectral imaging (HSI) approaches have found great utility[8] for separation of fluorescence signals from background autofluorescence[9–11] and for enhancing the ability to perform multilabel imaging[12–14]. Supporting these applications, a range of spectral filtering and dispersion technologies have been developed, including traditional band-pass filtering, tunable filters, dispersive optics, interferometry, and spectral snapshot detectors. Each of these approaches presents certain instrumentation advantages as well as limitations. However, one common feature of each of these approaches is that they have all been implemented to allow spectral sampling of the fluorescence emission spectrum.

We have worked to develop an alternative spectral imaging modality (Figure 1) that allows scanning of the fluorescence excitation spectrum[15–17], called Hyperspectral Imaging Fluorescence Excitation Scanning (HIFEX). We have found that the HIFEX modality provides a similar ability to discriminate signals from multiple fluorescent labels as well as an

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ability to discriminate fluorescence labels from cellular and tissue autofluorescence. Importantly, for a subset of scenarios, the HIFEX imaging modality provided greatly enhanced signal-to-noise characteristics when compared to traditional fluorescence emission-scanning approaches. However, our initial implementations of the HIFEX technology required mechanical rotation of filters to switch wavelengths, and had relatively slow wavelength switching speed, precluding the ability to perform live-cell signaling studies.

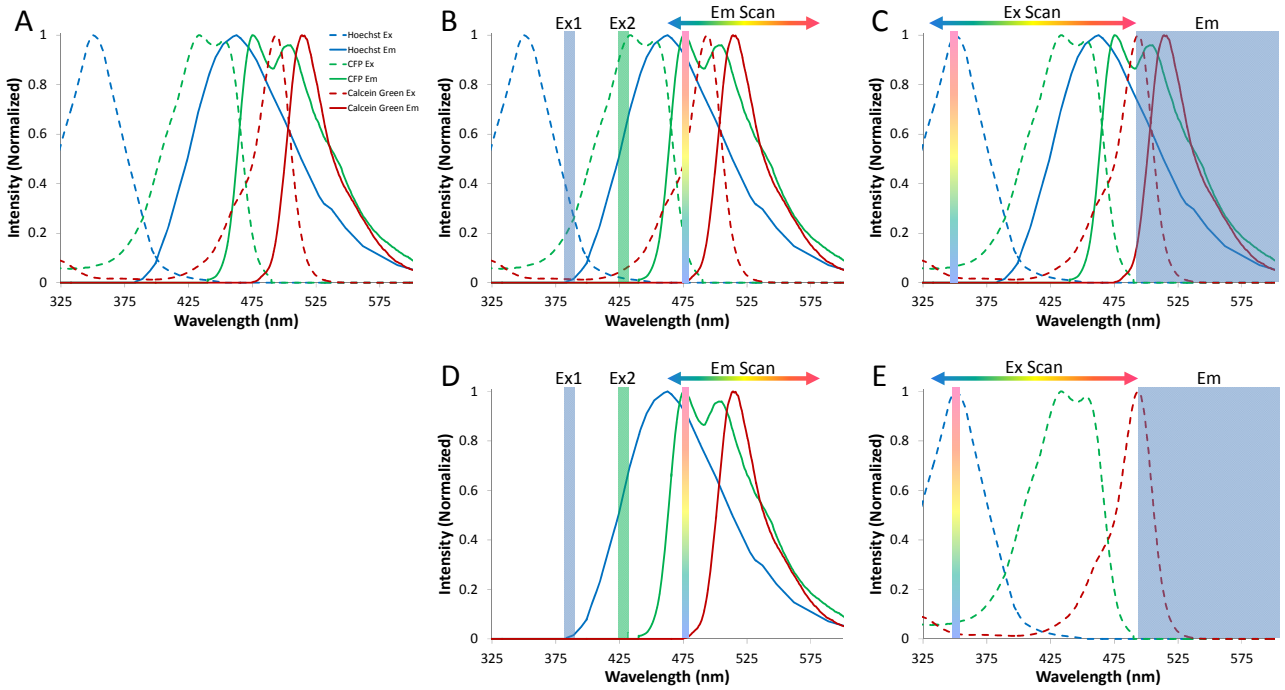

Figure 1: Schematic representation of traditional emission-scanning fluorescence hyperspectral imaging (HSI) approaches and the hyperspectral imaging fluorescence excitation scanning (HIFEX) approach. A) Excitation and emission spectra from 3 example fluorescent labels: Hoechst 33342 (blue lines), CFP (green), and Calcein Green (red). Dashed lines represent excitation spectra while solid lines represent emission spectra. B) Traditional emission-scanning HSI approaches operate by providing fluorescence excitation at one or several wavelengths and sampling the fluorescence emission across a range of wavelengths, whether through dispersion, tunable filter, snapshot, interferometry, or other method. C) The HIFEX approach operates by providing narrow-band excitation across a range of excitation wavelengths while detecting the fluorescence emission using a broad band-pass or long-pass filter. Excitation wavelengths are scanned sequentially. D) An equivalent representation of panel B, but where only the emission spectra of the labels are shown to better visualize the emission-scan range. E) An equivalent representation of panel C, but where only the excitation spectra of the labels are shown to better visualize the excitation-scan range. Spectroscopic data were obtained through the Semrock SearchLight spectral viewer, while original sources for spectroscopic data for Hoechst are (http://www.spectra.arizona.edu/), for CFP are (https://www.thermofisher.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html), and for Calcein Green are (https://www.thermofisher.com/order/catalog/product/C34852?ICID=search-product).

We have recently implemented an improved version of the HIFEX imaging technology that uses a tiltable filter wheel (VF-5, developed by Sutter Instruments, no commercial interests involved) that allows improved wavelength switching speeds with high repeatability. While still not high-speed, the system allows for acquisition of a spectral image stack in 20-30 seconds, depending on acquisition settings. This temporal resolution has been sufficient to perform preliminary live-cell imaging studies, such as for quantifying subcellular signaling responses. Here, we report initial results from cell signaling studies to investigate intracellular distributions of second messengers such as cyclic nucleotides and Ca^{2+}.

2. METHODS

2.1 Sample preparation

Three cell lines were evaluated for this study: human embryonic kidney (HEK 293) cells, rat pulmonary microvascular endothelial cells (PMVECs), and human airway smooth muscle cells (HASMCs). For each, cells were cultured as reported previously[11,12] and grown so as to achieve confluent monolayers on 25 mm round cover slips. To assess intracellular cAMP signaling, cells were transfected with a Turquoise-Epac-Venus H188 cAMP reporter 2 days prior to imaging, as described previously[11]. Coverslips were incubated with NucBlue (Invitrogen) nuclear label and were then secured in Attofluor cell chambers and bathed with 1 mL phosphate buffered saline (PBS) prior to imaging. Single-label controls were also prepared using HEK 293 cells labeled with either NucBlue, mTurquoise, or mVenus alone.

2.2 Hyperspectral imaging

Spectral images were acquired using a custom inverted microscope (TE2000, Nikon Instruments) equipped with a 300W Xe arc lamp and tunable excitation filter (VF-5, Sutter Instrument Co., equipped with VersaChrome filters, Semrock), a set of custom long-pass dichroic beamsplitters placed at 458, 495, and 555 nm wavelengths (Semrock), and an EMCCD camera (Rolera em-c^2, QImaging). Image acquisition was coordinated using custom scripts in MicroManager. Timelapse spectral images were acquired at excitation wavelengths ranging from 340-485 nm, in 5 nm increments, every 30 seconds. For a subset of experiments, 50 μM forskolin (an adenylyl cyclase activator) was added after 2 minutes to stimulate cAMP production. Single (non–time-lapse) spectral images were acquired from single labeled samples for use in building spectral libraries.

2.3 Image analysis

Spectral image data were evaluated using ENVI (Harris Geospatial Solutions) software and custom scripts in MATLAB (MathWorks). Regions with well-labeled cells were selected within the single-label control images and the average spectrum of each fluorescent label was extracted as the end member spectrum. End member spectra were normalized to a peak value of unity and stored in a spectral library. Spectral image data from multi-labeled samples were linearly unmixed using custom MATLAB scripts, as described previously[10,12,14], for each time point. Where appropriate, unmixed time-lapse images were false-colored and exported to a movie format for visualization.

3. RESULTS AND DISCUSSION

The HIFEX fluorescence excitation-scanning approach enabled measurement of characteristic excitation spectra for a range of molecules, such as NucBlue, mTurquoise, and mVenus. To separate spectral contributions to mixed pixels, a spectral library of the pure end member signatures was first formed (Figure 2).

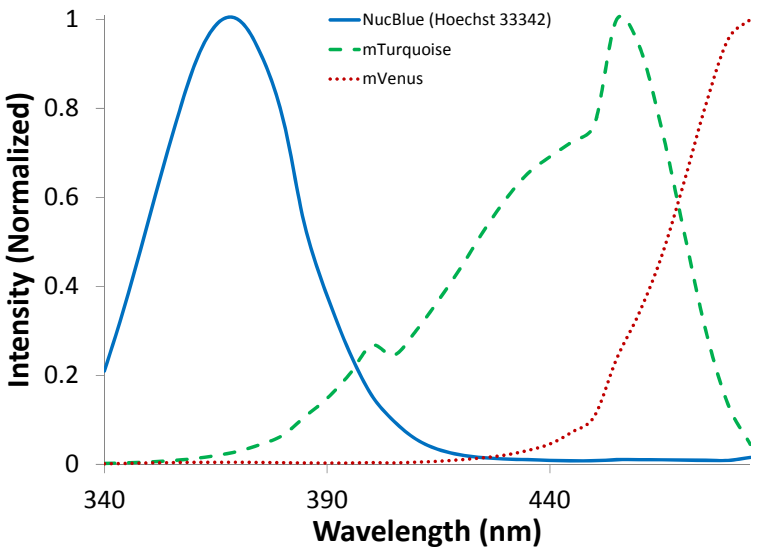

Figure 2: A fluorescence excitation spectral library was formed by acquiring spectral images from single-labeled cell samples, defining regions corresponding to well-labeled cells, and extracting the pixel-averaged spectrum from each region. This example spectral library contained fluorescence excitation spectra for 3 fluorescent labels: NucBlue (blue solid line), mTurquoise (green dashed line), and mVenus (red dotted line).

To assess the ability to acquire live-cell, time-lapse spectral image data, live cell samples were prepared and spectral images acquired every 30 seconds for 10 minutes. In a subset of experiments, the maximum imaging speed of the system was evaluated by adjusting acquisition settings, which allowed a spectral image stack to be acquired as fast as every 5 s. To visualize raw spectral image data, single wavelength bands were identified, linearly scaled by a fixed amount per band, converted to 8-bit image data, and monitored over time (Figure 3). Preliminary data indicate little-to-no effects of photobleaching, as can be seen by examining the intensity of the 3 representative wavelength bands shown. Interestingly, the 360 nm band increased in intensity, possibly due to continued internalization of the NucBlue probe during the experiment, subtle movement of the coverslip over time, or sample cooling (a heated stage was not available for these experiments). At the 360 nm spectral band, clear contributions from the NucBlue label were visible, as well as subtle contributions from mTurquoise. At the 390 nm spectral band, mTurquoise contributions were more pronounced, while NucBlue signals were weakly visible. At the 450 nm spectral band, the majority of contributions were attributed to mTurquoise and mVenus.

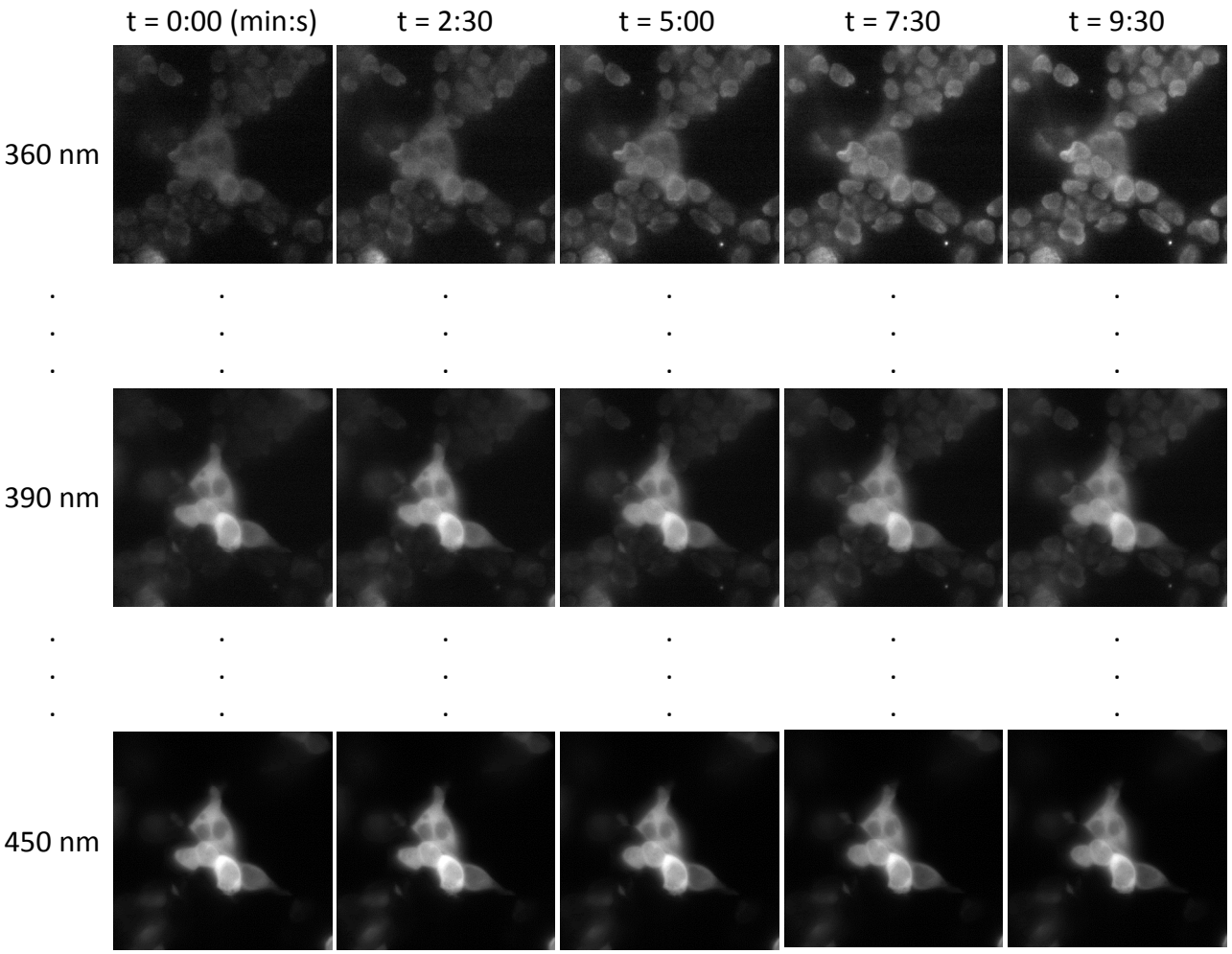

Figure 3: A visualization of 3 representative wavelength bands selected from the 30 wavelength band spectral image at 5 representative time points. For each wavelength band, a linear range was selected to scale all time points for visualization (0-200 greyscale levels for 360 nm, 0-500 for 390 nm, and 0-3500 for 450 nm). 16-bit images were rescaled linearly and converted to 8-bit greyscale format for visualization. Of note, the NucBlue label is the primary contributor in the 360 nm band, while mTurquoise is the primary contributor in the 390 nm band, and mVenus the primary contributor in the 450 nm band.

HIFEX spectral timelapse image data were linearly unmixed to visualize the estimated contribution of each end member component in the spectral library (Figure 4). As could be seen in the raw spectral data, NucBlue signals were seen to increase in label intensity during the time lapse experiment, possibly due to a change in focus of the microscope or due to increased internalization of the label over time (Figure 4, top row). mTurquoise and mVenus signals showed less variation in intensity with time (Figure 4, middle and bottom rows).

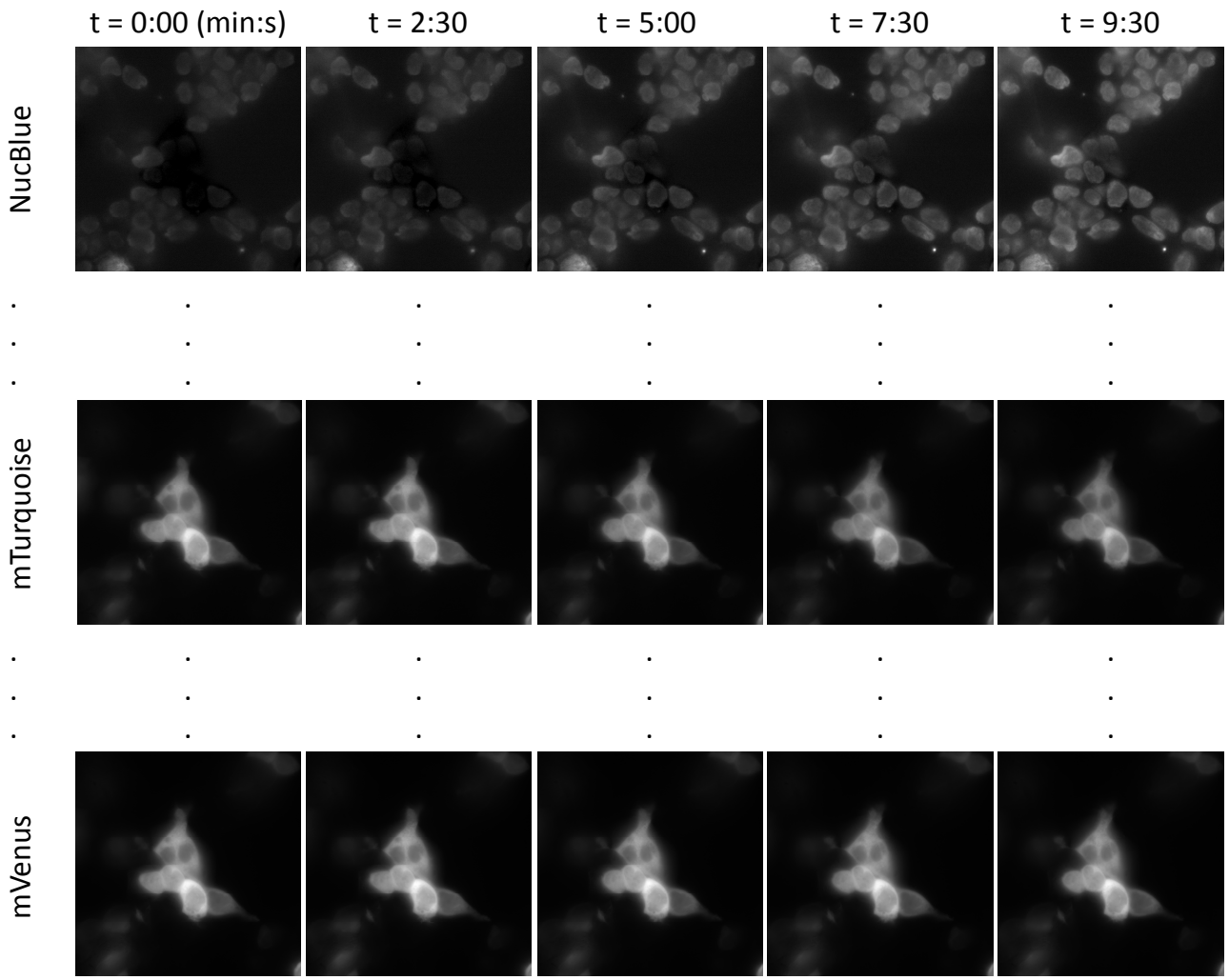

Figure 4: Linearly unmixed excitation-scanning (HIFEX) spectral images corresponding to the raw images shown in Figure 3, and unmixed using the spectral library shown in Figure 2. NucBlue end member images were seen to increase in intensity over the course of the experiment, while mTurquoise and mVenus image intensities remained relatively constant. For each unmixed image, a linear range was selected to scale all time points for visualization (0-300 greyscale levels for NucBlue, 0-3500 for mTurquoise, and 0-11000 for mVenus). 16-bit images were rescaled linearly and converted to 8-bit greyscale format for visualization.

4. CONCLUSIONS AND FUTURE WORK

Hyperspectral imaging fluorescence excitation scanning (HIFEX) is a relatively new approach for fluorescence microscope spectral imaging that allows acquisition of the fluorescence excitation spectrum and separation of mixed spectral signatures, similar to traditional emission-scanning spectral imaging approaches. Here, we have demonstrated that HIFEX imaging can be implemented for time-lapse live-cell imaging studies, albeit with only a moderate temporal sampling of 30 s per spectral image stack. Future work will focus on developing alternative hardware approaches to further increase the speed of HIFEX for live cell imaging and cell signaling studies.

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