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14. ABSTRACT
The goal of this proposal is to design a portable bronchoscope that can determine the health status of the tracheal epithelial lining cells by analyzing changes in their metabolic profile. The device is intended to be used to quickly single out which individuals is showing injury and inflammation in the trachea and lung and therefore to provide them with the most adequate and early care. In this closing year 2 funding period, we have tested and tuned up the bronchoscope we built in year 1 and we developed proprietary algorithms to speed up data acquisition and analysis. We performed extensive testing of our device on mouse trachea samples exposed to chemicals known to injure the tracheal epithelium and further confirmed that our approach can, indeed, separate injured tracheal tissues from the healthy ones without the use of additional reagents or chemicals. The size of the bronchoscope is quite small and will allow its transport and use by the military in the field or remote locations, while the reduced diameter of the instrument could permit its use on patients who are conscious. We recently submitted and IRB protocol and we plan to test this device on human subjects next year.

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

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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).

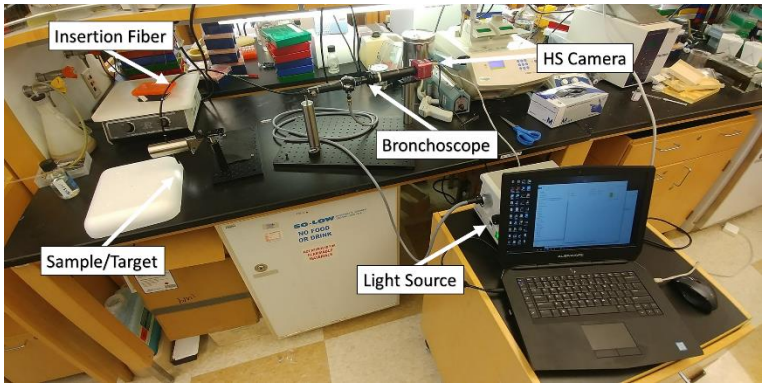


Figure 1: *Seek* modality setup for snapshot Hyperspectral Phasors reflectance. Endoscopic light source provides excitation spectrum between 400nm and 800nm. Bronchoscope fiber delivers excitation light and collects reflected light directed to a low profile hyperspectral camera mounted on distal side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

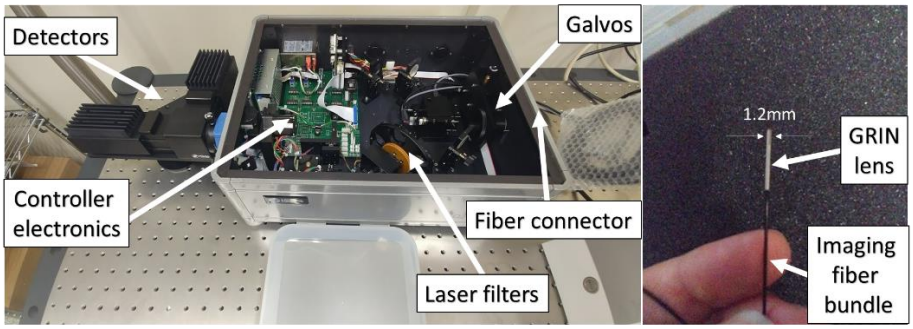


Figure 2: *Focus* modality prototype setup for SpectraFLIM measurements. Laser filters sort excitation light to be raster scanned using Galvos onto an imaging fiber bundle. This fiber bundle contains 30k fibers and is factory fused to a GRIN lens that will focus light onto the sample. The small diameter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

In YEAR 2 we have further improved the seek mode producing a more compact and resistant instrument.

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: 95%

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: 70% completed. We have:

- optimized the protocol for sample preparation. Krt5-Er-Cre/DFT-Stop-fl/+ mice were generate 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.

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.

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.

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

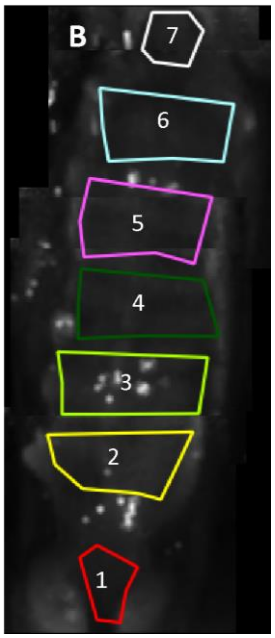


Figure 3: Preliminary results for *Focus* modality SpectraFLIM measurements. During experimental procedures we developed an analysis pipeline. Data is first acquired as separate hyperspectral images as a "fly-over" on an open mouse throat incision (A). Using the position of trachea (B,7) we create a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

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 3,4).

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.

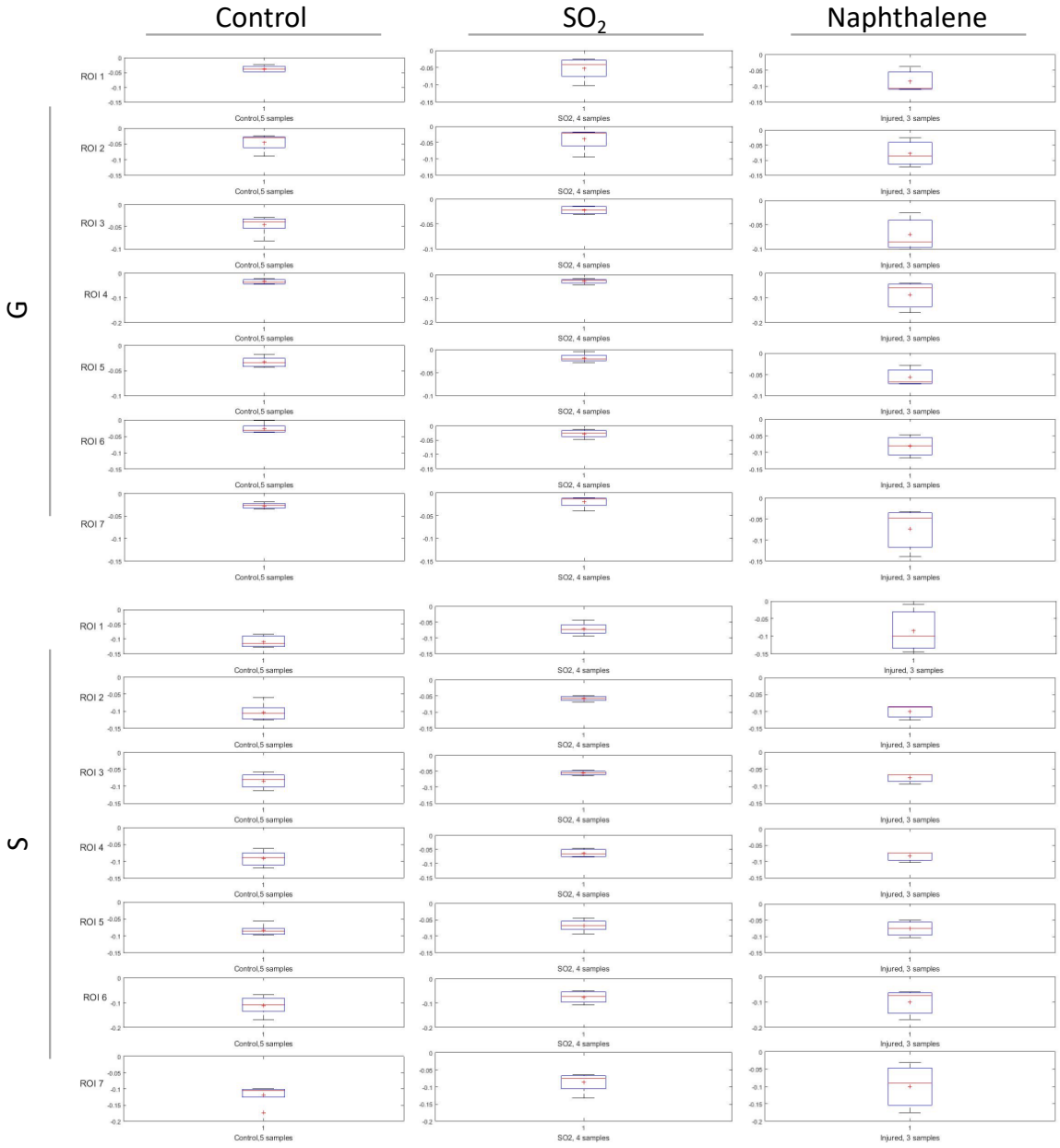


Figure 4: Preliminary results for *Focus* modality SpectraFLIM measurements. In this large map we report the preliminary results in terms of G and S coordinates of the phasor plot. The box plots represent mean (+), median (red line), minimum and max values as well as standard deviation (box) over multiple 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).

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.

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 exhibits 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 5). 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.

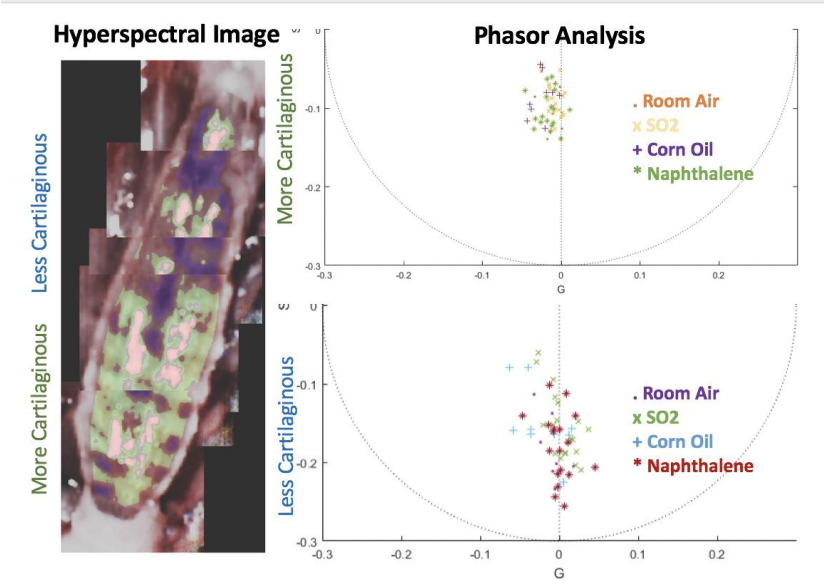


Figure 5: Phasor maps for 77 samples including controls (room air), SO2, Corn Oil, Naphthalene. The hyperspectral image is subdivided in two regions based on the amount of cartilaginous tissue. The analysis is performed separately on the two regions. The phasor plots show greater separation between injuries 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
- To account for biological variability we have developed a generalized cross-correlative approach for comparing different hyperspectral phasor datasets. The

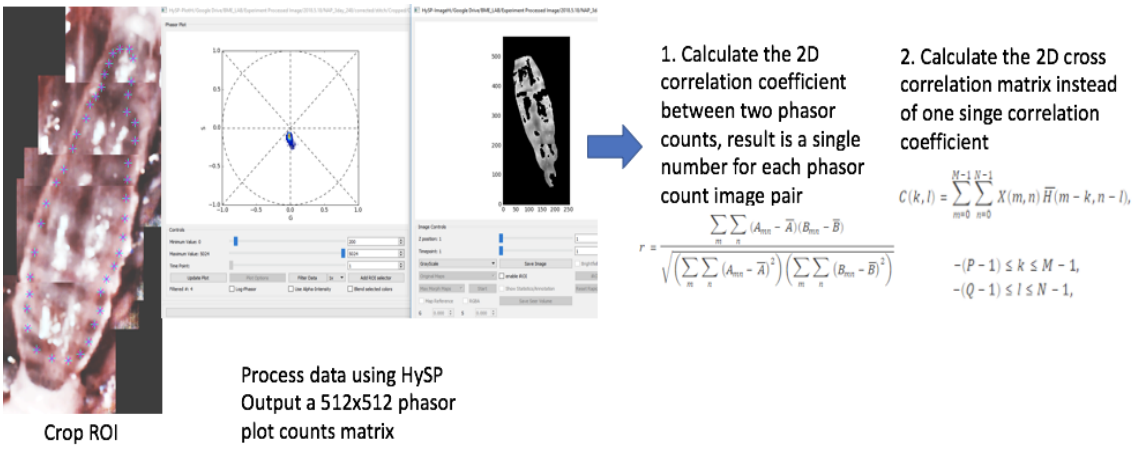


Figure 6: generalized cross-correlative approach for comparing different hyperspectral phasor datasets. Area of the book-prepped trachea is segmented and processed using the phasor approach. The resulting histogram plot represents the counts per spectrum. This plot is treated as an image and used for 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.

process is reported in figure 6. The cross correlation is performed against the average control sample, utilizing room air as a non-injury. In Figure 6 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.

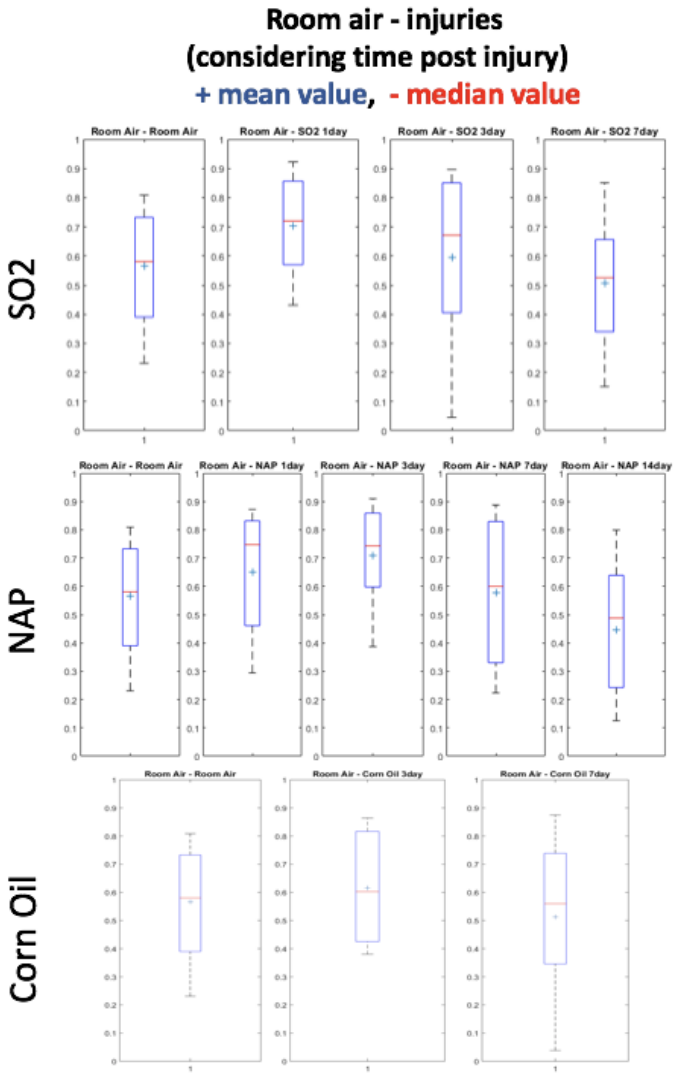


Figure 7: Phasor correlation coefficients for a set of 50 (of 77) samples. The reference value is correlation between two room air samples. The correlation is calculated between room and SO2, Naphthalene and Corn Oil. We observe a pattern in the cross-correlation, the highest difference compared to control 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.

4) other achievements

<u>YEAR 1</u>

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 5 (left) and 6 (below). We are currently testing the algorithm performance and limits in preparation of a scientific manuscript.

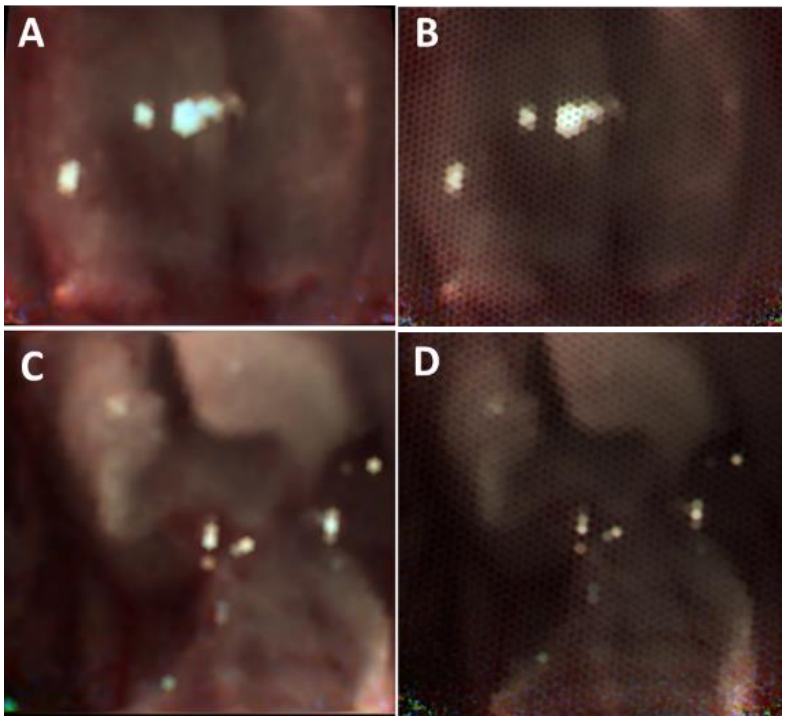


Figure 8: Hyperspectral fiber pattern correction algorithm. This preliminary result shows application of a novel algorithm that corrects patterns on the image resulting from fiber bundles (A) while maintaining good confidence on spectral information. Steps for pattern removal involve fiber detection (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.

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.

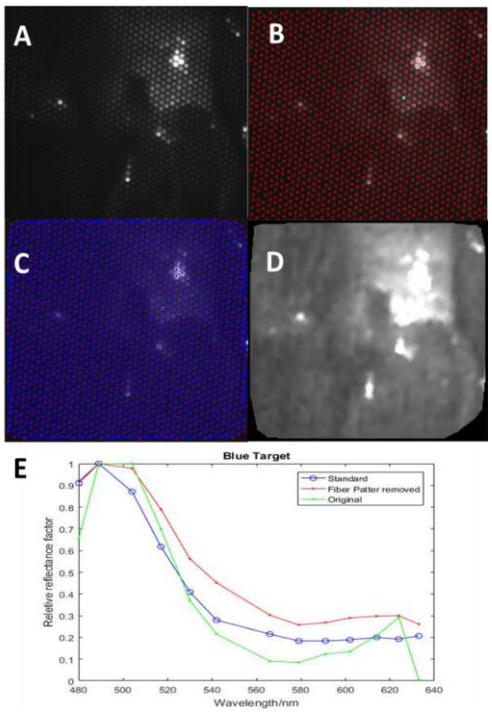


Figure 9: Example application for Hyperspectral fiber pattern correction algorithm. This example shows the result of such algorithm applied hyperspectrally and represented as a "color" image. A) corrected image of mouse airway from corresponding B) raw image with honeycomb pattern. Similarly C) shows a hyperspectrally corrected mouse trachea, reconstructed from pattern D) raw image.

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.

What opportunities for training and professional development has the project provided?

YEAR 1

This project created great opportunities for training and professional development both for key personnel and students involved in the work. Two students received mentoring, one key personnel developed mentoring skills.

One USC master student, Mr. Pu Wang, had the chance to greatly improve his knowledge in hyperspectral imaging and camera programming, as well as learning how a translational project can be designed, tested and optimized. This opportunity resulted in one novel fiber pattern correction and one manuscript in preparation.

One graduate student also received mentoring, Ms. Wen Shi, where she acquired deep knowledge of the phasor analysis and aided in the development of novel visualization algorithms and performed experiments. This mentoring resulted in one novel compressive algorithm and one manuscript in preparation.

One key personnel, Dr. Francesco Cutrale, had the chance to mentor the students and attain a greater proficiency in performing one-on-one mentoring with a clear project, target and results.

YEAR 2

The project continues creating great opportunities for training and professional development both for key personnel and students involved in the work.

Niki Noe was hired as a part time technician at CHLA and she was mentored by Dr. Turcatel and Dr. Warburton. She was in charge of the collection, storage and analysis of the trachea samples. She performed immuno-histological stainings and she has become quite familiar with the several lung injury models and the Spectra-FLIM technology. This project's training and professional development provided her the knowledge and inspiration to pursue a medical career. She successfully accessed medical school where she currently is pursuing a path to MD.

Pu Wang, USC master student involved in this project during Year 1 was inspired in pursuing a research career. He applied for graduate school and was accepted in the Biomedical Engineering program at USC. He is currently in his first year of graduate school and still expanding his knowledge on the field through this project.

Wen Shi, graduate student at USC, has developed a number of complex algorithms aimed at simplifying the analysis of multimodal datasets. The strong motivation that drives her derives from the aims of this project and the potential impact of her work on the well-being of humans.

One key personnel, Dr. Francesco Cutrale, has refined his mentoring skills with the students Pu Wang and Wen Shi above. The necessary team building and work has resulted in 2 submitted publication and 2 high impact articles. Dr. Cutrale has been promoted to Assistant Professor of Research at the Biomedical Engineering Department at USC.

How were the results disseminated to communities of interest?

YEAR 1

Results were disseminated at the "Unraveling Vascular Inflammation: From Immunology to Imaging" Conference organized by National Heart, Lung, and Blood Institute, NIH, DHHS in Bethesda, MD. During this meeting a poster was presented with preliminary results, receiving particular interest by the prevalently MD crowd.

Results were also presented at a Children's Hospital Los Angeles retreat organized in Pasadena, CA where the method and purpose in this work were reported to a mixed group of scientists and medical specialists.

YEAR 2

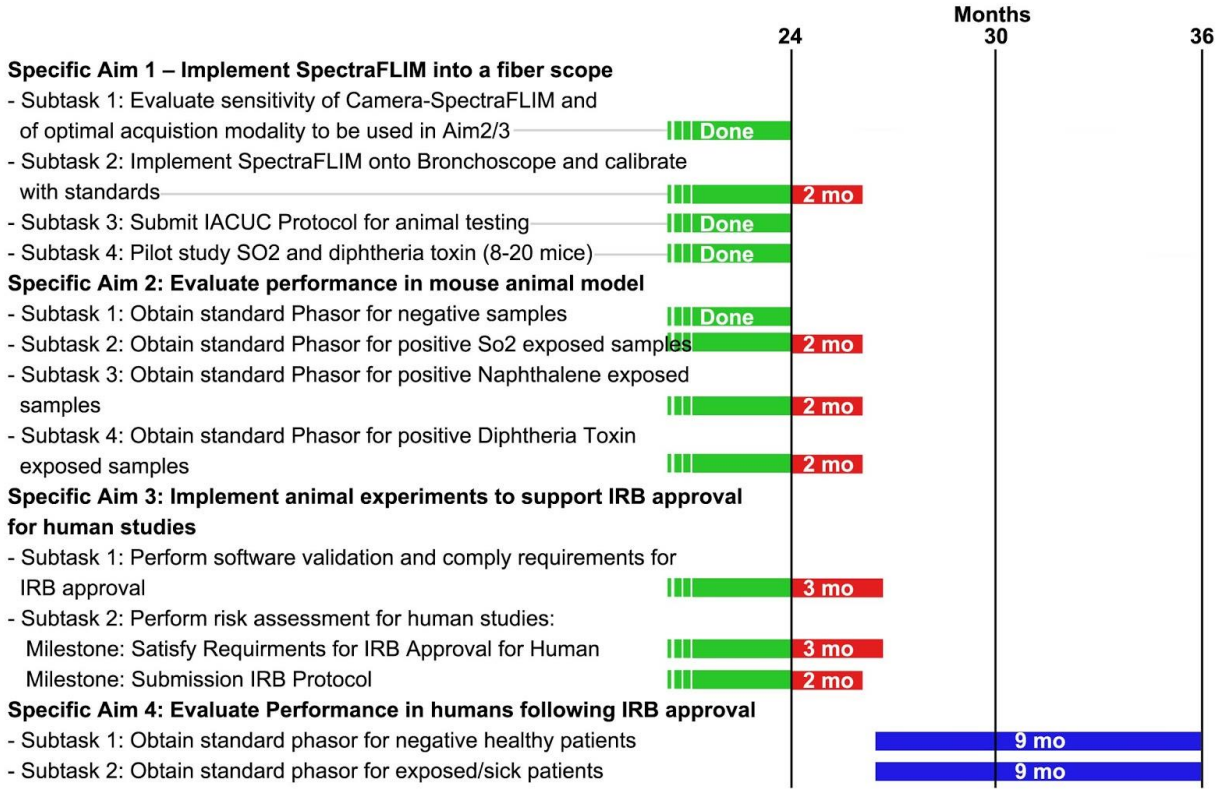
Results were divulged in the form of a poster presentation at the FASEB conference (July 2018 - St. Bonaventure University). Work was well received by the audience.

The compressive algorithm was presented in December 2017 at the American Society of Cell Biology. Industry leaders and audience expressed strong interest in the technology.

Our work was selected as an invited oral presentation for a symposium for Micro- and Nanotechnologies for medicine: emerging frontiers and applications, held at UCLA in July, 2018. The presentation was very well received by the audience, especially the MDs specializing in bronchoscopy and angiography.

What do you plan to do during the next reporting period (year-3) to accomplish the goals?

To accomplish all the goals and objectives for the next reporting period we will make sure we're on schedule with our Gantt chart (below)



More specifically we will:

- Complete the data collection and analysis for the animal studies.
- Complete tune up of SpectraFLIM instrument
- Address the comments we received for our IRB protocol.
- Utilize our instrument on a cohort of patients enrolled at the Children's Hospital of Los Angeles to assess its ability to detect metabolic alterations of the tracheal epithelium in order to reveal pathological changes of the upper airways.

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 really 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.

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.

What was the impact on society beyond science and technology?

Nothing to report

Animals usage

Species: Mice

Animals used: 150

Category: 52 (USDA D), 98 (USDA B, C).

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

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.

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

<u>YEAR 2</u>

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

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)

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers, and presentations.

Nothing to report

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)

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.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	David Warburton
Project Role:	Project Director/Principal Investigator
Researcher Identifier (ORCID ID):	0000-0002-4605-1298
Nearest person month worked:	1.20 calendar months
Contribution to Project:	Dr. Warburton is the PI of this project and is responsible for coordinating the overall project and making sure that milestones are met.
Funding Support:	DOD, NIH, CHLA

Name:	Rex Moats
Project Role:	Key Collaborator / CHLA
Researcher Identifier (ORCID ID):	0000-0002-5448-6988
Nearest person month worked:	1.20 calendar months
Contribution to Project:	Dr. Moats is responsible for coordinating the biological with the imaging aspects of the project.
Funding Support:	DOD, NIH, CHLA

Name:	Gianluca Turcatel
Project Role:	Key Collaborator / CHLA
Researcher Identifier (ORCID ID):	0000-0002-4178-5081
Nearest person month worked:	7.80 calendar months
Contribution to Project:	Dr. Turcatel wrote the mouse protocol and amendments and led the communication between CHLA and DOD regarding the use of animals for experimentation. He purchased the transgenic animals and bred them to obtain the desired genotype. He performed the genotyping of the pups for each offspring. He designed and tested the SO2 exposure chamber to selectively injure the tracheal epithelium. He performed the injury experiments (naphthalene injection and SO2 exposure), collected the tracheal tissue and performed the histological analysis. He supervised both Nikki Noe and Sue Buckley. He coordinated with Dr. Hochstim and Dr. Osterbauer the writing and approval of the IRB protocol. He contributed to the writing of two manuscripts.
Funding Support:	DOD, American Heart Association

Name:	Nikki Noe
Project Role:	Research Specialist
Researcher Identifier (ORCID ID):	0000-0000-0000-0000
Nearest person month worked:	6.00 calendar months
Contribution to Project:	Ms. Noe has contributed to the collection of the reflectance data, tissue collection, tissue embedding, paraffin block sectioning, lung sections staining and imaging.

Name:	Sue Buckley
Project Role:	Research Specialist
Researcher Identifier (ORCID ID):	0000-0000-0000-0000
Nearest person month worked:	2.40 calendar months
Contribution to Project:	Ms. Buckley has contributed to the histological analysis of the mouse tracheal samples (paraffin blocks sectioning, H&E staining, immunofluorescence staining for lung epithelial cell markers)
Funding Support:	DOD

Name:	Scott E. Fraser
Project Role:	Principal Investigator / USC
Researcher Identifier (ORCID ID):	0000-0002-5739-4026
Nearest person month worked:	1.20 calendar months
Contribution to Project:	Dr. Fraser has overseen the SpectraFLIM project, providing solutions and suggestions on innovative options for performing this Translational Microscopy. He helped designing the SpectraFLIM microscope setup, choice of hardware. He contributed designing experiments.
Funding Support:	DOD, NIH, University of Southern California internal funding

Name:	Francesco Cutrale
Project Role:	Key Collaborator / USC
Researcher Identifier (ORCID ID):	0000-0003-0517-3069
Nearest person month worked:	6.00 calendar months
Contribution to Project:	Dr. Cutrale is the expert in Hyperspectral Phasors and Multispectral Imaging Microscopy. He helped designing the SpectraFLIM microscope setup, choice of hardware and performing extensive market research for evaluating devices for hyperspectral acquisitions. He designed experiments for bronchoscope imaging and performed the experiments. He wrote software for analysis, has overseen experimental analysis

	pipeline and mentored two students on parts of this project, particularly Hyperspectral fiber pattern correction and Compressive Spectral algorithm.
Funding Support:	DOD, University of Southern California internal funding, University of Southern California Coulter Foundation.
Name:	Cosimo Arnesano
Project Role:	Key Collaborator / USC
Researcher Identifier (ORCID ID):	0000-0002-8843-2961
Nearest person month worked:	6.00 calendar months
Contribution to Project:	Dr. Arnesano is the expert in Fluorescence Lifetime Imaging Microscopy. He helped designing the SpectraFLIM microscope setup, choice of hardware and performing extensive market research for evaluating devices for FLIM acquisitions. He contributed designing experiments for bronchoscope imaging and performing the experiments.
Funding Support:	DOD, NIH, University of Southern California internal funding
Name:	Wen Shi
Project Role:	Graduate Student
Researcher Identifier (ORCID ID):	0000-0002-6624-2331
Nearest person month worked:	6.00 calendar months
Contribution to Project:	Ms. Shi has contributed coding and development for the Compressive Spectral Algorithm (Phasor Maps) under the mentorship of Dr. Cutrale. She optimized algorithms for compressive visualization of data in close-to-realtime.
Funding Support:	University of Southern California internal funding
Name:	Pu Wang
Project Role:	Master Student
Researcher Identifier (ORCID ID):	0000-0002-8664-6886
Nearest person month worked:	3.00 calendar months
Contribution to Project:	Mr. Wang has contributed coding and development for the hyperspectral camera under the mentorship of Dr. Cutrale. He contributed calibration of the bronchoscope system as well analysis of data.
Funding Support:	University of Southern California internal funding, University of Southern California Coulter Foundation, University of Southern California Viterbi Fellowship

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. David Warburton – Nothing to Report

Dr. Rex Moats – Nothing to Report

Dr. Gianluca Turcatel – Nothing to Report

Dr. Scott E. Fraser – Nothing to Report

Dr. Francesco Cutrale – Nothing to Report

Dr. Cosimo Arnesano – Nothing to Report

What other organizations were involved as partners?

USC is a leading private research university located in Los Angeles, CA since 1895. The Keck School of Medicine at USC has been affiliated with Children's Hospital Los Angeles since 1934. The faculty of USC staffs both the university and the hospital. Dr Fraser is the Provost Professor at USC. His laboratory is located in a purpose built Convergent Imaging Sciences building on the University Park Campus adjacent to the Coliseum. Dr Warburton the PI of this project is a tenured and endowed Professor of Pediatrics, Surgery and Craniofacial Biology at USC and directs a large Developmental Biology and Regenerative Medicine Research Program at the Saban Research Institute, which is located at CHLA in Hollywood, CA. These campuses of USC are connected by high bandwidth ecomms. Drs Warburton, Moats and Fraser communicate weekly either in person or on ecomms and there is a monthly in person all hands grant progress coordination meeting at Saban.

Organization Name: University of Southern California

Location of Organization: Los Angeles, CA

Partner's contribution to the project (identify one or more)

- Facilities
- Collaboration
- Personnel exchanges

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not Applicable

QUAD CHARTS: Not Applicable

APPENDICES:

Nothing to Report