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14. ABSTRACT: The available technologies to assess lung function in the upper airways are time consuming and lack of proper sensitivity, therefore the identification of novel medical tool for the analysis of lung cells health with satisfying sensitivity and specificity will have significant impact for health care system. In this proposal we propose a hybrid imaging system Camera & Raster with two modalities: seek and focus. Seek modality is a large field, camera-based reflectance hyperspectral, that provides an intermediate accuracy for measuring lung injuries. This modality is coupled with a standard bronchoscope and already greatly enhances its capabilities. It acquires the reflectance spectrum of lung tissues and provides an intermediate accuracy result on tissue health using Hyperspectral Phasor analysis. Areas of interest with a higher likelihood of lung injury will be imaged using the focus modality, that provides high accuracy on a small field of view. The focus modality allows us to identify the different cell types in the lung and precisely detect the metabolic and bimolecular footprint of the cell and tissue.

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INTRODUCTION

Military personnel can be exposed by inhalation to dusts and toxicants in the field that may contribute to lung disease. The available technologies to detect early stage alterations of lung function in patients require a long processing time and lack satisfactory sensitivity and resolution. These existing medical technologies can only visualize, but not precisely measure the metabolic health of individual airway lining cells. We will build and tune a new fiber-optic system that can be deployed alone or as a modification to an existing upper or lower airway scope instrument, that will detect airway lining cell injury with a greater specificity, sensitivity and speed.

KEYWORDS

Lung injury, endoscopy, hyperspectral, spectraFLIM, fluorescence, autofluorescence, metabolic profile, non-invasive, basal cells, clara/club cells, SO2, naphthalene

ACCOMPLISHMENTS

What were the major goals of the project?

AIM 1

Subtask 1: Evaluate sensitivity of Camera-SpectraFLIM

Status: Completed 2017-03

Result: We performed research enquiries on the actual sensitivity of current camera based tools for acquiring low intensity autofluorescent data. We evaluated the latest generation camera sensors designed by PCO AG GmbH (Kelheim, Germany) with frequency domain 40MHz rate and highest quantum efficiency peak on the market (39%@peak). After discussing with multiple industry developers and with the university research laboratory that spearheaded that product application[1], we concluded that, as of March 2017, this camera based technology is not mature for measuring autofluorescence. However, in our further researches we found that recent snapshot hyperspectral camera sensors (reflectance based), that use super-bayer filter technology, has reached a sufficient sensitivity for our application. We have chosen one of these cameras by PhotonFocus (Lachen, Switzerland) to pair with wide-field bronchoscopic observation.

Milestone # 1: Selection of optimal acquisition modality between Camera- and Raster-SpectraFLIM to be used in Aims 2-3

Status: Completed 2017-03

Result: After careful consideration we decided to develop a hybrid system Camera and Raster with two modalities: *seek* and *focus*. *Seek* modality is a large field, camera-based reflectance hyperspectral, that provides an intermediate accuracy for measuring lung injuries. This modality is coupled with a standard bronchoscope and already greatly enhances its capabilities. It acquires the reflectance spectrum of lung tissues and provides an intermediate accuracy result on tissue health using Hyperspectral Phasor analysis. Experimental results using this modality are reported below. Areas of interest with a higher likelihood of lung injury will be imaged using the *focus* modality, that provides high accuracy on a small field of view. This modality uses Raster scan SpectraFLIM to acquire autofluorescence, coupled with high efficiency GRIN lenses mounted on high density optical fiber bundles.

Subtask 2: Implement SpectraFLIM onto Bronchoscope and calibrate with standards

Status: 95% complete

Result: Two acquisition modalities are to be implemented on the instrument: *seek* and *focus*. *Seek* modality utilizes snapshot reflectance hyperspectral imaging combined with HyperSpectral Phasor. This modality has been implemented on a bronchoscope using low profile camera (Figure 1).

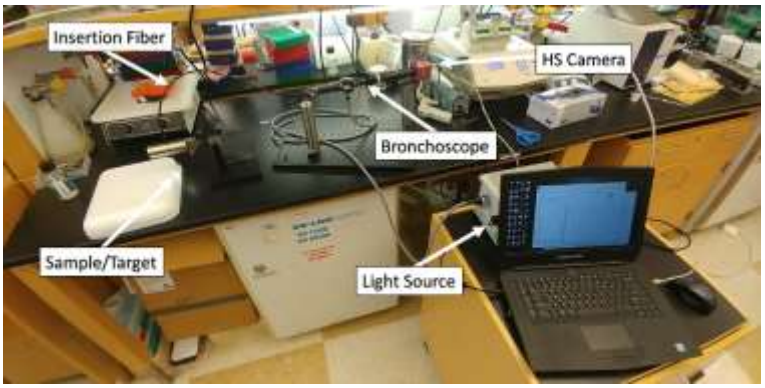


Figure 1: *Seek* modality setup for snapshot Hyperspectral Phasors reflectance. Endoscopic light source provides excitation spectrum between 400nm and 800nm. Bronchoscope fiber delivers excitation light and collects reflected light directed to a low profile hyperspectral camera mounted on distal side of bronchoscope. Data is processed using HySP software.

Focus (Figure 2) modality is still under implementation, currently has scanning unit, laser filters, electronics controllers, imaging fiber bundles with GRIN lens. All these components are custom designed and made.

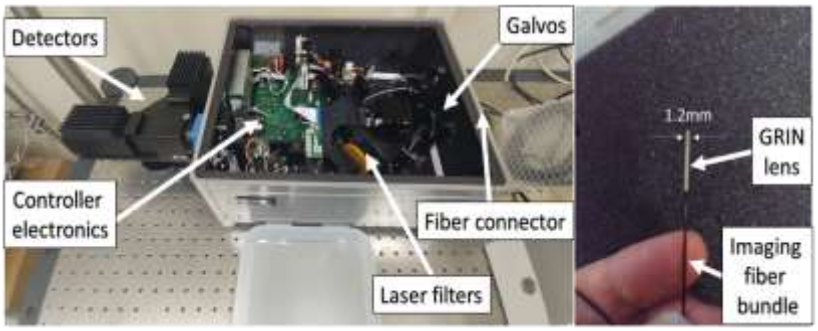


Figure 2: *Focus* modality prototype setup for SpectraFLIM measurements. Laser filters sort excitation light to be raster scanned using Galvos onto an imaging fiber bundle. This fiber bundle contains 30k fibers and is factory fused to a GRIN lens that will focus light onto the sample. The small diameter of the lens allows access to the auxiliary port of a bronchoscope like the one in used in *Seek* modality.

Subtask 3: Submit IACUC Protocol for animal testing

Status: Completed 2017-01

Result: IACUC Protocols were drafted, submitted 2017-01. Minor amendments for transport of samples from Children's Hospital Los Angeles to USC University park campus were submit 2017-08.

Milestone # 2: SpectraFLIM implemented onto Bronchoscope and calibrated

Status: 95%

Result: Design of SpectraFLIM unit was prepared, optimized and submitted to manufacturers. Some custom components required longer than expected for fabrication and assembly. Components have been delivered, system is currently being aligned.

Milestone #3: Approval of IACUC protocol

Status: Completed 2017-03

Result: IACUC Protocols were approved by USC Board in 2017-03. Minor amendments for transport of samples from Children's Hospital Los Angeles to USC UPC were accepted 2017-09.

Subtask 4: Pilot study SO2 and diphtheria toxin (8-20 mice)

Status: 100%

Result: We performed pilot studies on mice with naphthalene and SO2 using the Seek modality setup. Experiments allowed optimization of protocols for both image acquisition and sample preparation.

- The Naphthalene exposure protocol we used, efficiently depleted the Club cells in the tracheal epithelium. The loss of the Club cells was more prominent in the distal portion of the trachea (closer to the bronchi).
- To expose animals to SO2 gas we created a propylene exposure chamber to deliver SO2 (500ppm) mixed in 80% N2/20% O2 to the animals. Histological analysis showed marked loss of the tracheal Clara and ciliated cells at 1 and 3 days post exposure. The tracheal epithelium completely regenerately by around 7 days post injury.
- The last injury model entails the expression of the diphtheria toxin specifically in the lung basal cells using the KRT-5ER-CRE mouse transgenic line: the breeding and the testing of these animals have been delayed of about 2 months. This was caused by the necessity to write a full new IACUC protocol

to allow the transport of animals from CHLA to USC UPC to facilitate some of the tissue imaging procedures.

YEAR 2: We generated mice in which the diphtheria toxin can be expressed in Krt5+ cells (basal cells). 8 weeks old, double transgenic (Krt5-Er-Cre/DFT-Stop-fl/+) mice were injected with tamoxifen once and mouse trachea and upper airways were collected for histological analysis. We found that basal cells were completely gone by 36 hours post injection. Originicall, we planned to collect lung tissue until 10 days after tamoxifen injection. However, since Krt5 is expressed in multiple basal cell population in the body (e.g esophagus) we discovered that mice didn't tolerate the lack of basal cells as expected and we decide to collect tissues at 1, 2, 3- and 4-days post tamoxifen exposure (instead of 1, 3, 7 and 10 days). Control animals injected with tamoxifen did not show any alteration of the tracheal epithelium.

AIM 2

Subtask 1: Obtain standard Phasor for negative samples

Status: 98% completed. We have acquired a total of 9 samples and are currently in the process of analyzing the data. We plan on acquiring 21 more samples to test for environmental and experimental variability.

Subtask 2: Obtain standard Phasor for positive So2 samples

Status: 98% completed. We have:

- optimized the protocol for sample preparation. We designed a propylene exposure chamber to deliver SO2 (500ppm) mixed in 80% N2/20% O2 to the animals. Histological analysis showed marked loss of the tracheal Clara and ciliated cells at 1 and 3 days post exposure. The tracheal epithelium completely regenerated by around 7 days post injury. For imaging, once the mouse was euthanized the trachea was cut longitudinally to expose the epithelium.
- acquired a total of 27 samples and are currently in the process of analyzing the data at 1, 3 and 7 days post injury. We plan on acquiring 8 more samples to test for environmental and experimental variability.

Subtask 3: Obtain standard Phasor for positive Naphthalene exposed samples

Status: 98% completed. We have:

- optimized the protocol for sample preparation. Naphatale injection 250 mg/kg was injected in mice once. 1, 3, 7 and 14 days later mice were euthanized. Clara cells fully repopulated the epithelium by 14 day after Naphatale exposure. For imaging, once the mouse was euthanized the trachea was cut longitudinally to expose the epithelium.
- acquired a total of 29 samples and are currently in the process of analyzing the data at 1, 3, 7 and 14 days post injury. We plan on acquiring 11 more samples to test for environmental and experimental variability.

Subtask 4: Obtain standard Phasor for positive Diphtheria Toxin exposed samples

Status: 90% completed. We have:

- optimized the protocol for sample preparation. Krt5-Er-Cre/DFT-Stop-fl/+ mice were generated and, once reached 8 weeks of age, were injected with tamoxifen (0.25mg/g). For imaging, once the mouse was euthanized the trachea was cut longitudinally to expose the epithelium. Acquired a total of 12 samples and are currently in the process of analyzing the data at 1, 2, 3 and 4 days post tamoxifen injection. We plan on acquiring 36 more samples to test for environmental and experimental changes.

YEAR 3: We acquired phasor plot from trachea from Krt5-Er-Cre/DFT-Stop-fl/+ mutant and control animals injected with tamoxifen.

AIM 3

Subtask 1: Perform software validation and comply requirements for IRB approval

Status: 60 % completed. We have satisfied compliance for IRB and are performing software validation and improvements.

Subtask 2: Perform risk assessment for human studies

Status: 75% completed. We have assessed risks for the seek modality. We have completed approximately half of the assessment for the focus modality. We expect this part to be completed in 3 months.

What was accomplished under these goals?

1) major activities:

YEAR 1

1a) **Technology landscape assessment**: we performed a thorough analysis of currently existing technology both available commercially and in developmental stage. Performed meetings with multiple leader manufacturers in the field of:

- detectors: we compared Hamamtsu Photonics K.K., Hamamatsu City, Japan; Leica Microsystems, Wetzlar, Germany; Gpixel Inc, Changchun, China; Spectral Devices Inc., London, Canada.
- Optomechanics: we compared Optics Technology Inc, Pittsford, NY; ISS, Urbana-Chamapign, IL; Mirrorcle Technologies Inc, Richmond, CA.
- fiber optics and lenses: we compared Grintech Gmbh, Jena, Germany; Tag Optics Inc., Princeton, NJ; Mitsubishi Cable America Inc., Blue Bell, PA; Fujikura Ltd, Tokyo, Japan; US Fiberoptec Technology Inc, San Jose, CA.

1b) **Instrument design**: based on the information obtained through assessing the technological landscape we designed a hybrid raster scanning SpectraFLIM and snapshot reflectance hyperspectral system, *seek* and *focus* (described in milestone #1 above). This system leverages existing technology for bronchoscopy to gain access into airways and exploits auxiliary bronchoscope port to insert a custom made optical fiber bundle factory coupled to a medical grade gradient index objective.

1c) **Building and calibration**: we assembled the Seek modality of the instrument, that provides an intermediate lung injury assessment accuracy on a large field of view, utilizing low profile camera-based reflectance hyperspectral (Figure 1).

1d) **Preliminary testing on mice**: we performed 12 sessions of iterative optimization for injury protocol and experimental imaging. During this we performed injury assessment of lung airways of mice exposed to naphthalene and SO2 as well as control animals.

YEAR 2

1e) **Extended testing on mice**: we performed a total of 12 sessions of iterative optimization for injury protocol and experimental imaging. During this we performed injury assessment of lung airways of mice exposed to naphthalene and SO2 as well as control animals.

1f) **Building and calibration**: we assembled the *focus* modality of the instrument, that provides a high quality lung injury assessment accuracy on a small field of view, utilizing SpectraFLIM (Figure 1).

1g) **Instrument design**: progressing on the work in Year 1 we have tested different approaches for miniaturizing the profile of the endomicroscope for achieving compliance in portability and size for bronchoscopy room. We identified components, designed and commissioned an imaging-fiber / GRIN lens assembly for performing distal raster scanning. We designed a compact femtosecond pulse compressor for compensating for pulse dispersion of 2-photon laser along the imaging fiber.

1h) **Satisfy requirements for IRB approval**: progressing on the work in Year 1 we have improved our *seek* and *focus* prototypes to comply software validation, sterilization and requirements for obtaining IRB approval.

YEAR 3

1i) Expanded testing on mice: we obtained phasor plot on tracheas from Krt5-Er-Cre/DFT-Stop-fl/+ mutant and control animals injected with tamoxifen.

1l) Wrote human subject protocol: we submitted to the CHLA IRB a protocol for the testing of the hyperspectral camera on human subjects.

1m) Building and testing: we tested the fiber-based fluorescent endomicroscope designed in year 2 for measuring subtle fluorescent signals and autofluorescence.

1n) Design: we designed, assembled, tested a novel multispectral camera for fluorescent signals which considerably simplifies the design of the endomicroscope.

2) specific objectives

YEAR 1

The objective of year 1 was to design and establish an instrument and experimental pipeline for performance assessment through experimentation in year 2.

In summary:

2a) We evaluated sensitivity of Camera based approaches for measuring SpectraFLIM

2b) We designed an instrument that hybridizes Camera hyperspectral reflectance with Raster SpectraFLIM

2c) We implemented Seek modality on a bronchoscope and performed calibration with standards

2d) We submitted IACUC protocols and obtained approval

2e) We performed pilot studies of SO2 and naphthalene lung injuries

YEAR 2

The objective of year 2 was to evaluate the performance in mouse animal model for the dual purpose of improving design of the instrument and collecting supporting information for IRB approval for in human studies.

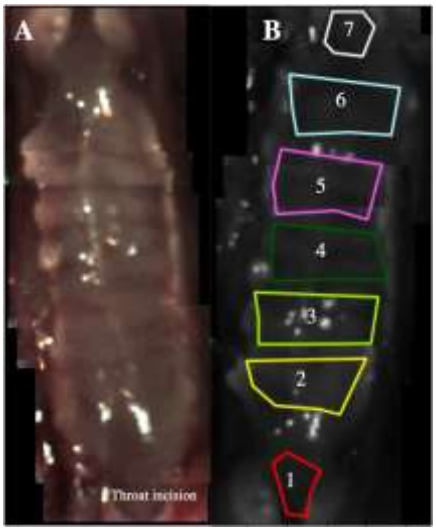


Figure 3: Preliminary results for *Focus* modality SpectraFLIM measurements. During experimental procedures we developed an analysis pipeline. Data is first acquired as separate hyperspectral images as a "fly-over" on an open mouse throat incision (A). Using the position of trachea (B,7) we create a series of ROI for performing analysis. With an approximately constant pixel size we repeated the analysis on multiple mice with different injuries to have a comparison of phasor signatures at different positions.

In summary:

2f) we performed experiments on:

- negative sample
- SO2 exposed samples
- Naphthalene exposed samples

2g) improved software stability for future utilization in human

2h) we submitted IRB for testing in human.

YEAR 3: The goals of year 3 were to complete animals experiments and to test the hyperspectral camera and SpectraFLIM apparatus on human subjects.

In summary:

2i) We generated phasor plots from Krt5-Er-Cre/DFT-Stop-fl/+ mutant and control tracheas.

2j) Submitted to the CHLA IRB a new stand-alone protocol for the testing of the hyperspectral camera reflectance "seek" on human subjects.

2k) We tested SpectraFLIM technology in detecting diphtheria toxin.

2l) We tested the "focus" microscope design against fluorescence and autofluorescent signals (Figure 4).

2m) We designed a new fluorescence multispectral camera which will considerably simplify the design of the "focus" fluorescent microendoscope.

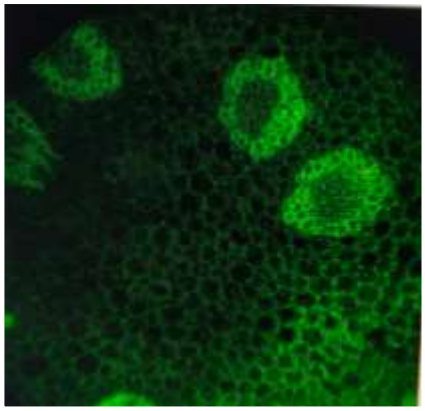


Figure 4: **SpectraFLIM "focus" endomicroscope test.** The fixed sample was imaged using the design made during year 2. High quality imaging fiber in combination with single photon modulated excitation was utilized to acquire a multicolor fluorescent fixed sample of convollaria.

3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative)

YEAR 1

3a) Significant Results: from preliminary experiments, lung injuries appear to have characteristic phasor signatures depending on the chemical used and the location of lung epithelial injury. Different positions in the lung are affected in different ways by chemicals. Preliminary assessment shows phasor differences in these lung positions (Figure 3,5).

3b) Development: Seek modality can be used as an intermediate assessment tool for determining areas of interest to be imaged with SpectraFLIM in greater detail.

3c) Technical assessment conclusion: After discussing with multiple industry developers and several university research laboratories that tested similar sensitivity applications, we concluded that, as of March 2017, camera-based technology is not mature for measuring autofluorescence. However, camera-based snapshot hyperspectral technology is a viable instrument for measuring reflectance hyperspectral.

Figure 5: Preliminary results for *Focus* modality SpectraFLIM measurements. In this large map we report the preliminary results in terms of G and S coordinates of the phasor plot. The box plots represent mean (+), median (red line), minimum and max values as well as standard deviation (box) over multiple samples. The experiment was repeated on control samples and samples exposed to SO2 as well as naphthalene. The plots show significant differences for the 3 samples, suggesting each injury has a specific phasor pattern. For each ROI selected in figure 3, we perform phasor analysis and characterized the average phasor coordinates of that region. Interestingly the regions closer to trachea (ROI 7) exhibit smaller differences compared to ROIs deeper in the lungs. This is an expected result as this injury is expected to be stronger in deeper positions in lungs (ROI1).

YEAR 2

3d) Significant Results: we have collected data from 77 total samples comprising SO2, Naphthalene, controls, corn oil. There are some interesting findings to report:

- tracheal regions with more visibly cartilaginous tissue exhibit stronger differences in spectral signatures across multiple injuries. We report this example in figure 5 where we segment areas inside the trachea based on the amount of cartilage. The cartilaginous/not areas are then analyzed separately (Figure 6). The analysis on phasor shows a larger difference between lung injuries in non-cartilaginous areas. This finding is reasonable as in these areas the epithelial thickness is larger, allowing for improved interaction of light with cells which are affected by the injury, compared to cartilage cells which would show more subtly the effects.

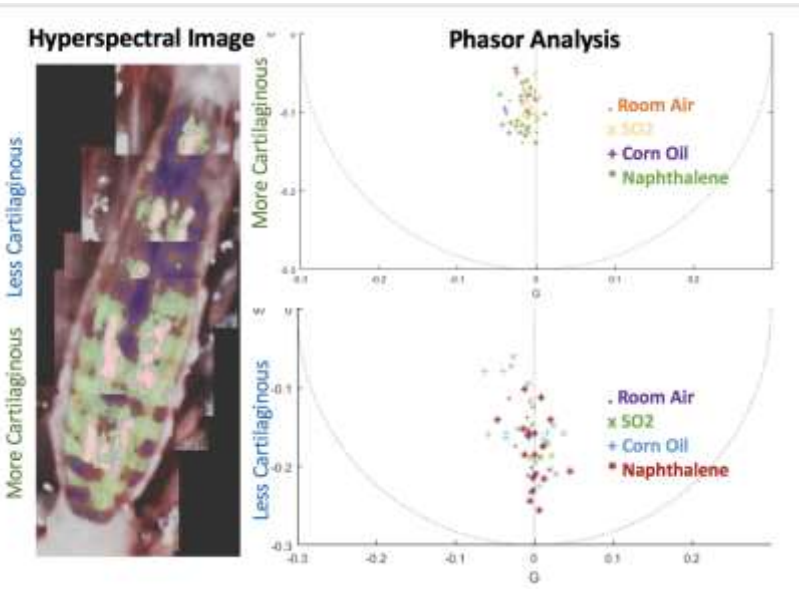


Figure 6: Phasor maps for 77 samples including controls (room air), SO2, Corn Oil, Naphthalene. The hyperspectral image is subdivided in two regions based on the amount of cartilaginous tissue. The analysis is performed separately on the two regions. The phasor plots show greater separation between injuries in the more cartilaginous areas.

- Phasor plots can be used to correlate the hyperspectral data with the injury, however there is an intrinsic variability related to the nature of the experiments. The variability can be used as a probability for a specific type of injury

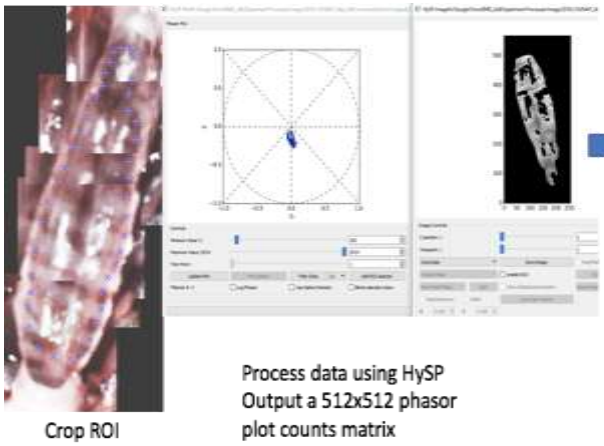


Figure 7: generalized cross-correlative approach for comparing different hyperspectral phasor datasets. Area of the book-prepped trachea is segmented and processed using the phasor approach. The resulting histogram plot represents the counts per spectrum. This plot is treated as an image and used for calculating 2D correlation coefficient and 2D cross-correlation in reference to other datasets' phasors. This approach allows for a simplified comparison between multiple multi-dimensional datasets.

- To account for biological variability, we have developed a generalized cross-correlative approach for comparing different hyperspectral phasor datasets. The process is reported in figure 7. The cross correlation is performed against the average control sample, utilizing room air as a non-injury. In Figure 7 we report the correlation against SO2, Naphthalene and Corn Oil on 1, 3, 7 and 14 days where available and compare it to room air self-correlation. We observe a pattern in the cross-correlation, the highest difference compared to control happens in the early days after injury.

After 7 days the injury returns to the same values of correlation as the control, overshooting after 14 days in the case of Naphthalene.

- After imaging tracheal tissues was collected and formalin-fixed/paraffin embedded for histological analysis. We performed staining for Cc10 and phopho-Rps6 to quantify regeneration and metabolic status, respectively, of the tracheal epithelium. Correlation studies will be performed to fit the hyperspectral phasor data sets with immunohistological findings.

3e) Development: we have improved portability of the seek hyperspectral bronchoscope and compacted the analysis to a portable cart. This should simplify its use in hospital rooms. The focus modality is currently being realigned. We have measured considerable loss of two-photon excitation along the imaging fiber resulting from two factor: absorption of the imaging fiber bundle (Fujikura, Tokyo, JP) along 780nm excitation wavelength, group velocity dispersion along the fiber bundle. The use of more powerful and considerably more expensive two-photon lasers could solve this problem, however it would reduce the portability of the instrument. These factors combined complicate the use of this laser for exciting autofluorescence. We have taken two actions to overcome these limits:

- designed a portable and low cost femtosecond pulse compressor for delivering sufficient laser power to the sample,
- co-excite utilizing single-photon lasers modulated at 20, 40 and 80 MHz.

We are currently in the process of aligning these lasers and compressors.

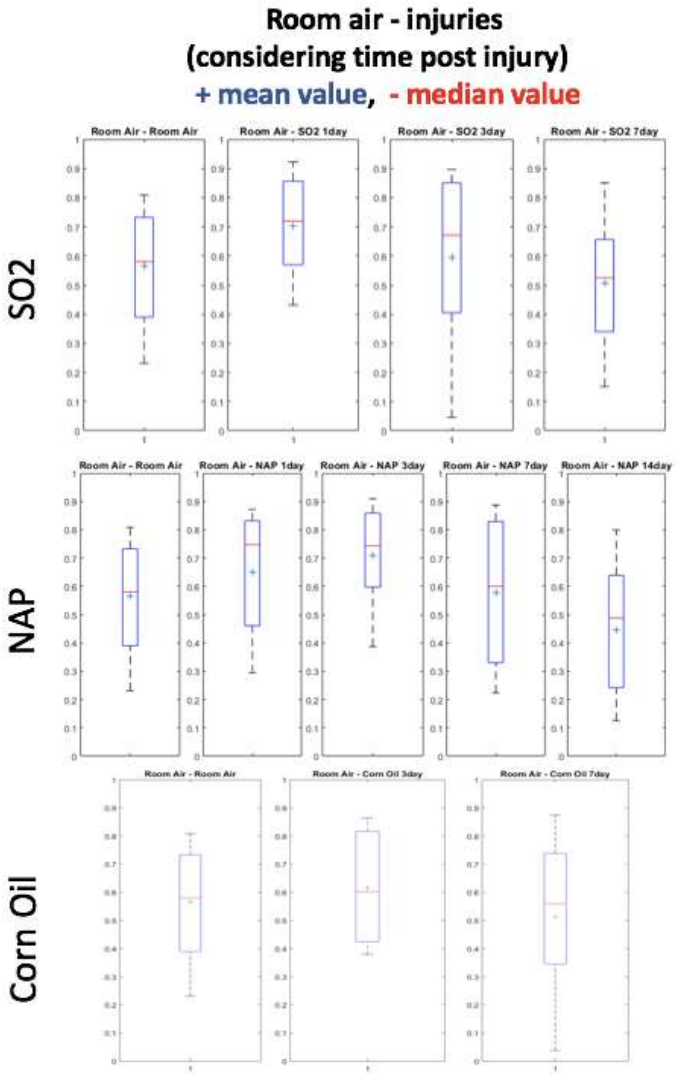


Figure 8: Phasor correlation coefficients for a set of 50 (of 77) samples. The reference value is correlation between two room air samples. The correlation is calculated between room and SO2, Naphthalene and Corn Oil. We observe a pattern in the cross-correlation, the highest difference compared to control happens in the early days after injury. After 7 days the injury returns to the same values of correlation as the control, overshooting after 14 days in the case of Naphthalene.

YEAR 3:

3f) Significant Results: we have produced and analyzed the phasor plot of the Krt5-Er-Cre/DFT-Stop-fl/+ mutant and control tracheas at 1 and 2 days post tamoxifen injection. Tamoxifen injection induces the expression of the diphtheria toxin in the tracheal basal cells and, consequently, their death. Loss of trachea basal cells is evident as early 24 hours post injection and was confirmed by histological analysis.

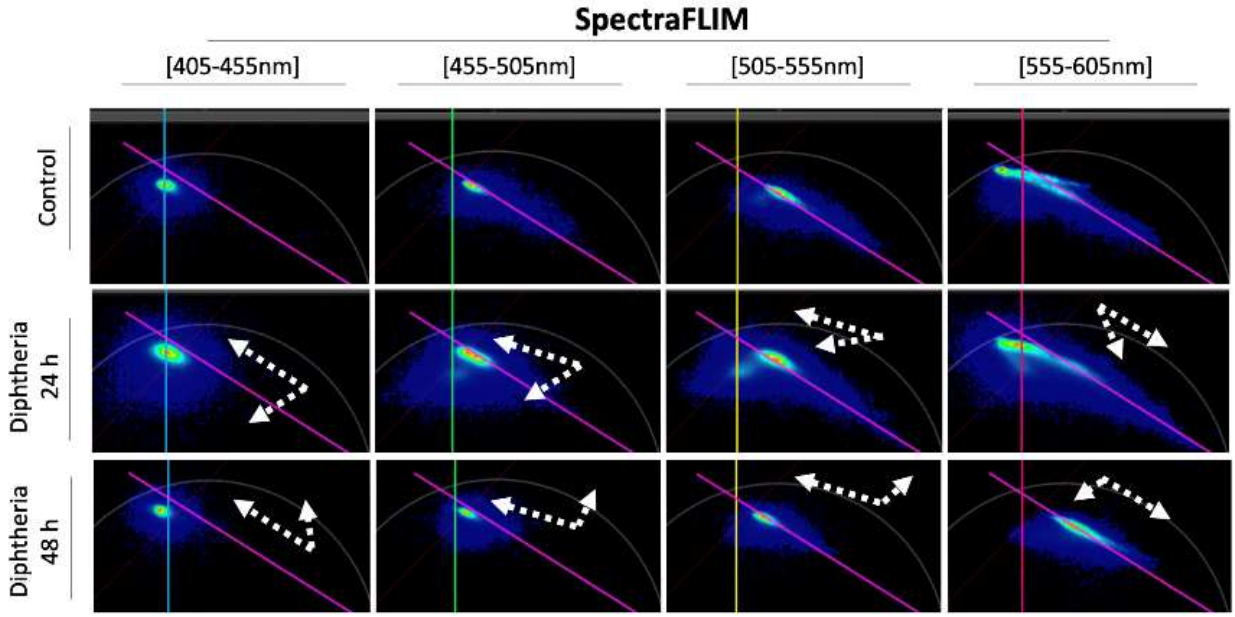


Figure 9. SpectraFLIM autofluorescence phasors comparison for unlabeled freshly isolated mouse tracheal explant affected with diphtheria toxin. The live sample was imaged using multi-spectral two-photon microscopy (740nm excitation, 4 wavelength bins, 50nm bandwidth, 405-605 nm detection) with FLIM detection on each channel, to collect the fluorescence of intrinsic molecules including folic acid, retinoids and NADH in its free and bound states. The holistic view of phasor shows population changes for metabolites from control sample unaffected (first row) to samples affected by diphtheria toxin at 24 and 48 hours. White arrows indicate metabolic shift respect to control. Magenta line represents the metabolic trajectory adjoining fingerprinted positions of NADH free/bound (right/left side of plot). Vertical lines are placed for reference. The distribution change respect to control is evident, with both changes in shape, center of mass and area.

Autofluorescence changes affect the center of mass, shape, area of the phasor plot, suggesting a pattern which can consistently be used to identify the state of progression. The major implication of this finding is that spectraFLIM based autofluorescence imaging is able detect biomolecular changes in the lung during injury and repair without the use of dies and contract reagents and despite the injured tracheal epithelium appears macroscopically normal and indistinguishable from the control (Figure 9).

3g) Development: we tested the design raster-scan SpectraFLIM endomicroscope. The instrument utilizes an optimized Fujikura Imaging Fiber connected with grin lens. The test on fluorescent samples was successful (Figure 4 above), with considerable loss along the fiber, we were able to collect visible (480-600nm) fluorescent signals. However, upon testing acquisition of autofluorescent signals, mainly between 400-470nm, we encountered a dual problem: autofluorescence from the fiber cladding and excessive loss of signal along the fiber (in this case a 2m fiber bundle). Utilizing 2-photon excitation in combination with a custom designed femtosecond pulse compressor we could not achieve sufficient photon density on sample when utilizing a 2 meters fiber bundle. We tested 470nm laser with 20MHz modulated excitation and could excite fluorescence but not autofluorescence. We then tested a simplified design (Figure 10) in an effort to debug the fiber bundle limitations. Utilizing different wavelength LED we tested the efficiency of the fiber to transmit and collect autofluorescent signals. The conclusion was that utilizing wavelengths more suitable for exciting intrinsic signals (380nm-405nm LEDs) the autofluorescence from the fiber would exceed that of the intrinsic signals by at least 10 fold. We then separated the LEDs and excited the sample laterally, without introducing the excitation wavelength into the fiber, obtaining Figure 10B.

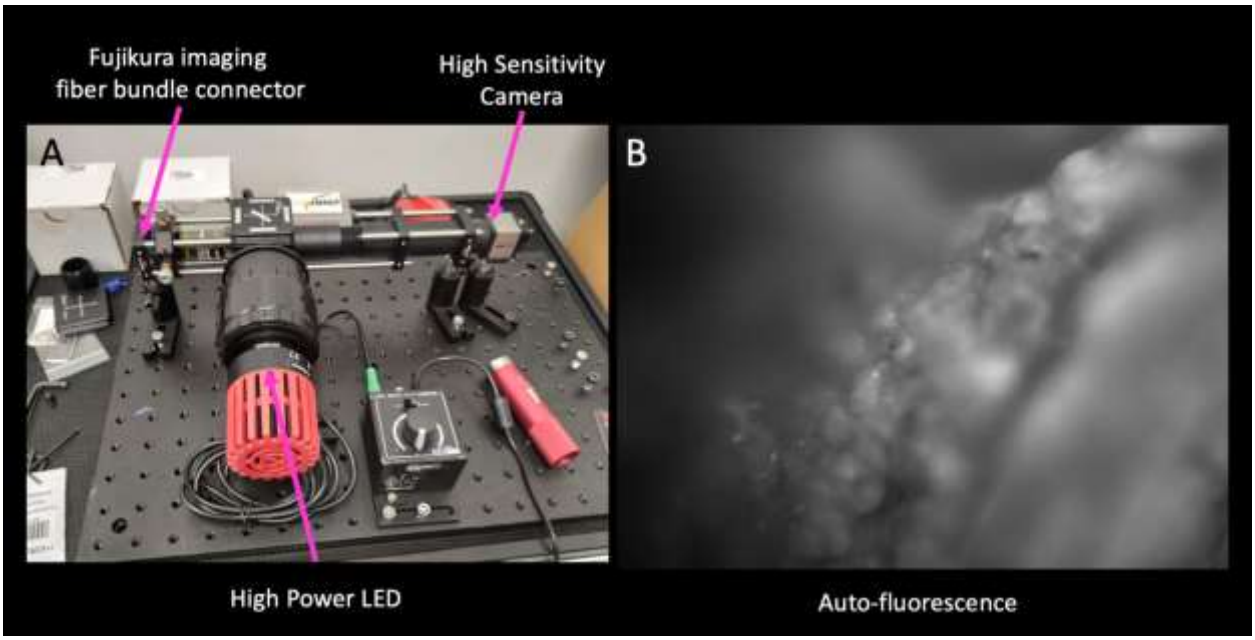


Figure 10. Simplified Endomicroscope design. A) This streamlined design utilizes a high sensitivity camera in combination with a high-power LED to excite fluorescent signals in tissues. The autofluorescence deriving from the fiber at low UV excitation would exceed the signal strength saturating the detector. B) We separated the high power LED from the optical path through the fiber and illuminated the sample laterally, collecting the autofluorescent signals through GRIN lens and fiber, obtaining the image here reported.

This suggests that the limitation of the fiber relates mainly to the excitation wavelength and, with a sufficiently strong excitation, the biomarkers' signal can be collected. This can be designed if excitation is transported utilizing a hollow core single fiber and detection is performed utilizing the imaging fiber bundle or utilizing a camera mounted in the distal end of the endoscope. The latter approach has become the principal for major endoscopes companies like Olympus and Pentax.

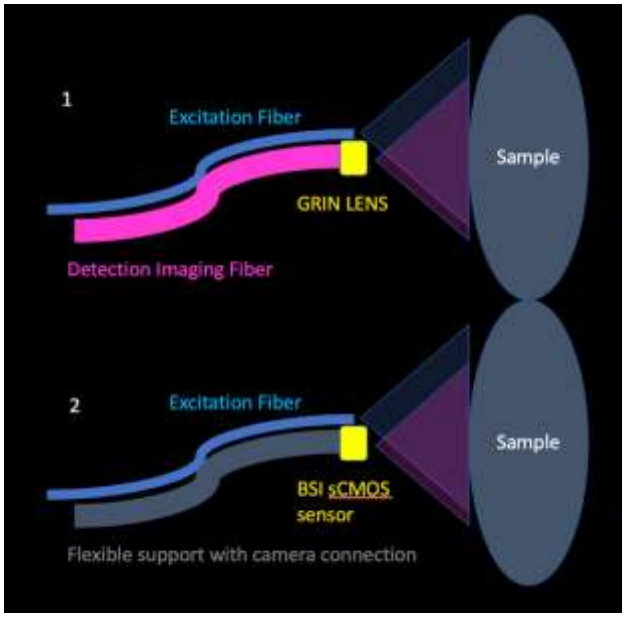


Figure 11. Improved wide field fluorescence endomicroscope designs. 1) This design utilizes a high sensitivity fluorescent camera scientific CMOS (sCMOS) in the proximal end. Excitation is delivered to the sample through an excitation hollow core fiber, designed for UV wavelengths. Excitation at sufficiently strong power is delivered to the sample exciting intrinsic biomarkers autofluorescence. Detection is performed through a GRIN lens at the distal end, connected to an imaging fiber bundle. 2) This "chip-on-tip" design exploits the same approach for excitation but eliminates the need of an imaging fiber bundle. Recent advancements in manufacturing technology for sCMOS sensors has increase their sensitivity up to 95% Quantum Efficiency and reduced sizes down to $1mm^2$ sensors. These sensors are sufficiently small to be installed at the distal end of the endomicroscope, maximizing signal collection.

We have prepared two future wide-field designs for our "focus" endomicroscope, utilizing a wide-field approach. The two designs are reported in Figure 11. These designs take advantage of recent technological advancements in the manufacturing of scientific CMOS sensors (sCMOS). The latest generation of sensors has reached Quantum Efficiency (QE) in detection of 95% and can be manufactured with small sizes down to $1mm^2$. This wide field approach was not available for our application in year 1 and yields high potential for application.

However, the new generation of sCMOS detectors is manufactured as a grayscale sensor, collecting indiscriminately different wavelengths. For us to pursue this approach there is a need for a simple, sensitive and snapshot approach to collect multispectral fluorescent data. There is currently no wide-field, snapshot multispectral fluorescent image camera. The hyperspectral camera we utilize for the "seek" modality works well in reflectance mode, however it does not possess sufficient sensitivity (max 14% QE) for measuring the subtle fluorescent signals.
We designed a camera which acquires HyperSpectral Phasors fluorescent data in a snapshot, high throughput and high sensitivity manner. This novel approach is shown in Figure 12 and uses custom made beamsplitters to optically apply a Sine and Cosine Fourier transform to the image, collecting a snapshot image of wavelength-continuous HyperSpectral Phasor. The sensor is divided in 4 parts for large sensors, can be adapted to utilize 4 separate small sensors.

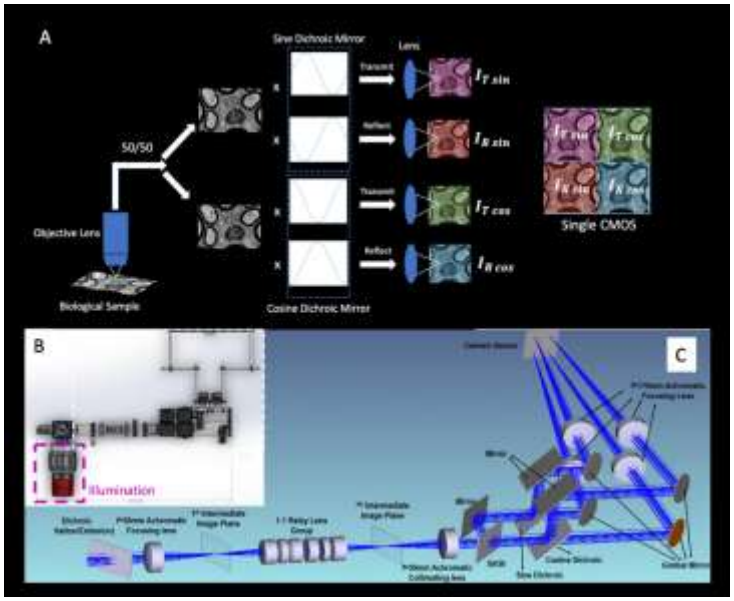


Figure 12. Design for a high-throughput, snapshot Hyperspectral Phasor Camera. A) Principle of hyperspectral phasor camera. Fluorescent signal is equally split and sent to two custom made beam splitters. These beam splitters resemble a sine and a cosine function in the range 400-700nm. This resembles the sine and cosine Fourier transforms utilized in the phasor approach. The reflected anti-sine and anti-cosine, together with sine and cosine are reimaged on a 4-way split high sensitivity sCMOS sensor. This approach has an estimated throughput of signal above 90%, collecting majority of the light. The signals from the 4 quadrants are utilized to calculate phasor coordinates and then analyzed utilizing SpectraFLIM algorithms. B) Top view of solidworks assembly. C) Zemax calculations of optics for reducing chromatic aberration.

After designing the optics, hardware, electronics and simple FPGA code we tested the HyperSpectral Phasor camera. The setup and example data are shown in Figure 13 and 14. The high sensitivity of the sensor allowed for very high speed acquisitions (8ms exposure), increasing ~20 fold the acquisition speed compared to the raster scan approach. The technology is extremely promising for application with the designs proposed in Figure 11 and is considerably less expensive than a confocal raster scan endomicroscope. We are currently preparing a manuscript and a US Provisional Patent for the technology. The current setup is still large for simplicity of alignment, however, with more advanced optical design, the whole system could be reduced to form-factor compatible with chip-on-tip endoscopy, providing the first ever multispectral fluorescence endoscope.

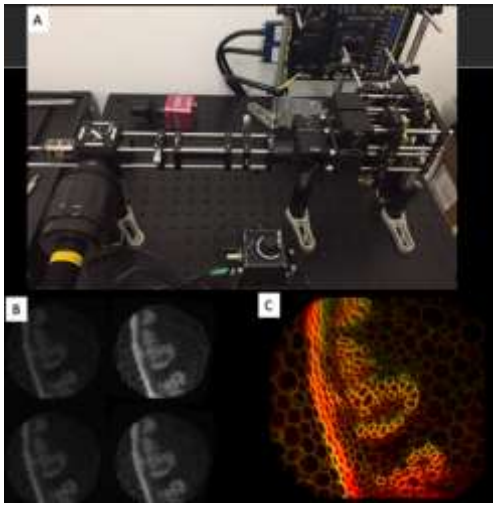
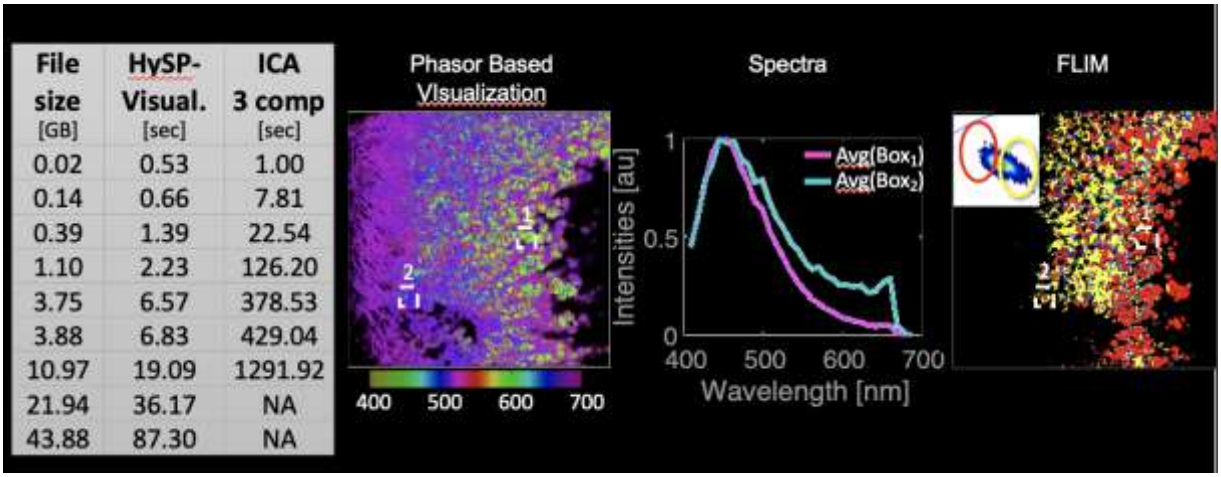


Figure 13. Testing of high-throughput, snapshot Hyperspectral Phasor Camera. A) Hyperspectral phasor camera setup. The setup is designed to collect low Signal to Noise Fluorescent spectral signals in the range of 400-700nm. B) A test sample is imaged in fluorescent mode, exposure time 8ms, the four quadrants represent sine, cosine, anti-sine and anti-cosine. The values are used for calculating a normalized Sine and Cosine Fourier Transform, basis for the phasor approach. C) The fluorescent signal is then visualized using SEER approach designed in Year 2.



File size [GB]	HySP-Visual. [sec]	ICA 3 comp [sec]
0.02	0.53	1.00
0.14	0.66	7.81
0.39	1.39	22.54
1.10	2.23	126.20
3.75	6.57	378.53
3.88	6.83	429.04
10.97	19.09	1291.92
21.94	36.17	NA
43.88	87.30	NA

Figure 14. SEER hyperspectral visualization performance. A) Comparison of SEER (HySP-Visual) with Independent Component Analysis (ICA) for different datasets size. Phasor Based Visualization. The gradient descent morphed map shows differences between apical and basal layers, suggesting different metabolic activities of cells based on the distance from the tracheal airway. Cells on the apical and basal layer (dashed boxes) are rendered with distinct color groups. Average spectra for the cells in dashed boxes (1 and 2 in panel c) show a blue spectral shift in the direction of the apical layer. (e) Fluorescence Lifetime Image Microscopy (FLIM) of the sample, acquired using a frequency domain detector validates the interpretation from SEER.

We further characterized the performance of the visualization approach we named Spectrally Encoded Enhanced Representations (SEER). Particularly we tested SEER's performance against the state-of-the-art Independent Component Analysis for autofluorescence data of mouse trachea.

4) other achievements

YEAR 1

4a) Developed a **fiber pattern correction for hyperspectral imaging** based on Delaunay triangulation. During our preliminary testing using Hyperspectral Phasors in combination with a commercial bronchoscope we observed presence of fiber patterns in the image. Multiple algorithms have been presented in literature for "color" and "monochrome" fiber pattern correction. However, no algorithm has been presented yet that can correct these fiber patterns in combination with hyperspectral data acquisition (Figure 15 (left) and 16 (below). We are currently testing the algorithm performance and limits in preparation of a scientific manuscript.

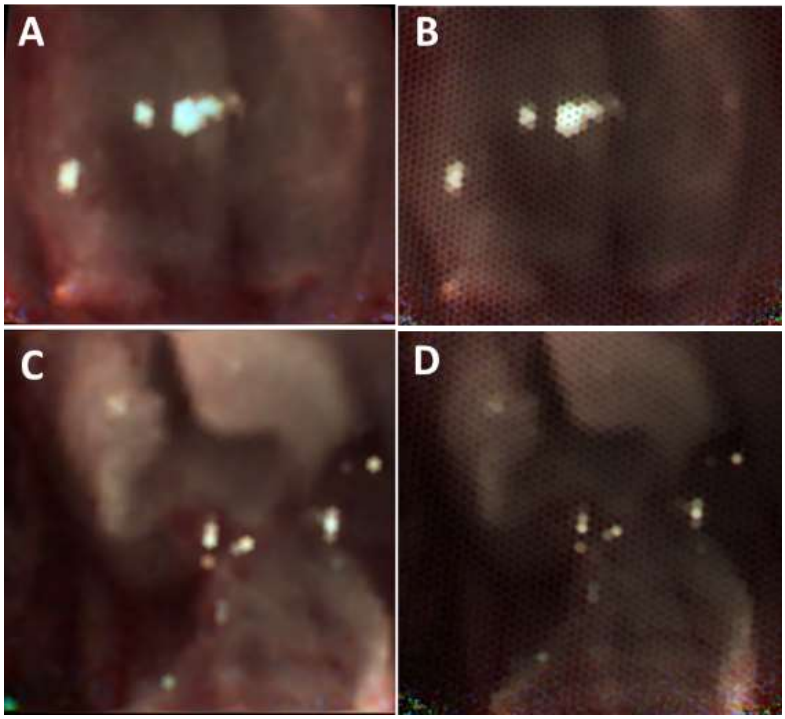


Figure 15: Hyperspectral fiber pattern correction algorithm. This preliminary result shows application of a novel algorithm that corrects patterns on the image resulting from fiber bundles (A) while maintaining good confidence on spectral information. Steps for pattern removal involve fiber detection (B), hyperspectral Delaunay triangulation (C) and corrected image (D). The advantage and novelty of this approach is the close confidence in spectral domain information when snapshot hyperspectral images are acquired. (E) shows comparison spectra acquired using a calibrated blue target. The reference calibrated spectrum is represented in (green); before fiber pattern removal data is represented in (blue). After fiber pattern removal is represented in (red). Removal process fits within 10% error of raw data, outperforming in the lower and higher wavelengths the standard and approximating values closer to the original.

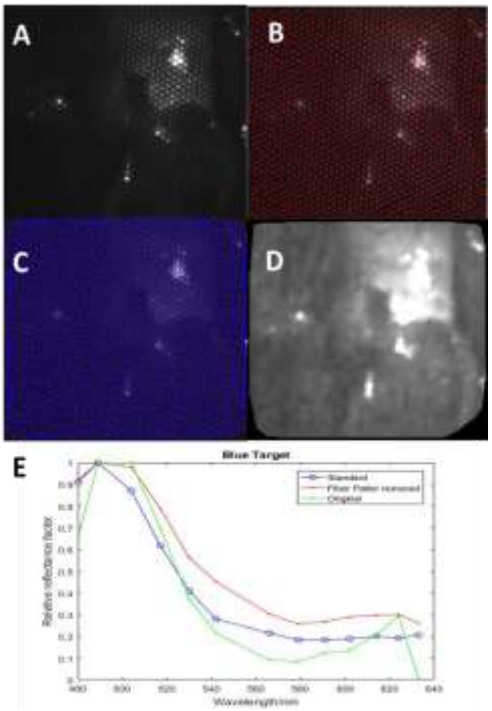


Figure 16: Example application for Hyperspectral fiber pattern correction algorithm. This example shows the result of such algorithm applied hyperspectrally and represented as a "color" image. A) corrected image of mouse airway from corresponding B) raw image with honeycomb pattern. Similarly C) shows a hyperspectrally corrected mouse trachea, reconstructed from pattern D) raw image.

4b) **Compressive spectral algorithm (Phasor-Maps)**: in order for us to understand the health status of the lungs, we need to perform accurate analysis. The *seek* and *focus* strategy places the accurate analysis in a specific region of interest identified during *seek*. However, we foresee a time limitation in how long a doctor or medic can spend seeking these regions of interest. For this purpose, we have developed a compressive spectral algorithm that represents, using special color maps (Phasor-Maps), the spectral dimension as a color. Differently from remote sensing approaches, our approach is directed at enhancing and highlighting spectral differences, from very subtle to very wide, for spectra with different Fourier phase and magnitude. A scientific manuscript about this is in preparation.

SpectraFLIM implementation on a bronchoscope has been completed at 80%. The reason behind this delay is the amount of custom components we needed for this setup. In particular fiber systems,

spectral and lifetime were designed by us and custom made at factory. Delivery is expected in approximately one month.

YEAR 2

4c) Developed a compact version of the seek modality bronchoscope which can be safely handled by a doctor. The setup has been reduced in size and stabilized for ease-of-use.
4d) We obtained encouraging results for recovery and fingerprinting of lung injuries on mouse model. The damage caused with different chemicals appears in separate distinguishable areas of the phasor, suggesting different spectral shifts during the epithelium injury.
4e) Published a novel article on Biomedical Optics Express titled " Fiber pattern removal and image reconstruction method for snapshot mosaic hyperspectral endoscopic images". The article has been already downloaded 192 times, inspired work from other groups in Cambridge (UK) and Chinese Academy of Sciences (China).
4f) Submitted a high impact article to Nature Communications titled "Visualization of hyperspectral fluorescent data with Spectrally Encoded Enhanced Representations (SEER)" which describes a powerful tool for preprocessing and visualizing with high speed and sensitivity the multimodal datasets. The article is currently in the review process, has been seen by 3 reviewers with very positive comments and minor revisions. We expect this major work to be in press by year end.

YEAR 3
4g) Published on Nature Communication an article titled "Visualization of hyperspectral fluorescent data with Spectrally Encoded Enhanced Representations (SEER)" (accepted in principle)

IMPACT

What was the impact on the development of the principal discipline(s) of the project?
YEAR 1
The main techniques that were developed in this first year of funding explore the dimension of color using hyperspectral imaging. We assembled a portable instrument capable of acquiring multidimensional images of the lungs in either humans or animals. The preliminary experiments we perform suggest that lung injuries appear to have characteristic phasor signatures depending on the chemical used and the location of lung epithelia injury. Identifying these phasor signatures will move us one step closer to noninvasively and quantitatively classifying lung injuries.
During the development of the main instrument, we also created novel algorithms aimed for biomedical imaging but, truly, applicable to a large variety of fields. Looking through a fiber bundle produces a characteristic pattern that reduces the quality of the image. Multiple approaches have been proposed in literature for correcting this pattern. However, no algorithm is available that can perform this task in hyperspectral or multi-dimensional images. We developed an algorithm that combines Delaunay triangulation, pattern removal and hyperspectral imaging that improves the quality of data while maintaining the spectral information. This work is likely to result in Intellectual Property filing, one publication is currently being drafted.
Another technical advance is a compressive algorithm for multidimensional data, that can visualize hyperspectral and lifetime datasets as a "color" image while enhancing the hyperspectral content information. This algorithm is also generic and can be applied to different fields of compressive sensing. It utilizes special color maps that evolve from Fourier-phasor approach, which we named Phasor-maps. We are preparing IP disclosure in parallel to a technical scientific manuscript.
YEAR 2
The work performed in year 2 has been inspiring for the field of multimodal endoscopy and imaging. The work published in Biomedical Optics Express received a wide interest. We were contacted by researchers at the Chinese Academy of Sciences and at the Cambridge University inquiring on the technology. The group in Cambridge has made a follow up publication confirming the efficacy of our approach compared to others.

The second publication currently under review has received positive comments during the review process. The innovation described in this work is posed to have a high impact on the community of both Spectral, Hyperspectral and FLIM.

YEAR 3

The work performed in year 3 has been focused on improving the software and endomicroscope "focus" design. The novel SEER compressive hyperspectral visualization has been impactful in the field, due to its improved performance in comparison to the state of the art. The novel hyperspectral fluorescent phasor camera is posed to be a game-changer in the field, as it is the first device of its type on the market, with sufficiently small form factor and high sensitivity to integrate with endomicroscopes.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

YEAR 1

The project is likely to produce 2 IP disclosures within the first year of funding. We anticipate interest from industry for these algorithms, particularly in the field of medical imaging. We are currently designing a business model for initiating a start-up company based on IPs translating from this project.

YEAR 2

We have disclosed two separate IPs based on the publications:

1. Wang P., Fraser S.E., Cutrale, F, Hyperspectral fiber pattern removal and reconstruction, US Patent Provisional 62/593,079
2. Spectrally encoded enhanced representations (SEER), (Shi, W., Koo, D.E.S., Fraser, S.E., Cutrale, F) US Patent pending EIR 7636101-18-0051

Patent 1 is currently being marketed by the Stevens Center at USC. It appears Olympus has expressed interest in the technology.

Patent 2 is being licensed by PhaseSpec Corp., a startup that focuses on hyperspectral analysis.

YEAR 3

We are preparing two IPs based on the current state of the project:

3. Hyperspectral Phasor Camera – in preparation
4. Method for early stage disease detection utilizing multimodal fluorescence - in preparation

What was the impact on society beyond science and technology?

Nothing to report

Animals usage

Species: Mice
Animals used: 192
Category: 70 (USDA D), 122 (USDA B, C).

CHANGES/PROBLEMS

Changes in approach and reasons for change

YEAR 1

1- SpectraFLIM implementation on a bronchoscope has been completed at 80%. The reason behind this slight delay is the amount of custom components we needed for this setup. In particular fiber systems, spectral and lifetime were designed by us and custom made at factory. Delivery is expected in approximately one month.

2- The diphtheria toxin pilot experiments have been delayed for about 2 months. This was caused by the necessity to write and obtain approval for a new IACUC protocol that allowed the transport of animals from CHLA to USC UPC. We plan to perform these tests in October-November 2017. This delay, as also explained above, won't materially affect the over-all project timing as the diphtheria toxin injury model will be used in the second part of the next reporting year (see original statement of work and Gannt chart), which will give us ample time to tune up the injury models.

YEAR 2

1- For the diphtheria toxin injury model we had to change the data collection timepoints as we discovered that this genetic injury model is not well tolerated by the animals.
2- We encountered greater than expected variability of the metabolic data across samples. From our experimental results it appears that data collection from the expired mouse is particularly time sensitive. We also believe that other variables (such as organic and inorganic contaminants, level of stress of the animals, circadian factors) may play a significant role in modulating the metabolic measurements by our instrument. We are currently working in limiting the effects of these variables in our measurements.
3- The SpectraFLIM setup had some further delays. We have measured considerable loss of two-photon excitation along the imaging fiber resulting from two factor: absorption of the imaging fiber bundle (Fujikura, Tokyo, JP) along 780nm excitation wavelength, group velocity dispersion along the fiber bundle. The use of more powerful and considerably more expensive two-photon lasers could solve this problem, however it would reduce the portability of the instrument. These factors combined complicate the use of this laser for exciting autofluorescence. We have taken two actions to overcome these limits:

- designed a portable and low cost femtosecond pulse compressor for delivering sufficient laser power to the sample
- co-excite utilizing single-photon lasers modulated at 20, 40 and 80 MHz
- utilize LED based laser sources that can be modulated at MHz range to create excitation

we are currently in the process of aligning these lasers and compressors.

YEAR 3

Switching from raster-scan to wide-field approach for "focus" modality. Reason for change is the inability of imaging fiber to deliver excitation wavelength at a sufficient strength to excite autofluorescence. Thanks to recent technological advancement in manufacturing of advanced scientific CMOS sensors, we were able to design, assemble and successfully test a hyperspectral fluorescent phasor camera capable of high-throughput, snapshot, high-sensitivity data collection, compatible with the desgins of "focus" microendoscopy in this project.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

YEAR2

Preliminary experiments with the diphtheria mouse model showed that depletion of the basal cells in the mouse is not well tolerated by the animals. Mutant animals were clearly lethargic and in pain 5 days after injection with tamoxifen. Therefore, we decided, for this injury model to collect the trachea at 1, 2, 3 and 4 days post tamoxifen injection (instead of 1, 3, 7 and 10 days, as originally planned)

PRODUCTS

Publications, conference papers, and presentations

YEAR 1

Multi-modal fluorescence as an insight tool for imaging vasculature; Francesco Cutrale, Cosimo Arnesano, Le A. Trinh, Gianluca Turcatel, David Warburton, Scott E. Fraser; Poster; Unraveling Vascular Inflammation: From Immunology to Imaging Conference; National Heart, Lung and Blood Institute, NIH, DHHS

YEAR 2

1. Enhancing visualization of hyperspectral data with Phasor-Maps; Wen Shi, Eun Koo, Le A. Trinh, Benjamin Steventon, Scott E. Fraser, Francesco Cutrale; Poster; American Society of Cell Biology (ASCB) Meeting 2017
2. SpectralFLIM imaging of the lung epithelium. Gianluca Turcatel, Francesco Cutrale, Cosimo Arnesano, Scott Fraser and David Warburton. Federation of American Societies for Experimental Biology (FASEB) conference 2018.
3. Translating multi-domain fluorescence imaging to medicine, Francesco Cutrale. Invited talk. Symposium for Micro- and Nanotechnologies for medicine: emerging frontiers and applications, (UCLA, Los Angeles) July, 2018
4. Wang P., Turcatel G., Arnesano C., Warburton D., Fraser S.E., Cutrale F#. Fiber pattern removal and image reconstruction method for snapshot mosaic hyperspectral endoscopic images. Biomed. Opt. Express 9, 780-790 (2018)
5. Shi, W.*, Koo, D.E.S.,* Kitano, M., Chiang, H.J., Trinh L.A., Turcatel, G., Steventon, B., Arnesano, C., Warburton, D., Fraser, S.E., Cutrale, F.#, Visualization of hyperspectral fluorescent data with Spectrally Encoded Enhanced Representations (SEER). Nature Communications (in review, minor edits)
6. Wang P., Fraser S.E., Cutrale, F, Hyperspectral fiber pattern removal and reconstruction, US Patent Provisional 62/593,079
7. Shi, W., Koo, D.E.S., Fraser, S.E., Cutrale, F., Spectrally encoded enhanced representations (SEER), US Patent pending EIR 7636101-18-0051

YEAR 3

1. Gianluca Turcatel, Pu Wang, Wen Shi, Cosimo Arnesano, Scott Fraser, Francesco Cutrale, David Warburton. Reflectance And SpectraFLIM Imaging of The Lung Upper Airways; Poster; Military Health System Research Symposium (MHSRS) 2019
2. Francesco Cutrale, Gianluca Turcatel, Pu Wang, Wen Shi, Cosimo Arnesano, David Warburton, Scott E. Fraser. Translating multi-domain fluorescence imaging to medicine. SPIE Photonics West BiOS 2019. (Invited Speech)
3. Wang P., Fraser S.E., Cutrale, F, Hyperspectral snapshot phasor camera - in preparation
4. Wen Shi, Daniel E.S. Koo , Masahiro Kitano, Hsiao J. Chiang, Le A. Trinh, Gianluca Turcatel, Benjamin Steventon, Cosimo Arnesano, David Warburton, Scott E. Fraser, Francesco Cutrale. "Visualization of hyperspectral fluorescent data with spectrally encoded enhanced representations" , Poster, SPIE Medical Imaging 2019 (San Diego, CA)

Journal publications.

YEAR 1

Nothing to report

YEAR 2

1. Wang P., Turcatel G., Arnesano C., Warburton D., Fraser S.E., Cutrale F#. Fiber pattern removal and image reconstruction method for snapshot mosaic hyperspectral endoscopic images. Biomed. Opt. Express 9, 780-790 (2018)
2. Shi, W.*, Koo, D.E.S.,* Kitano, M., Chiang, H.J., Trinh L.A., Turcatel, G., Steventon, B., Arnesano, C., Warburton, D., Fraser, S.E., Cutrale, F.#, Visualization of hyperspectral fluorescent data with Spectrally Encoded Enhanced Representations (SEER). Nature Communications (in review, minor edits)

YEAR 3

1. Shi, W.*, Koo, D.E.S.,* Kitano, M., Chiang, H.J., Trinh L.A., Turcatel, G., Steventon, B., Arnesano, C., Warburton, D., Fraser, S.E., Cutrale, F.#, Visualization of hyperspectral fluorescent data with

Spectrally Encoded Enhanced Representations (SEER). Nature Communications (accepted in principle)

Website(s) or other Internet site(s)

YEAR 1

Nothing to report

YEAR 2

http://bioimaging.usc.edu/software.html : the website reports the latest version of the Hyperspectral/FLIM software with the Spectrally Encoded Enhanced Representations (SEER)

YEAR 3

http://bioimaging.usc.edu/software.html : the website reports the latest updated version of the Hyperspectral/FLIM software with the Spectrally Encoded Enhanced Representations (SEER)

Technologies or techniques

YEAR 1

Nothing to report

YEAR 2

Sample preparation: metabolic imaging of the lung epithelium is time-sensitive and we established a solid protocol that improves consistency and reduces measurement biases.
Hyperspectral bronchoscopy: we have refined the acquisition, analysis, assembly and experimental design for performing hyperspectral bronchoscopy
SpectraFLIM calibration: we have designed a SpectraFLIM calibration algorithm which we are going to present in a novel publication.

Inventions, patent applications, and/or licenses

YEAR 1

The project is likely to produce 2 IP disclosures within the first year of funding. IP disclosures are under preparation for:

- hyperspectral fiber pattern removal algorithm
- compressive spectral algorithm (Phasor-maps)

YEAR 2

As we anticipated in year 1, we have disclosed 2 Intellectual Properties:

1. Wang P., Fraser S.E., Cutrale, F, Hyperspectral fiber pattern removal and reconstruction, US Patent Provisional 62/593,079
2. Shi, W., Koo, D.E.S., Fraser, S.E., Cutrale, F., Spectrally encoded enhanced representations (SEER), US Patent pending EIR 7636101-18-0051

Patent 1 is currently being marketed by the Stevens Center at USC. It appears Olympus has expressed interest in the technology.
Patent 2 is being licensed by PhaseSpec Corp., a startup that focuses on hyperspectral analysis.

Other Products

YEAR 1

During the first year of this project we report the following research tools:

Software:

- Hyperspectral fiber pattern correction: improves hyperspectral imaging when performed through a fiber bundle
- Compressive spectral algorithm (Phasor-Maps): compresses spectral information for fast visualization that enhances different types of spectral differences

Instruments or equipment:

- Seek Hyperspectral Bronchoscope: Phasor powered system capable of interfacing with existing bronchoscopes and acquiring hyperspectral reflectance data of wide areas for identifying areas of interest to be imaged with SpectraFLIM.
- Focus SpectraFLIM system: system has been designed and is in its final stage of assembly.

YEAR 2

Software:

- Hyperspectral fiber pattern analysis: corrects for distortion in spectra due to fiber light transport
- Spectrally Encoded Enhanced Representations (SEER): fast compressive approach for visualizing multimodal (spectral, FLIM) data close to real time.

Instruments or equipment:

- Seek Hyperspectral Bronchoscope: improved design, increased resistance to use and handling, improved instrument stability.

YEAR 3

Software:

- Spectrally Encoded Enhanced Representations (SEER): fast compressive approach for visualizing multimodal (spectral, FLIM) data close to real time.
- SpectraFLIM calibration algorithm for frequency domain lifetime acquisition
- SpectraFLIM analysis and quantification algorithms

Instruments or equipment:

- Focus Hyperspectral fluorescence endomicroscope: widefield design, reduced costs, form factor.
- Novel Snapshot Hyperspectral Phasor Camera: designed for very low Signal to Noise ratio, ideally fluorescence, could be applied for very low-light or high-speed acquisitions.

PARTECIPANTS AND COLLABORATING INSTITUTIONS

Site 1: Children's Hospital of Los Angeles / Saban Research Institute
Drs: David Warburton, Gianluca Turcatel and Rex Moats
Site 2: University of Southern California, Bioimaging Departments
Drs. Scott Fraser and Francesco Cutrale.

SPECIAL REPORTING REQUIRMENTS

Nothing to report

APPENDICES

References

1. Multi-modal fluorescence as an insight tool for imaging vasculature; Francesco Cutrale, Cosimo Arnesano, Le A. Trinh, Gianluca Turcatel, David Warburton, Scott E. Fraser; Poster; Unraveling Vascular Inflammation: From Immunology to Imaging Conference; National Heart, Lung and Blood Institute, NIH, DHHS

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11. Wang P., Fraser S.E., Cutrale, F, Hyperspectral snapshot phasor camera - in preparation

12. Wen Shi, Daniel E.S. Koo , Masahiro Kitano, Hsiao J. Chiang, Le A. Trinh, Gianluca Turcatel, Benjamin Steventon, Cosimo Arnesano, David Warburton, Scott E. Fraser, Francesco Cutrale. "Visualization of hyperspectral fluorescent data with spectrally encoded enhanced representations" , Poster, SPIE Medical Imaging 2019 (San Diego, CA)

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