AWARD NUMBER: W81XWH-17-2-0015

TITLE: Real-Time Detection of Cellular Respiratory Biomarkers of Early-Stage Infections Using Terahertz Sensing

PRINCIPAL INVESTIGATOR: Medvedev, Ivan

CONTRACTING ORGANIZATION: Wright State University

REPORT DATE: Feb 2020

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE
Feb 2020

2. REPORT TYPE
Final

3. DATES COVERED
05/01/2017 - 10/31/2019

4. TITLE AND SUBTITLE
Real-Time Detection of Cellular Respiratory Biomarkers of Early-Stage Infections Using Terahertz Sensing

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-2-0015

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)
Ivan Medvedev (PI), Katherine Excoffon (co-PI), Jennifer Martin (co-PI)

E-Mail: ivan.medvedev@wright.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Wright State University
Datyon, OH 45435

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Early detection and prevention of respiratory infections is a critical mission of military and civilian medical facilities. For example, adenoviruses are a major cause of acute respiratory illness in the military. The hypothesis that different types of cells produce unique profiles of respiratory chemicals (gaseous biomarkers) is substantiated by apparent differences in cell-specific metabolic biochemistry. The research team comprised of Drs. Ivan Medvedev (WSU-Physics), Katherine Excoffon (WSU-Biology), and Jennifer Martin (AFRL) is studying the variability of respiratory chemical profiles for a variety of cellular species, as well as a range of metabolic, homeostatic, and infectious conditions. The analysis is done using THz chemical sensing facility at WSU and Gas Chromatography-Mass Spectrometry (GC-MS) facility at AFRL. We are studying variability of respiration associated with viral infections (such as adenovirus). We are studying the effects of variability of cellular metabolism in response to varying concentrations of glucose, insulin, melatonin, and cortisol intended to simulate a range of cellular stress conditions. An exhaustive range of respiratory biomarkers from cellular models will be collected to create biomarker libraries and investigate the feasibility of using this information for pre-symptomatic detection of related pathologies, unadulterated by competing bio-processes in human body.

15. SUBJECT TERMS
Adenovirus, Virus, Infection, Cellular, Metabolism, Bio-markers, Terahertz, Spectroscopy, Breath, Analysis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	42	9

Table of Contents

	Page
1. Introduction……………………………………………………….	3
2. Keywords……………………………………………………...…..	3
3. Accomplishments………..……………………………………………	3
4. Impact…………………………..…………………………………..……	35
5. Changes/Problems...…………………………………………….………	35
6. Products…………………………………….……….……………...….	36
7. Participants & Other Collaborating Organizations……………	36

1. INTRODUCTION:

Early detection and prevention of respiratory infections is a critical mission of military and civilian medical facilities. For example, adenoviruses are a major cause of acute respiratory illness in the military. The hypothesis that different types of cells produce unique profiles of respiratory chemicals (gaseous biomarkers) is substantiated by apparent differences in cell-specific metabolic biochemistry. The research team comprised of Drs. Ivan Medvedev (WSU-Physics), Katherine Excoffon (WSU-Biology), and Jennifer Martin (AFRL) studied the variability of respiratory chemical profiles for a variety of cellular species (primary epithelial and calu-3) infected with adenovirus (AD-5). The analysis was done using Terahertz (THz) chemical sensing facility at WSU and Gas Chromatography-Mass Spectrometry (GC-MS) facility at AFRL. Based on GC-MS analyses the study has tentatively identified several gaseous compounds (4-ethyltoluene, acetophenone, styrene) as likely biomarkers of adenovirus (Ad-5) infections. THz and GC-MS sensing has identified a variance in the rate of ethanol and acetaldehyde metabolism between healthy and infected primary epithelial cells. Variability of respiration associated with the level of viral infection, cell types, across cell derived from multiple donors were studied. The study lays the foundation to investigate the feasibility of using this information for pre-symptomatic detection of related pathologies, unadulterated by competing biochemical processes in the human body.

2. **KEYWORDS:** Adenovirus, Virus, Infection, Cellular, Metabolism, Biomarkers, Terahertz, Spectroscopy, Breath, Analysis

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**

The main objective of this research program was to determine if Adenovirus infections cause detectable changes in gaseous metabolites produced by in-vitro cell cultures. The quantitative analysis of gaseous metabolites is performed using complementary Terahertz and GC-MS sensing approaches.

This objective is being reached through the following three major tasks.

Major Task 1: To grow primary airway epithelia and liver cell and collection of related Respiratory Volatile Organic Compounds

Major Task 2: Collect all relevant Terahertz spectral libraries

Major Task 3: To infect airway epithelia with adenovirus and identify unique VOC bio-signature

- **What was accomplished under these goals?**

The list of major achievements during the entire period of performance (Year 1-3) of the research program:

Culture Identifier	Cell Type	Donor	Period of performance	Gaseous Analysis	Multiplicity of Infection	Exposure system	Condition
Cp0	primary	D1	Year 1	THz	10	custom	inf
Cp1	primary	D1	Year 2	THz	100	custom	inf
Cp2	primary	D1	Year 2	THz, GC-MS	100	custom	inf
Cp3	primary	D1	Year 2	THz	100	custom	inf
Cp4	primary	D1	Year 2	THz, GC-MS	100	Cultex	inf
Cp5	primary	D1	Year 3	GC-MS	1000	custom	inf
Cp6	primary	D1	Year 3	GC-MS	1000	custom	inf
Cp7	primary	D2	Year 3	GC-MS	1000	custom	inf
Cp8	primary	D2	Year 3	GC-MS	1000	custom	inf
Cp9	primary	D3	Year 3	GC-MS	1000	custom	ctrl, inf
Cp10	primary	D3	Year 3	GC-MS	1000	custom	ctrl, inf
Cp11	primary	D3	Year 3	GC-MS	1000	custom	ctrl, inf
Cp12	primary	D3	Year 3	GC-MS	1000	custom	ctrl, inf
Cc13	Calu-3	-	Year 3	GC-MS	1000	custom	inf
Cc14	Calu-3	-	Year 3	GC-MS	1000	custom	inf
Cc15	Calu-3	-	Year 3	GC-MS	1000	custom	inf
Cc16	Calu-3	-	Year 3	GC-MS	1000	custom	inf
Cc17	Calu-3	-	Year 3	GC-MS	1000	custom	inf
Cc18	Calu-3	-	Year 3	GC-MS	1000	custom	inf

Table 1. Summary of cell types, methods of analysis, multiplicity of infection, exposure system used (Cultex – Figure 2, custom – Figure 3) and condition (ctrl – control, inf – infected) for cell cultures grown and infected in this research project.

1. In Year 1 the primary focus was to establish the experimental facility to collect gaseous metabolites produced by human cell cultures. A commercial gas collection system (Cultex – shown in Figure 2) was purchased and modified to allow the collection of cellular metabolites. In experiments the system was deemed rather prone to bacterial contaminations, thus we resorted to design a custom bio-exposure system (Figure 3) which was used in most of our experiments in Years 2 and 3.
2. In Year 2 we studied biosignatures of 3 independent cultures (referred to as Cp1 - 3 in what follows) of human primary epithelial cell (each growth cycle lasting 4-6 weeks) using THz sensing and 2 culture (Cp2, Cp4) using GC-MS sensing. All cultures were derived from Donor 1.
3. In Year 3 we studied 2 cell cultures derived from Donor 1 (Cp5, Cp6), 2 cell cultures (Cp7-8) derived from Donor 2, 4 cell cultures derived from Donor 3 (Cp9-12), and 6 cultures Calu-3 cancerous lung cell (Cc13-18). All analyses were done using GC-MS sensing at AFRL.
4. In Year 1 using THz sensing we tentatively determined that primary epithelial cell (Cp0) gaseous metabolites manifested systematic trends in ethanol and acetaldehyde concentrations. Year 1

results shown in Figure 4 exhibit rather high concentration of ethanol (~1 ppm), which was traced in Year 2 to a contamination associated with the use of ethanol as a cleaning agent in the incubation facility. In Years 2-3 we took extra precautions to minimize this contamination. All cell cultures were handled in a dedicated fume hood with hydrogen peroxide used as a cleaning agent.

5. In Year 2 four successfully grown cell cultures we infected with adenovirus. These are referred to as Cp1-4 in what follows. The multiplicity of infection used in Year 2 runs was increased to 100 from 10 used in Year 1.
6. In Year 3 multiplicity of infection was further increased to 1000 for cultures Cp5-12, Cc13-18.
7. To better differentiate gaseous metabolites associated with viral infections we sampled same culture as ‘control’ and ‘infected’ during consecutive weeks to look for biomarkers of adenovirus infection (see Table 2 for a typical schedule of sample acquisition).
8. Gaseous metabolites for cultures Cp1-4 were studied with THz sensing.
9. Gaseous metabolites of Cp2 for infected and control cells at 48 hours after infection were analyzed with GC-MS instruments at AFRL chemical sensing facility.
10. Gaseous metabolites for Cp4 infected cells were analyzed with GC-MS instruments at AFRL chemical sensing facility. Six samples were studied: 1. Pre-infected cells; 2. Stock media; 3. 4-hours after infection; 4. 24-hours after infection; 5. 48-hours after infection; 6. 72-hours after infection.

Time relative to infection in hours	Sample
-24	Cell Media – sample 1
-22	Cell Media – sample 2
-21	Cell baseline – sample 1
-20	Cell baseline – sample 2
2	Cell - 2h
4	Cell - 4h
10	Cell - 10h
24	Cell - 24h
34	Cell - 34h
48	Cell 1 - 48h
72	Cell 1 - 72h
144	Cell 1 - 144h

Table 2. Representative timeline of sample acquisition for cultures Cp5-12 and Cc13-18 for infection and control runs. Time is listed relative to infection at 0h.

11. Gaseous metabolites for Cp5-12 and Cc13-18 infected/control cells were analyzed with GC-MS instruments at AFRL chemical sensing facility. For each culture 12 samples were studied. Table 2 summarizes the schedule of sample acquisition relative to infection time point. The 'control' run for cultures Cp9-12 was taken the week prior to infection and followed the schedule shown in Table 2. At the end of a control run cells were checked for wellbeing and placed into new media at the end of the control week run, which was followed with an infection run the next week. In total 180 samples were collected in Year 3.

12. THz analyses of gaseous metabolites revealed systematic decrease of ethanol and acetaldehyde concentrations along the timeline of the experiment. Both control and infected cells manifest this effect. The initial concentration of ethanol and acetaldehyde is thought to be present in the cell nutritional media. The concentration of ethanol and acetaldehyde is gradually decreasing (Figure 8) due to evaporation and possible metabolic consumption/production by cells. We detected a slight difference between concentration decay time constant for ethanol and acetaldehyde between control and infected cells (Figure 11). The statistically significant margin of variance can be due to metabolic differences between control and infected cells, but the magnitude of the difference does not allow us to state with confidence that ethanol and acetaldehyde can be used as biomarkers of viral infection.

13. Primary component analysis of concentrations obtained with GC-MS sensing for cultures Cp5-12 and Cc13-18 identified Acetophenone, 4-Ethyltoluene, Styrene as likely biomarkers of adenovirus infection that allowed us to differentiate infected primary cells with specificity of ~90% and sensitivity of 80% (Figures 25,26). In addition, these chemicals differentiate infected and control calu-3 cultures Cc13-18 from the primary cells. The size of the data set (180 samples) as well as the redundancy of sampling across cell types and donors make this chemical set a likely biomarker of adenovirus infection in primary epithelial cells.

Goals not met in the during period of performance:

The original proposal called for a study of cellular biomarkers in response to varying concentrations of glucose, insulin, melatonin, and cortisol intended to simulate a range of cellular stress. We also intended to develop additional THz spectral libraries for the chemicals deemed as biomarkers of viral infections by means of GC-MS sensing, to facilitate the adoption of THz sensing towards biosensing. Due to

unforeseen and significant reduction of the number of researchers working on the project at WSU in Year 3 we did not have sufficient resources to complete these two tasks.

A detailed account of our achievements and approaches follows.

Biocontainment/exposure system utilized for gaseous sample collection.

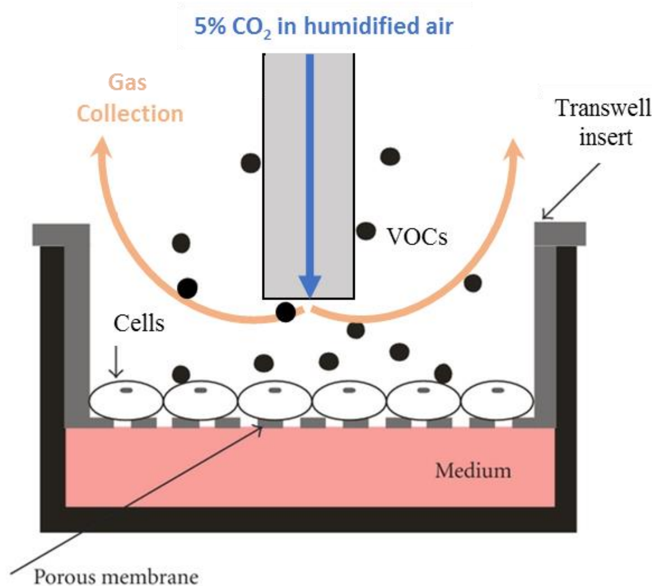


Figure 1. In-vitro THz identification of biomarkers of adenovirus. Organotypic cell cultures grown on a semi-permeable membrane inside a transwell insert and placed at the air-liquid interface (ALI).

Figure 1 shows our approaches to collection of metabolic VOCs from in-vitro cell cultures. In this project we focused on the development of an experimental system capable of collecting metabolic VOCs from organotypic cell cultures grown at the air-liquid interface (ALI) shown in Figure 1. Cells are grown on top of semi-permeable membrane located at the bottom of a transwell. The transwell is placed on top of the medium. Nutrients from the medium are delivered to cell cultures through the membrane, but the medium surface is not directly exposed to the flow of buffer gas (in our case we use 5% CO_2 in humidified air). When cell cultures are fully polarized permeation of VOCs dissolved in medium is further reduced. This facilitates selective collection of metabolic VOCs from cell cultures. Buffer gas has two roles: 1) it creates the optimum environment for cell wellbeing (for epithelial lung cells it mimics gas composition in human lungs); 2) it transports metabolic VOCs to gas collection hardware. In our case we used Tedlar bags and sorbent tubes containing Tenax TA for gas sampling, both certified by EPA for applications related to

sampling and analysis of environmental gas mixtures. Tedlar bags are routinely used in PI's laboratory for collection and THz analysis of human breath and have proven as a robust method of breath storage, with reliable sample retention up to 72 hours after sample collection.

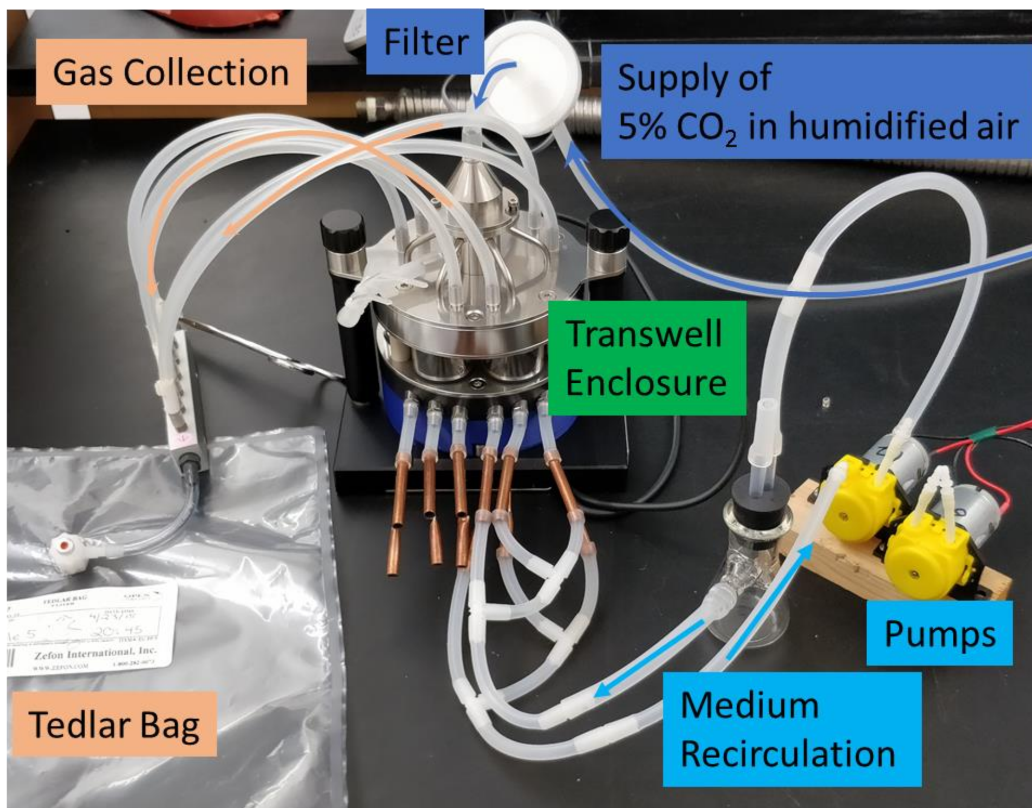


Figure 2. Cell exposure and gas collection system. Cultex exposure system shown in the center, is capable of housing 6 transwell insert. In Blue – A mixture of 5% CO_2 in humidified air flows through Cultex from a pressurized tank at a rate of 10 cc/min controlled by a flow mass controller (not shown in figure). In Orange – Buffer gas enriched with metabolic VOCs is collected into a Tedlar bag. In Cyan - Nutritional medium is recirculated by a water pump. The set-up shown provides medium recirculation to 3 out 6 enclosures.

Figure 2 shows the modified Cultex cell exposure system procured during this research. Figure 2 shows the custom media recirculation system comprised of 2 peristaltic pumps (shown in yellow), a Buchner flask for medium storage, and custom copper adapters to implement nested tubing for supply and return of media to individual chambers through a single inlet port. A custom base for Cultex system was 3-D printed (blue bottom of Cultex visible in Figures 2 and 3) to accommodate the additional tubing for the recirculation system. Cultex system has proven to be hard to clean, with and without modification, and has resulted in bacterial contamination in some of the cell growth/infection runs. Cultex system was used in collecting samples for culture Cp4 (Table 1) with minimal contamination but was not used in following data collections out of fear of bacterial contaminations.

The results presented in this report for cultures Cp1-3, Cp 5-12, Cc13-18 have been obtained using a custom-built exposure system shown in Figure 3. The system shown in Figure 3 allowed us to sample gases from cell cultures with greater surface area. The system consists of an aluminum base with a 4 inch opening for dishes with cell cultures. An air-tight lid has ports for buffer gas supply and return. The system is temperature controlled at 37 degrees Celsius by means of two heaters controlled by two Omega temperature controllers, with temperature readout provided by two thermocouples. The heaters and thermocouples are placed on two sides of the well for better temperature uniformity.

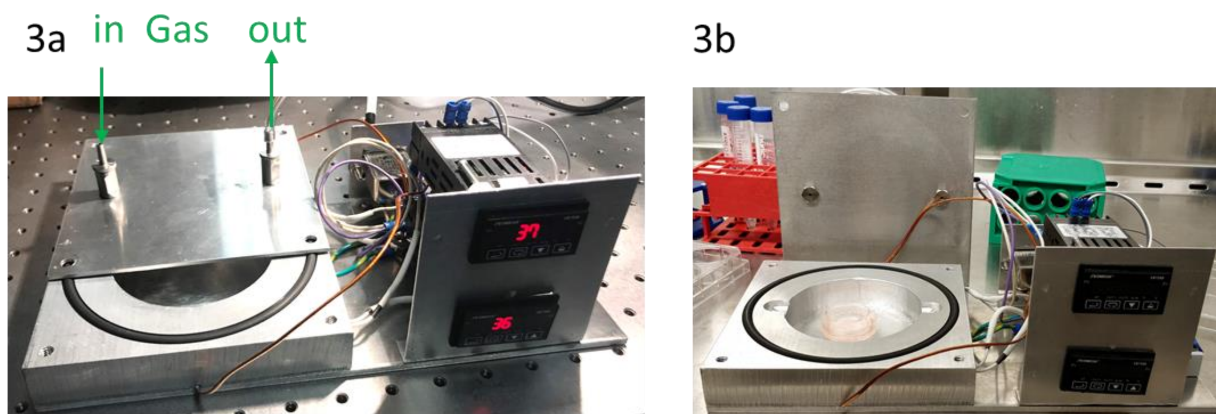


Figure 3. Custom bio-containment/exposure system designed to collect gaseous VOCs from cell cultures. 3a - Bio-containment/exposure system is temperature stabilized at 37°C. 4b - shows dish containing a 2" transwell with medium.

The results presented below for cultures Cp5-12, Cc13-18 were obtained with cells placed into a 2" transwell made of polystyrene inside the custom exposure system shown in Figure 3. The cells were grown on a semi-permeable membrane made out of polycarbonate which facilitated medium isolation from the buffer gas stream.

Primary Epithelia Cell Culture Growth

The growth of primary human airway requires feeder fibroblast irradiated cells and a unique cocktail of nutrients (medium). The production of sufficient number of feeder fibroblasts and primary human airway epithelial cells requires 4-6 weeks for each run. At that time primary cells can be seeded in transwells as described below and polarized for a minimum of 7 days prior to experiment.

For primary cultures Cp1-3 three million primary human airway epithelial cells from healthy donors were seeded onto transwells (3 cm diameter, 0.4 um pore size). Cells were allowed to polarize over 7-14 days

by incubating at 37ºC with humidity of 5% CO_2 and polarization medium (1:1 mix of Dulbecco's modified Eagle's medium (DMEM) and F12 medium, 2% Ultroser G serum substitute, 1% penicillin-streptomycin) on the basolateral surface. Medium was changed every 2-3 days. This resulted in polarized epithelia containing roughly 1.4 million cells. Epithelia were infected with wild-type human adenovirus (AdV5), suspended in phosphate buffered saline, at multiplicity of infection of 100 plaque forming units per cell for 2 hours at the apical and basolateral surfaces of the polarized epithelium. During this infection period, baseline gaseous sampling of the medium was performed in order to obtain a profile of the medium for background subtraction. Epithelia were then returned to the medium and gaseous samples were collected from uninfected control epithelia and infected epithelia at 4, 24, 48, and 72 hours after the end of mock (control) or viral exposure. Gaseous sampling of the conditioned media was performed along the timeline of the experiment in order to obtain a profile of the medium post-infection for background subtraction. THz analyses of gaseous samples for control and infected cell/media are shown in Figures 7,8, and 9. The 48h sample for culture Cp2 was analyzed with GC-MS with results shown in Figure 20.

For primary culture Cp4 one million primary human airway epithelial cells from healthy donors were seeded onto transwells (0.6 cm diameter, 0.4 um pore size). Cells were allowed to polarize over 7 days by incubating at 37ºC with humidity of %5 CO_2 and polarization medium (1:1 mix of Dulbecco's modified Eagle's medium (DMEM) and F12 medium, 2% Ultroser G serum substitute, 1% penicillin-streptomycin) on the basolateral surface. Medium was changed every 2-3 days. This resulted in polarized epithelia containing roughly .8 million cells. Epithelia were infected with wild-type human adenovirus (AdV5), suspended in phosphate buffered saline, at multiplicity of infection of 100 plaque forming units per cell for 2 hours at the apical and basolateral surfaces of the polarized epithelium. During this infection period, baseline gaseous sampling of the medium was performed in order to obtain a profile of the medium for background subtraction. Epithelia were then returned to the medium and gaseous samples were collected from uninfected control epithelia and infected epithelia 4, 24, 48, and 72 hours after the end of mock (control) or viral exposure. Gaseous sampling of the conditioned media was performed along the timeline of the experiment in order to obtain a profile of the medium post-infection for background subtraction. THz analyses of gaseous samples for control and infected cell/media are shown in Figure 12. The 4, 24, 48, and 72 hours samples for culture Cp4 were analyzed with GC-MS with results shown in Figures 20.

For primary culture Cp5-6, primary human airway epithelial cells from healthy donors were seeded onto transwells (0.6 cm diameter, 0.4 um pore size). Cells were allowed to polarize over 7 days by incubating at 37ºC with humidity of %5 CO_2 and polarization medium (1:1 mix of Dulbecco's modified Eagle's medium (DMEM) and F12 medium, 2% Ultroser G serum substitute, 1% penicillin-streptomycin) on the basolateral surface. Medium was changed every 2-3 days. The polarized epithelial were infected via apical and basolateral transduction with wild-type human adenovirus (AdV5), suspended in eagle's minimum essential media (EMEM), at a multiplicity of infection of 1000 for 1hour.

For calu-3 culture Cp7-12, primary human airway epithelial cells from healthy donors were seeded onto transwells (0.6 cm diameter, 0.4 um pore size). Cells were allowed to polarize over 7 days by incubation at 37ºC with humidity of %5 CO_2 and polarization medium (1:1 mix of Dulbecco's modified Eagle's medium (DMEM) and F12 medium, 2% Ultroser G serum substitute, 1% penicillin-streptomycin) on the basolateral surface. Medium was changed every 2-3 days. The polarized epithelial were infected via apical and basolateral transduction with wild-type human adenovirus (AdV5), suspended in minimum essential media (MEM), at a multiplicity of infection of 1000 for 1hour.

For culture Cc13-18, .4 million Calu-3 cells were seeded onto transwells (0.6cm diameter, 0.4 um pore size). Cells were allowed to polarize over 10-14 days by incubating at 37ºC with humidity of %5 CO_2 and polarization medium (Roswell Park Memorial Institute 1640 medium (RPMI) supplemented with 10% Fetal Bovine Serum (FBS) and .5% penicillin-streptomycin) on the basolateral surface. The medium was changed every 2-3 days. The polarized epithelial were infected via apical and basolateral transduction with wild-type human adenovirus (AdV5), suspended in minimum essential media (MEM), at a multiplicity of infection of 1000 for 1hour

Terahertz Molecular Sensing

A novel Terahertz gas sensor utilized in this program for chemical analysis has been recently developed in the PI's laboratory with funding from AFOSR[1] (Figure 4). It can detect a wide range of gaseous

---

[1] "Terahertz Spectroscopic Chemical Sensor for Analysis of Fatigued Human Breath", STTR Phase II, Air Force Research Laboratory, FA8650-16-C-6693

chemicals at a part per billion to part per trillion level of dilution. The distinct advantage of this technology is the near ‘absolute’ specificity (extremely low probability of a false alarm) stemming from the highly redundant spectroscopic signatures being used for chemical detection.

THz spectral range (millimeter/sub-millimeter) spans frequencies 10^{11} Hz (100 GHz) – $2{\cdot}10^{12}$ (2 THz) and lies just above the band used by cell phones and radars and below far infrared spectral range. The potential of the THz range for analytical gaseous sensing has long been recognized[2] but wide adoption of THz sensors for this application has been precluded by the lack of convenient and robust radiation sources and detectors. Recent advancement in semiconductor devices facilitated the development of now commercially available THz components.

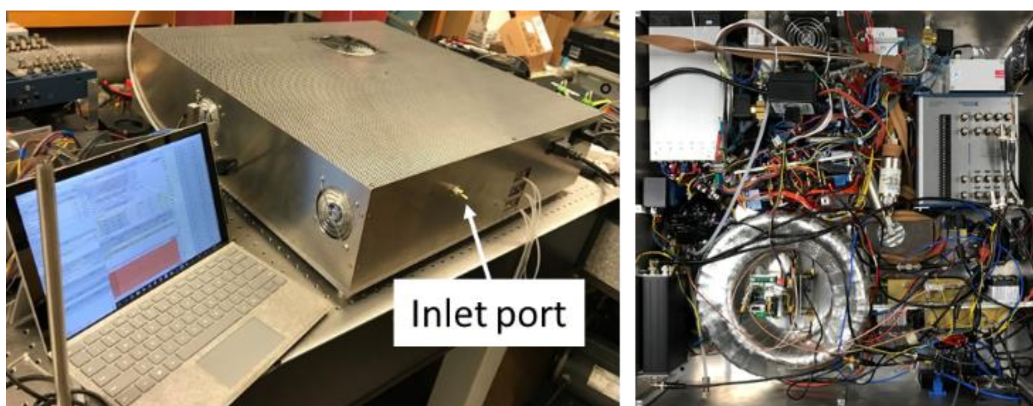
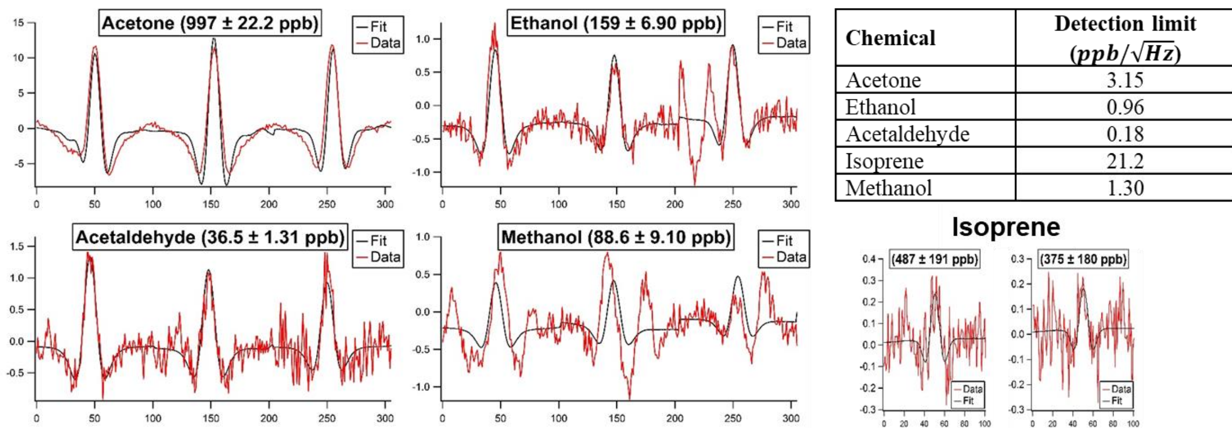


Figure 4. Air Force funded tabletop THz chemical sensor (28” x 27” x 7”) operated by an external table computer (left). Top view of the sensor components (right)

Light (<100 Dalton**)** polar species (molecules with a permanent electric or magnetic dipole moment) such as formaldehyde, acetone, ethanol, methanol, acetaldehyde, methyl cyanide, isoprene, hydrogen cyanide, hydrazine, toluene, acrolein are detectable with THz sensing with molecule specific detection limits spanning part per billion (ppb) to part per trillion (ppt) level of dilution (Figure 5). The complete list contains thousands of volatiles most of which are on various lists of Toxic Industrial Compounds with varying levels of toxicity.

[2] Townes, C.H., and Schawlow, A.L. (1955). "Microwave Spectroscopy" (New York, McGraw-Hill Dover Publications, Inc.)



Chemical	Detection limit ($ppb/\sqrt{Hz}$)
Acetone	3.15
Ethanol	0.96
Acetaldehyde	0.18
Isoprene	21.2
Methanol	1.30

Figure 5. Breath analysis with THz table top sensor. (left) typical spectra of breath acetone, ethanol, acetaldehyde, methanol, and isoprene obtained from analysis of 0.5 liter of breath in under 10 minutes (10 ms of integraton per spectal data point). (right) THz sensor detection limits for these five chemicals in $ppb/\sqrt{Hz}$ (1 s of integration per spectral data point)

***The PI's group at WSU has recently developed a Terahertz (THz) gas sensor funded through an AFOSR Phase II STTR contract*[3]**. The THz sensor shown in Figure 4, has been recently delivered to the Wright-Patterson Air Force Base. The sensor utilizes molecular rotational spectra for chemical detection. Spectral transition frequencies are mainly a function of molecular moments of inertia, do not depend on temperatures, and are known to a very high level of precision. The line shapes contain information about sample temperature and concentration. In the THz spectral range, Doppler broadening starts to dominate spectral line shapes at pressures below 10^{-5} atm, when contribution from collisional broadening becomes less significant. When in the Doppler regime, the peak intensities of the spectral line are proportional to the concentration of the chemicals in the path of THz beam. Doppler line broadening depends linearly on frequency of the spectroscopic transition. Thus, optical sensors operating at higher spectral frequencies (infrared and visible) will generally have fewer detection channels per unit bandwidth when compared to THz sensors, resulting in an overall lower specificity of chemical identification. A typical THz spectrometer covers over 100 GHz of spectral bandwidth, thus facilitating sensors with 10^5-10^6 Doppler limited spectral channels. Due to this advantage, THz sensing has been used for chemical detection with 'near absolute'

[3] "Terahertz Spectroscopic Chemical Sensor for Analysis of Fatigued Human Breath", STTR Phase II, Air Force Research Laboratory, FA8650-16-C-6693

specificity in mixtures containing over 30 analytes[4,5]. In addition, THz spectra are exquisitely sensitive to isotopic and conformational changes of molecular structure, making THz sensors a potent tool for molecular identification.

Figure 5 shows molecular rotational spectra recorded with the tabletop THz chemical sensor during a 10-minute analysis of 0.5L breath sample. Five chemicals shown in Figure 5 were detected at levels ranging between 30 to 1000 ppb. The detection limits listed are shown on the right side of Figure 5 and range between 180 ppt for acetaldehyde to 21 ppb for isoprene. Figure 5 demonstrates the capabilities of THz sensing towards quantitative breath analysis and shows that ***detection limits are molecule dependent***. The recently developed THz tabletop system is a major development over our earlier sensor designs[6], as it incorporates a robust preconcentration module, capable of autonomous gas sampling and conditioning of sorbent tubes. It is that feature that makes this system a truly analytical device, capable of sampling a wide range of gaseous chemicals. It can do so with 'near absolute' specificity. It matches the sensitivity of GC-MS and can detect molecular species a *ppt-ppb* level of dilution. THz molecular sensing can be considered complementary to GC-MS in its ability to quantify gaseous mixtures of lighter gases. This THz system is a major step in advancing THz chemical sensing towards handheld and low-cost THz gas sensors.

THz sensing of gaseous biomarkers of infected primary epithelial for cultures Cp0-4

Figure 6 shows concentrations of gaseous samples produced by primary epithelial cells determined with THz sensing at WSU. The concentrations of ethanol and acetaldehyde appear to increase and peak at 24h after infection by adenovirus. The high concentration of ethanol (~1 ppm) exceeds the usual abundance of ethanol in human breath, which made us think that the most likely source of ethanol is an external contamination, as ethanol is used as a cleaning agent in biology experiments. In later experiments extra precautions were taken to minimize this contamination. All cell growth and manipulation hardware was moved to a separate hood and hydrogen peroxide was used as cleaning

---

[4] Medvedev, I.R., Neese, C., Plummer, G.M., and De Lucia, F.C. (2010). "Submillimeter spectroscopy for chemical analysis with absolute specificity". OPTICS LETTERS 35, 1533-1535.
[5] Neese, C.F., Medvedev, I.R., Plummer, G.M., Frank, A.J., Ball, C.D., and De Lucia, F.C. (2012). "A Compact Submillimeter/Terahertz Gas Sensor with Efficient Gas Collection, Preconcentration, and ppt Sensitivity". Sensors Journal, IEEE 12, 2565 - 2574
[6] Fosnight, A.M., Moran, B.L., and Medvedev, I.R. (2013). "Chemical analysis of exhaled human breath using a terahertz spectroscopic approach". Applied Physics Letters 103, 133703-133705.

agent. This yielded good results, as detected ethanol and acetaldehyde levels dropped significantly. Figures 7-9 show much lower levels of ethanol.

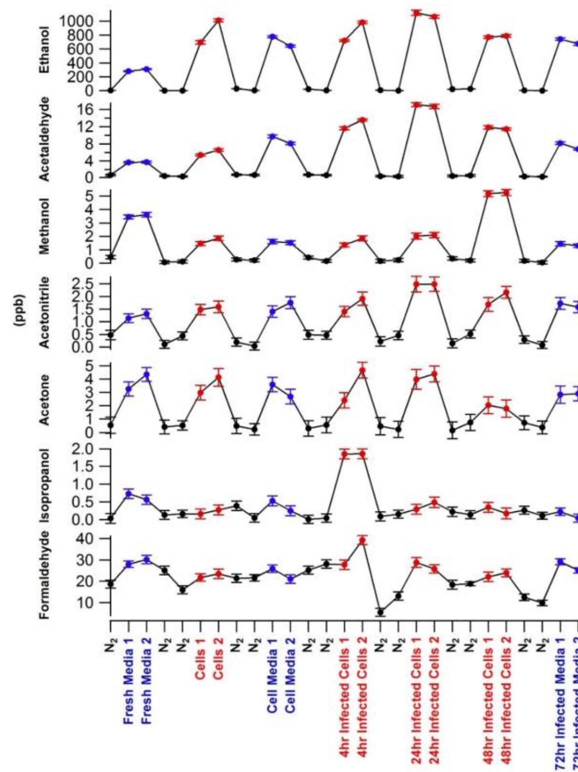


Figure 6. THz quantitative analyses of gaseous samples collected from a sample of healthy Primary human epithelial cell cultures in Year 1 (culture Cp0). Blue markers correspond to composition of media. Black markers correspond to composition of Tedlar bags filled with pure nitrogen. Red markers correspond to VOCs detected in presence of cell cultures. Consecutive unicolor markers shown correspond to sampling from the same Tedlar bag. All concentrations are measured in parts per billion.

In Year 2 we conducted 4 successful cycles of primary human epithelial cell growth and infection (cultures Cp1-4) referred. For cultures Cp1-3 gaseous metabolites were collected using custom biocontainment/exposure system shown in Figure 3. For culture Cp4 gaseous metabolites were collected using Cultex system shown in Figure 2.

Figures 7,8 and 9 show concentrations ethanol, acetaldehyde, acetone, methanol, methyl cyanide (also known as acetonitrile), isopropanol, n-propanol, and formaldehyde determined with THz sensing. For each time point we measured gaseous dilutions for cells and media in which cell resided (conditioned media). For conditioned media we removed the cells and collected gaseous samples evaporated by the media itself in absence of cell cultures.

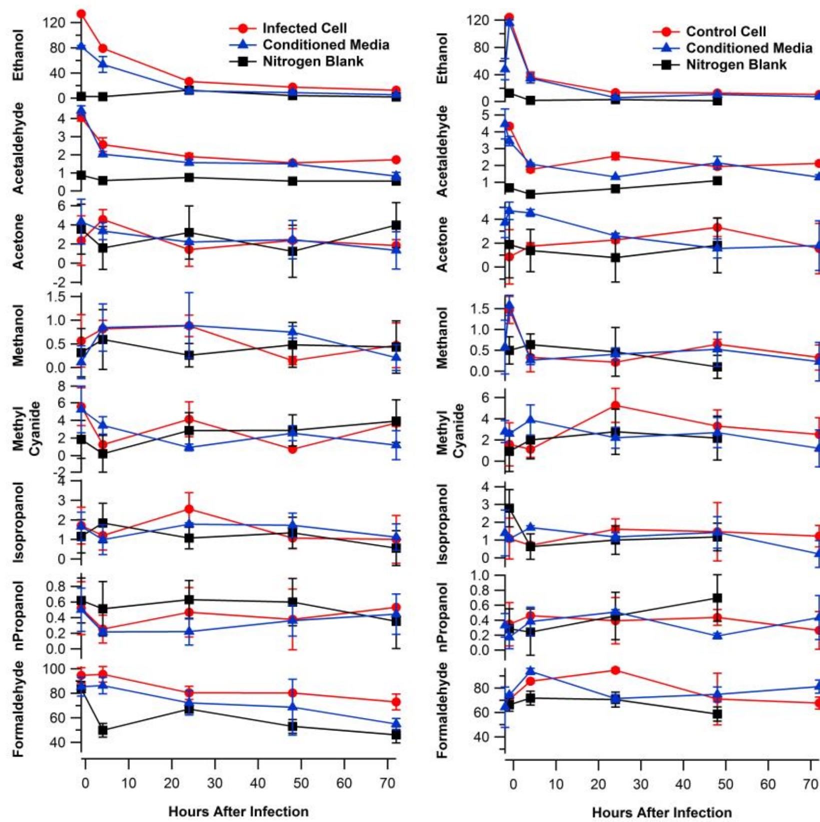


Figure 7. Gaseous metabolite concentrations determined with THz sensing for primary culture Cp1. Gaseous sample were collected using custom biocontainment/exposure system shown in Figure 3. Gaseous dilutions for Control (right) and Infected (left) cell, Conditioned Media (blue) and Pure Nitrogen Without cells or media (black) are shown in part per billion. Ethanol and Acetaldehyde initial concentrations show gradual reduction due to either evaporation or metabolic consumption. Other chemicals do not show any significant pattern.

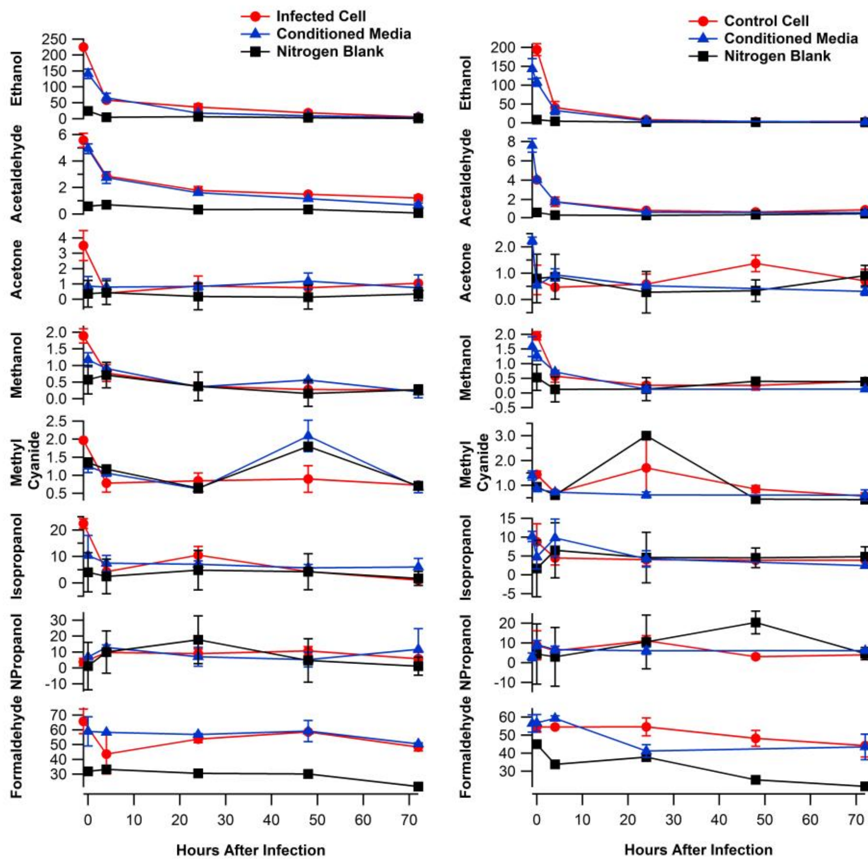


Figure 8. Gaseous metabolite concentrations determined with THz sensing for primary culture Cp2. Gaseous sample were collected using custom biocontainment/exposure system shown in Figure 3. Gaseous dilutions for Control (right) and Infected (left) cell, Conditioned Media (blue) and Pure Nitrogen Without cells or media (black) are shown in part per billion. Ethanol and Acetaldehyde initial concentrations show gradual reduction due to either evaporation or metabolic consumption. Other chemicals do not show any significant pattern.

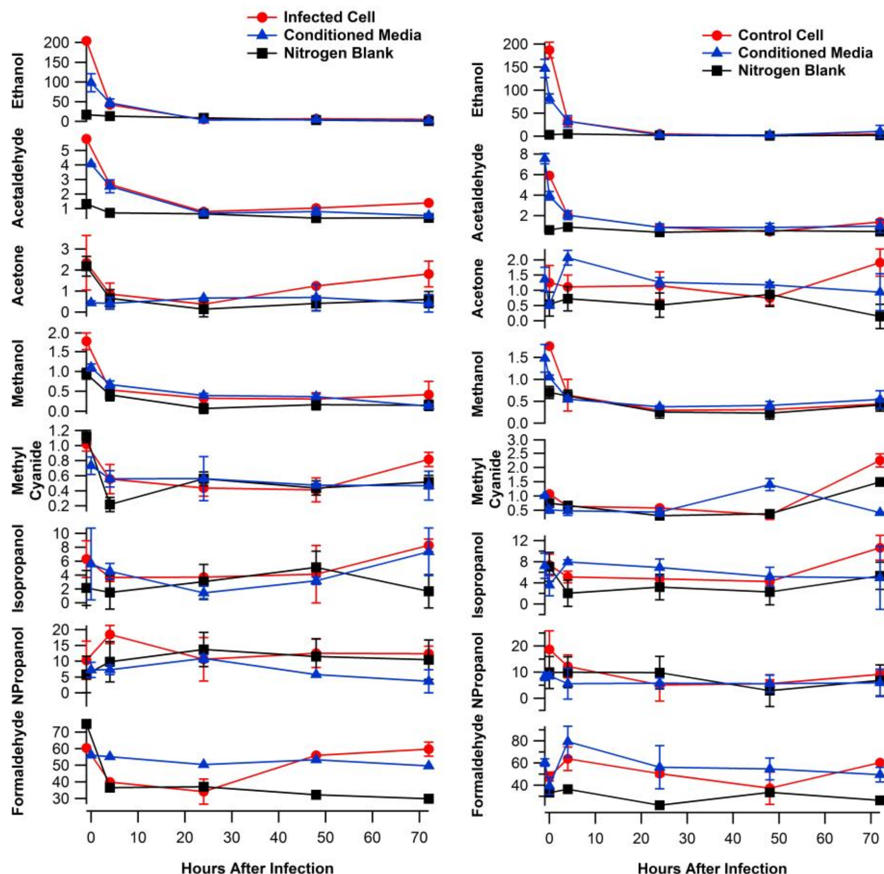


Figure 9. Gaseous metabolite concentrations determined with THz sensing for primary culture Cp3. Gaseous sample were collected using custom biocontainment/exposure system shown in Figure 3. Gaseous dilutions for Control (right) and Infected (left) cell, Conditioned Media (blue) and Pure Nitrogen Without cells or media (black) are shown in part per billion. Ethanol and Acetaldehyde initial concentrations show gradual reduction due to either evaporation or metabolic consumption. Other chemicals do not show any significant pattern.

For primary cultures Cp1-3 (Figures 7-9) ethanol and acetaldehyde show systematic reduction in dilution along the timeline of the experiment. The initial abundances of these chemicals are most likely due to their presence in nutritional media in trace amounts. Other chemicals show no prominent patterns. To further investigate the patterns of ethanol and acetaldehyde we fitted their concentration to exponential decay equation ($A\, e^{-t/\tau}$, where τ is a decay constant). Excessive scatter in acetaldehyde Cp1 control set did not allow us to get a good fit of this data. Figure 10 shows the fitting results while Figure 11 show the graphs of decay constants

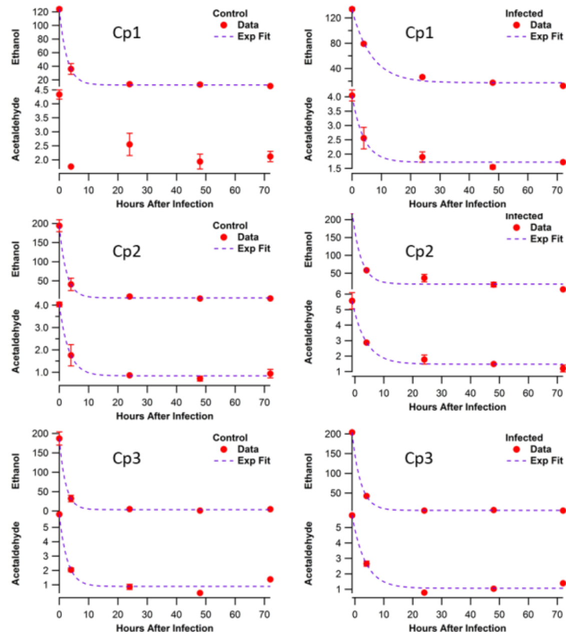


Figure 10. Fitting of ethanol and acetaldehyde concentrations (red) for primary cultures Cp1-3 to exponential decay equation. The fitted curves are shown in blue dashed lines. Infected cells exhibit consistently high decay constants.

for ethanol and acetaldehyde. The control (red trace in Figure 11) shown systematically lower decay constant than infected cells (green trace in Figure11). While the decay itself is most likely due to evaporation, the systematic differences in decay constant shown in Figure 11 can be an indication of metabolic differences between infected and control cells and deserve further investigation.

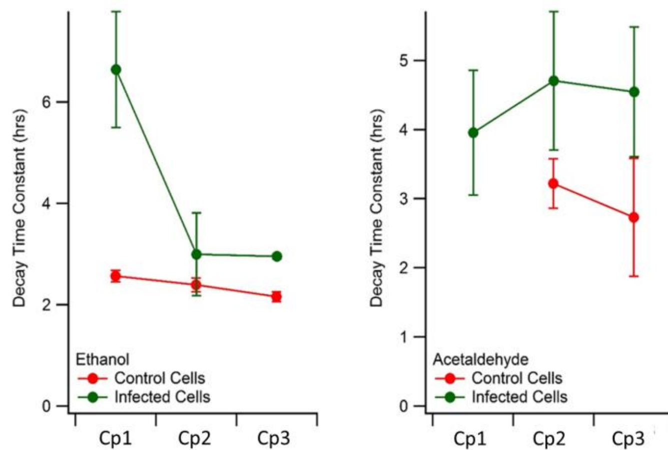


Figure 11. Decay time constants (τ) for ethanol (left) and acetaldehyde (right) dilutions determined with THz sensing. Control cells (red) exhibits systematically lower decay constant than infected cells (green).

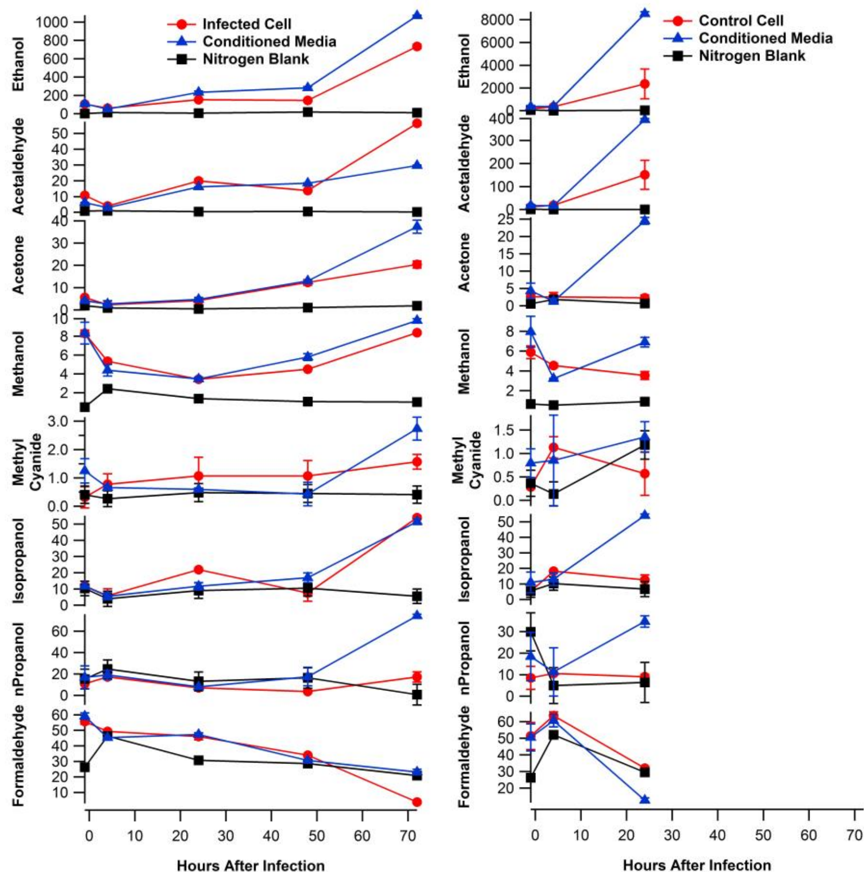


Figure 12. Gaseous metabolite concentrations determined with THz sensing for primary culture Cp4. Gaseous sample were collected using Cultex biocontainment/exposure system shown in Figure 2. Gaseous dilutions for Control (right) and Infected (left) cell, Conditioned Media (blue) and Pure Nitrogen Without cells or media (black) are shown in part per billion.

Figure 12 shows THz analyses of gaseous samples collected for culture Cp4, which was done on Cultex exposure system. Notably the concentrations of ethanol, acetaldehyde, acetone, methanol, methyl cyanide, and iso-propanol all appear to increase during infected cell run. No noticeable contamination was noticed during infected run, but the control cell run had to be abandoned after 24 hours due to obvious bacterial contamination of the control cell culture. The bacterial contamination most likely started during the infected cell run, which was done first, and got rather prominent during the control cell run despite our efforts to clean the Cultex system with hydrogen peroxide. Despite this we think the GC-MS data collected for culture Cp4 (see below) provides valuable insight into the gaseous metabolite of viral infection, as the bacterial contamination did not manifest itself up until 48 hours after the infection.

Gas chromatography mass spectrometry (GC-MS)

All sorbent tubes were thermally desorbed on a Markes International TD-100 thermal desorber and analyzed on a Trace Ultra-ISQ gas chromatograph in line with a single quadraple mass spectrometer (Thermo Scientific, Waltham, MA). Thermal desorption and GC-MS analysis of sample tubes were conducted as described previously[7]. Briefly, thermal desorption was carried out at 310 °C over 10 min. Trap settings were as follows: flow path temperature 160 °C, trap flow rate 50 ml min^{-1}, trap purge time 1 min, trap low temperature of 25 °C, trap high trap temperature 315 °C, trap heating rate 40 °C s^{-1} and post trap split 3.5 : 1. TO-14 A internal standards, bromocholoromethane, 4-bromofluorobenzene, chlorobenzene-d5, and 1,4-difluorobenzene, were applied automatically to the sorbent tube by the Markes TD-100 prior to thermal desorption (25 ppm, Linde Gas North America, Stewartsville, NJ). GC separations were carried out on a Restek Rxi-624Sil GC column (Bellefonte, PA, 60 m × 0.32 mm ID × 1.80 μm df), with a constant flow of helium (2 ml min^{-1}), over a temperature range of 40 to 240 °C at an increase of 10 °C min^{-1} with a 20 min hold time at the maximum temperature. Mass spectral analysis was carried out via electron impact ionization at 70 eV with an ion source of 275 °C while scanning over a 35–300 m/z range every 0.154 s. Data was acquired using the Thermo Scientific Trace finder EFS software package (v. 3.0). Raw data file conversion from .RAW to .CDF occurred using the File Conversion tool as part of the Xcalibur software package (v. 3.0, Thermo Scientific).

[7] Harshman, S.W., Geier, B.A., Fan, M., Rinehardt, S., Watts, B.S., Drummond, L.A., Preti, G., Phillips, J.B., Ott, D.K., and Grigsby, C.C. (2015). "The identification of hypoxia biomarkers from exhaled breath under normobaric conditions". Journal of Breath Research 9, 047103.

Culture Identifier	Condition	Flow Rate of Sample Collection in cc/min	Gas Volume Collected in cc	Number of Pre-infection Media Samples	Number of Pre-infection Baseline Samples	Time points after infection
Cp2	inf	10	500	0	0	48h
Cp4	inf	10	500	1	1	4, 24, 48, 72h
Cp5	inf	25	1500	2	2	2, 4, 8, 24, 48, 72h
Cp6	inf	25	1500	2	2	2, 4, 8, 24, 48, 72h
Cp7	inf	25	1500	2	2	2, 4, 8, 24, 48, 72, 144h
Cp8	inf	25	1500	2	2	2, 4, 8, 24, 48, 72, 144h
Cp9	ctrl	25	1500	2	2	2, 4, 8, 24, 48, 72h
Cp9	inf	25	1500	2	2	2, 4, 8, 24, 48, 72, 144h
Cp10	ctrl	25	1500	2	2	2, 4, 8, 24, 48, 72h
Cp10	inf	25	1500	2	2	2, 4, 8, 24, 48, 72, 144h
Cp11	ctrl	25	1500	2	2	2, 4, 8, 24, 48, 72h
Cp11	inf	25	1500	2	2	2, 4, 8, 24, 48, 72, 144h
Cp12	ctrl	25	1500	2	2	2, 4, 8, 24, 48, 72h
Cp12	inf	25	1500	2	2	2, 4, 8, 24, 48, 72, 144h
Cc13	inf	25	1500	1	3	4,20,24,90h
Cc14	inf	25	1500	1	3	4,20,24,90h
Cc15	inf	25	1500	1	2	2, 6, 24, 30, 52, 72h
Cc16	inf	25	1500	1	2	2, 6, 24, 30, 52, 72h
Cc17	inf	25	1500	1	2	2, 6, 24, 30, 52, 72h
Cc18	inf	25	1500	1	2	2, 6, 24, 30, 52, 72h

Table 3. Summary of data points of samples collected for GC-MS analysis. 'Condition' indicates the type of exposure: 'inf' represents infection, and 'ctrl' represents the data collection without infection - control. The time points after infection spanned 2-144 hours after infection for selected cultures.

The GC-MS analyses were conducted at AFRL using the state-of-the-art hardware described earlier. Instead of Tedlar bags, the gaseous metabolites were directly loaded onto sorbent tubes containing Tenax TA sorbent material. Table 3 summarizes the number of samples and timepoints for each cell culture studied in this project. A total of 0.5L of gas was drawn through sorbent tubes at a flow rate of 10 cc/min for cultures Cp2 and Cp4. For all other cultures a total of 1.5 L was drawn through sorbent tubes at a flow rate of 25 cc/min. For culture Cp2 we sampled gas at 48 hour mark for control and infected cultures. For culture Cp4 we collected 6 samples: pre-infected cell baseline (right before infection), pre-infected media (media conditioned by cell right before infection), 4 hours after infection, 24 hour after infection, 48 hours after infection, and 72 hours after infection.

For cultures Cp5-12 (primary cells) we collected 6 time points spanning 2-72h post infection. Cell cultures Cp9-12 were studied during 2 consecutive weeks. During Week 1 ('control') cells did not get infected but samples were collected on the same timeline to assess the baseline gaseous signatures. During Week 2 cells were infected to obtain biomarkers of viral infection. For infected cultures Cp7-10 we extended the timeline to 144 h post infection.

For cultures Cp2 and Cp4 GC-MS analyses were run in a tentative ('qualitative') identification mode, when chemicals are identified by fits against libraries. The software determines the match probability which needs to be used as guidance in determining the correctness of the measurement.

For the rest of cell cultures Cp5-12, Cc13-18 GC-MS analyses were performed in both 'qualitative' and 'quantitative' modes. In 'quantitative' mode the GC-MS instrument was calibrated prior to measurements using the following subset of chemicals from EPA TO-15/17 standard: Ethanol; Acetone; Isopropyl Alcohol; Methylene chloride; MTBE; trans 1,2-dichloroethylene; n-Hexane; 1,1-Dichloroethane; Methyl Ethyl Ketone; Ethyl acetate; Ethene, 1,1-dichloro-; cis 1,2-Dichloroethylene; Tetrahydrofuran; Chloroform; 1,1,1Trichloroethane; Cyclohexane; Carbon Tetrachloride; Benzene; 1,2-Dichloroethane; Heptane; Trichloroethylene; Methyl Methacrylate; 1,2-Dichloropropane; 1,4-Dioxane; Bromodichloromethane; cis 1,3-Dichloropropene; Methyl Isobutyl Ketone; Toluene; 1,1,2-Trichloroethane; Tetrachloroethylene; Butyl Methyl Ketone (2-Hexanone); Dibromochloro methane; 1,2-dibromo ethane,; Chlorobenzene; Ethylbenzene; m,p-xylene; o-xylene; Styrene; 1,1,2,2-tetrachloro ethane; 1,2,4-Trimethyl benzene; 1,3,5-Trimethyl benzene; 4-Ethyltoluene; 1,3-Dichloro benzene; 1,4-dichloro benzene,; Benzyl Chloride; 1,2-Dichloro benzene,; Naphthalene.

The qualitative reports for cultures Cp5-12, Cc13-18 contained several hundred VOCs for which probability of identification was used as discriminator for the inclusion into the predictive model of viral infections.

Primary Component Analysis

GC-MS analysis of cultures Cp5-12 and Cc13-18 yielded a wealth of data. We utilized Primary Component Analysis to tease out chemicals whose concentrations (or their linear combinations) result in greatest spread of data in the Primary Component Coordinate Space.

Principal Components Analysis (PCA) is a method to analyze multidimensional data sets in which linear combinations of each of the predictor variables are sorted into components which explain the variability of the predictor variables. Thus, the first principal component is the linear combination which explains the greatest amount of variability. The second principal component explains the 2nd most variability with the restriction that it be orthogonal to the first principal component, etc. PCA analysis is often used to reduce the dimensionality of a data set, since the first few components contain most of the information. PCA can also be used as a first pass in classifying data if one combines the graph with PCA components as axes

with data points classified according to some other metric.

Ethanol
Isopropyl Alcohol
Acetone
Styrene
Acetophenone
Methyl Ethyl Ketone
4-Ethyltoluene

Table 4. Chemicals Identified by Primary Component Analysis as chemicals that result in highest level of data variability.

Table 4 lists the top 7 chemicals sorted in the order of significance identified by PCA as variables that cause the highest variance in representation of the data. All these chemicals are known to be present in human breath[8] which gives additional validation to our findings.

Classification of Infected and Healthy Primary Cells based on Gaseous Metabolites.

Ethanol – an important endogenous metabolite present in human body. We demonstrated its detection with THz sensing. Figure 13 shows its concentration for cultures Cp5-12. The pattern is consistent with result of THz analysis shown in Figures 9 and 12 and corresponds to an exponential reduction of ethanol concentration associated with either evaporation or cellular metabolism. Infected cells manifest slightly higher time constant of the reduction in ethanol concentration which matches earlier THz observations (Figure 11). It is noteworthy that concentration of ethanol starts to grow towards 144h time, possibly indicating a change in cellular metabolism or presence of bacterial contamination which was observed in our earlier experiments. An alternative explanation is that the rise of ethanol concentration may serve as an indicator of viral infections 4 days after infection. This phenomenon deserves further investigation in later studies.

[8] de Lacy Costello, B., A. Amann, H. Al-Kateb, C. Flynn, W. Filipiak, T. Khalid, D. Osborne, and N.M. Ratcliffe, *A review of the volatiles from the healthy human body.* Journal of Breath Research, 2014. **8**(1): p. 014001.

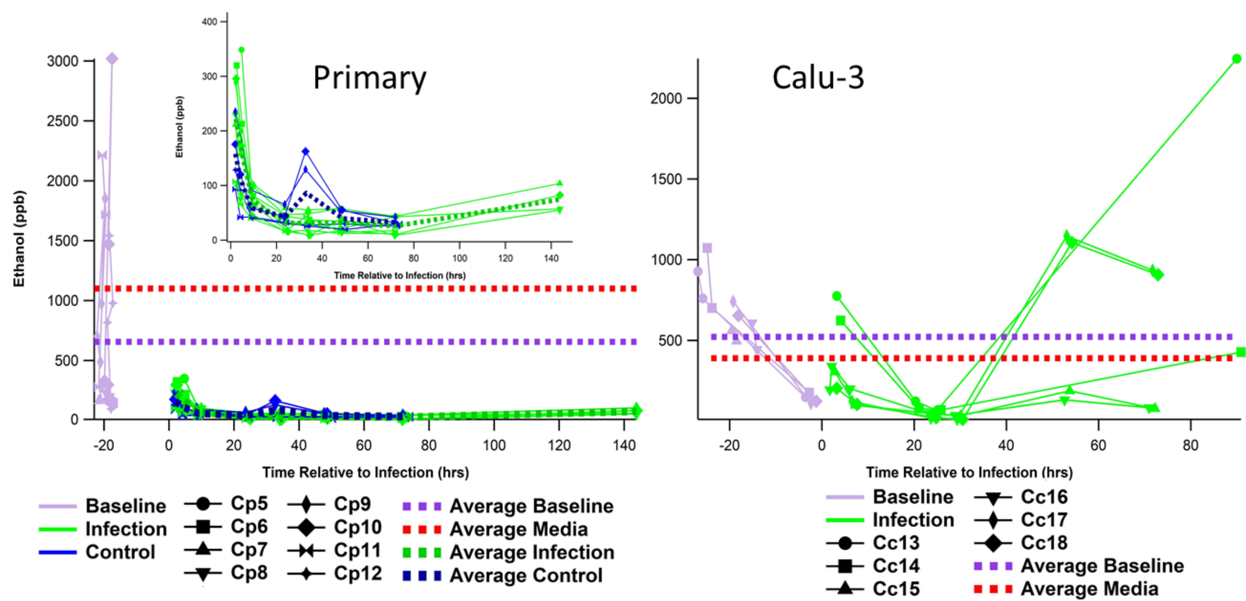


Figure 13. Concentrations of ethanol for primary cell cultures Cp5-12 (left) and calu-3 cultures Cc13-18 (right) determined with GC-MS. Purple traces represent pre-infection baseline values. Blue traces correspond to 'Control' measurements. Green traces depict post-infection values. Dashed traces represent averaged values for "media", "pre-infection baseline", "control", and "infection".

The initial concentration of ethanol is associated with trace amount of the compound in the cell nutritional media. Our experiments demonstrated our ability to monitor the rate of reduction in ethanol concentration. It is possible that this rate of reduction can be used as viral classifier. In this report we chose to focus on chemicals that are not present in media in excessive amount, which is clearly not the case for ethanol. Thus, we chose not to use it in classification presented below.

Isopropanol – another endogenous alcohol present in human breath. As with ethanol we chose to exclude it from our classification. Figure 14 shows its concentrations measures with GC-MS. It is important to note that decay time constant is higher for infected cells than 'control' which is consistent with the pattern for ethanol and acetaldehyde detected with THz sensing (Figure 11). Due it its initial high concentration of we chose not to use it in classification presented below.

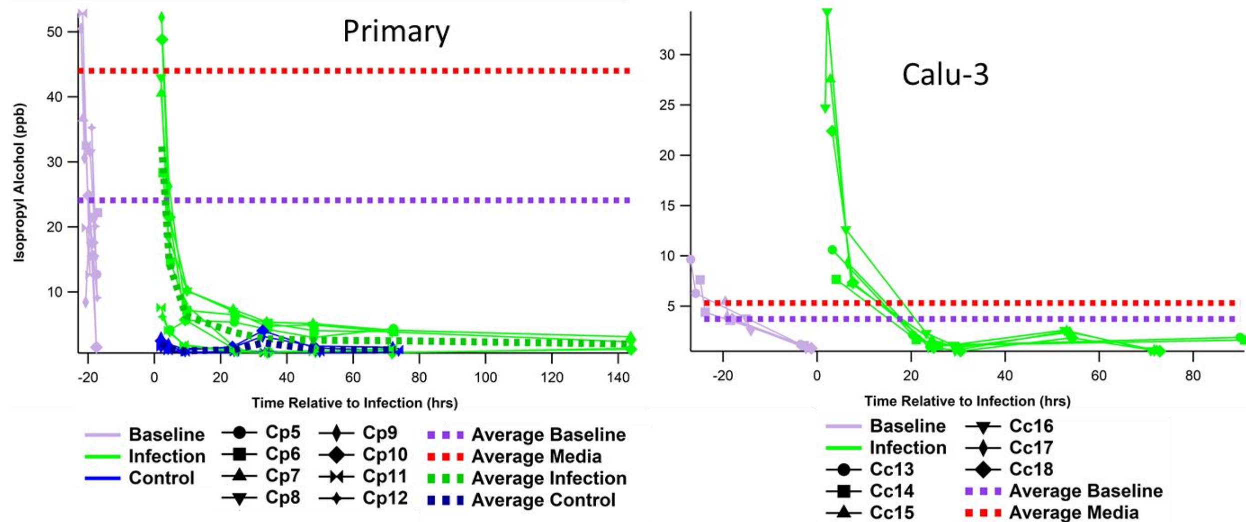


Figure 14. Concentrations of isopropanol for primary cell cultures Cp5-12 (left) and calu-3 cultures Cc13-18 (right) determined with GC-MS. Purple traces represent pre-infection baseline values. Blue traces correspond to 'Control' measurements. Green traces depict post-infection values. Dashed traces represent averaged values for "media", "pre-infection baseline", "control", and "infection".

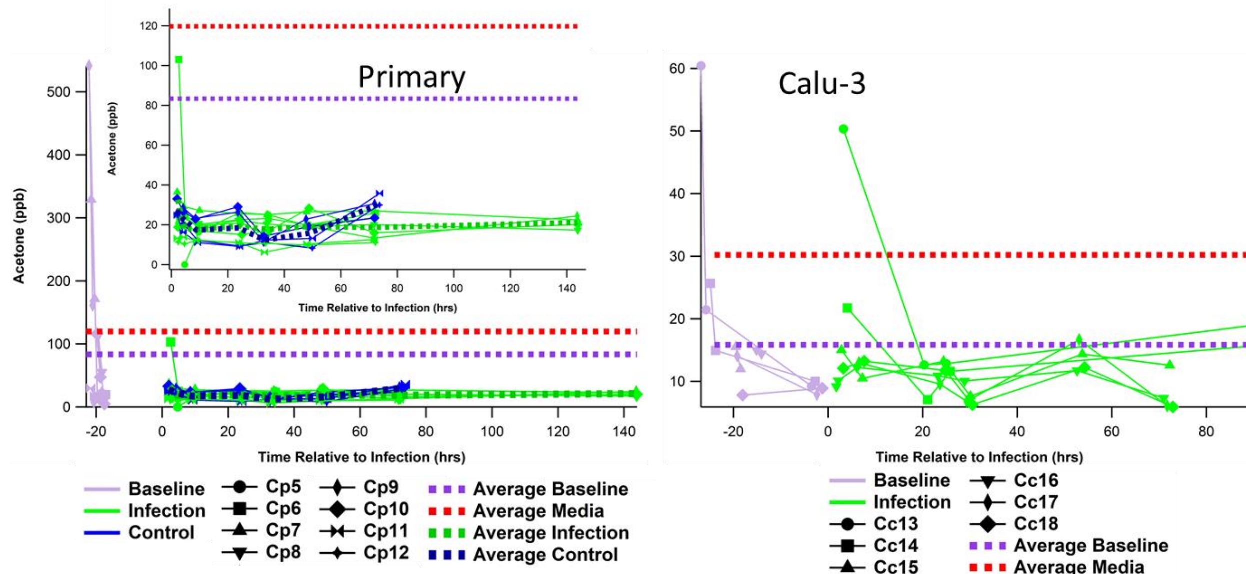


Figure 15. Concentrations of acetone for primary cell cultures Cp5-12 (left) and calu-3 cultures Cc13-18 (right) determined with GC-MS. Purple traces represent pre-infection baseline values. Blue traces correspond to 'Control' measurements. Green traces depict post-infection values. Dashed traces represent averaged values for "media", "pre-infection baseline", "control", and "infection".

Acetone is a simplest ketone and a prominent bio-marker present in human breath associated with fat metabolism. Figure 15 shows its concentration for cultures Cp5-12 (left) and Cc13-18 (right). No major difference was observed between concentrations of acetone in 'infected' and 'control' experiments. Its high

ranking in primary component analysis is most likely due to a few high values present in some measurements (not shown in Figure 15), likely associated with contaminations in those experiments. Thus, we chose not to use acetone in classification presented below.

Styrene – an aromatic carbohydrate ($C_6H_5CH{=}CH_2$, 104.15 amu), is known to be present in human breath, although its origin is not entirely understood. Figure 17 shows the concentration of styrene for cultures Cp5-12 and Cc13-18. Of note, both in 'control' and 'infected' experiments levels of styrene are reduced compared to the 'pre-infection' baseline which might be an indication of styrene consumption by cells.

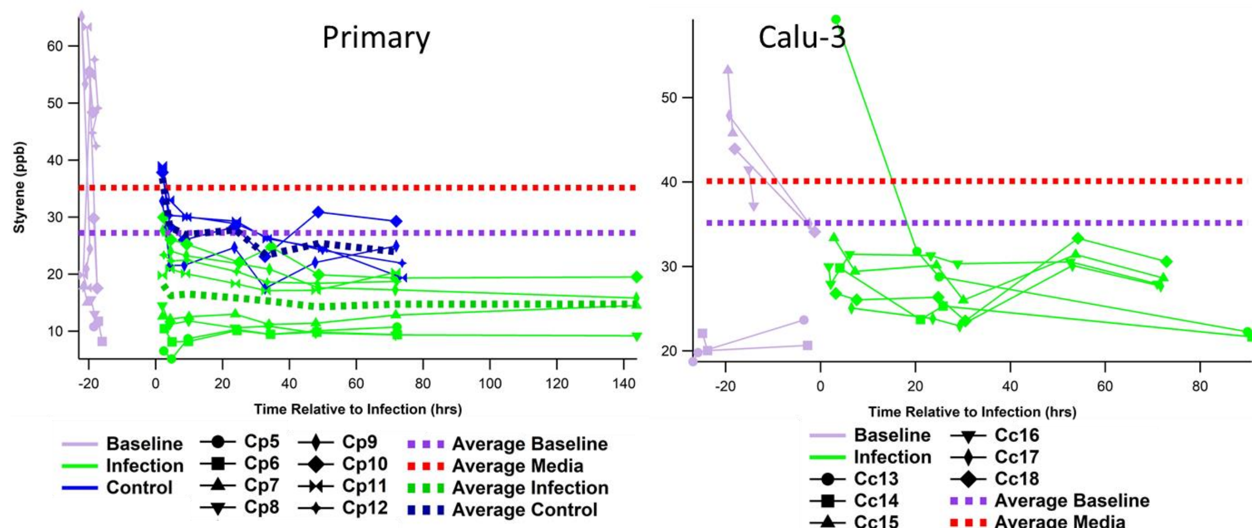


Figure 16. Concentrations of styrene for primary cell cultures Cp5-12 (left) and calu-3 cultures Cc13-18 (right) determined with GC-MS. Purple traces represent pre-infection baseline values. Blue traces correspond to 'Control' measurements. Green traces depict post-infection values. Dashed traces represent averaged values for "media", "pre-infection baseline", "control", and "infection".

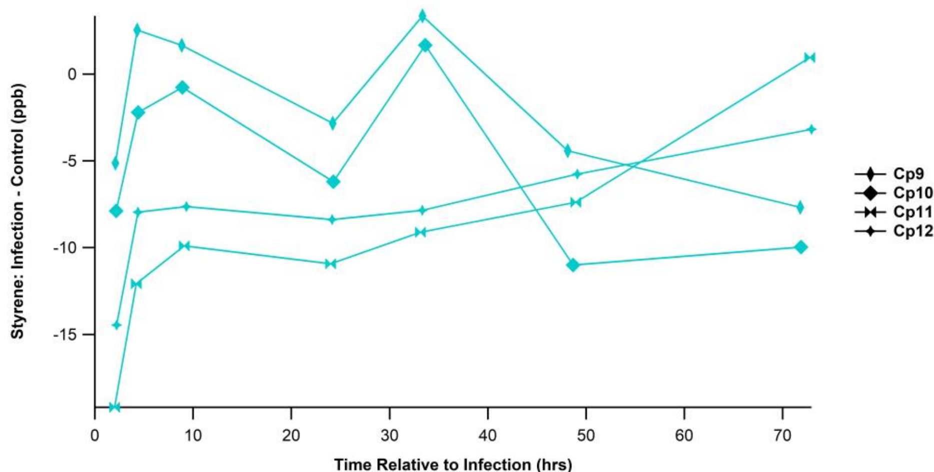


Figure 17. Difference between concentrations of acetophenone for 'Infection' and 'Control' experiments for primary cell cultures Cp5-12.

Figure 16 (right) shows reduction of the styrene level for infected calu-3 cells below pre-infection values. Figure 17 shows a difference between 'infection' and 'control' measurements of styrene in primary culture Cp5-12 with majority of values being negative, which indicated a reduction of styrene concentration associated with infection. 'Control' measurement was conducted on a culture a week prior to 'infection' run to establish basis for metabolic comparison. The average value in 'infected' calu-3 runs (Figure 16 – right) is comparable to 'control' measurements for primary cell cultures. This could be an indication of metabolic variance between cell types associated with adenovirus infection. In most of our measurements the average value of chemical concentrations (dashed red trace in Figures 13-16,18,21,22) is above the average value of pre-infection baseline (dashed purple trace in Figures 13-16,18,21,22). This difference can be explained by the reduction of evaporation rate of chemical with cell cultures placed on top of media, thus restricting chemical transport into gas phase. For styrene, the average value of 'control' measurement is very close to the average value of pre-infection baseline, while the average value of 'infection' measurement is nearly 10 ppb lower. The effect is persistent across multiple cultures and donors; thus, we hypothesize that reduction of styrene is caused by altered cellular metabolism associated with adenovirus infections. Through this analysis we chose styrene as one of the classifiers of adenovirus infection.

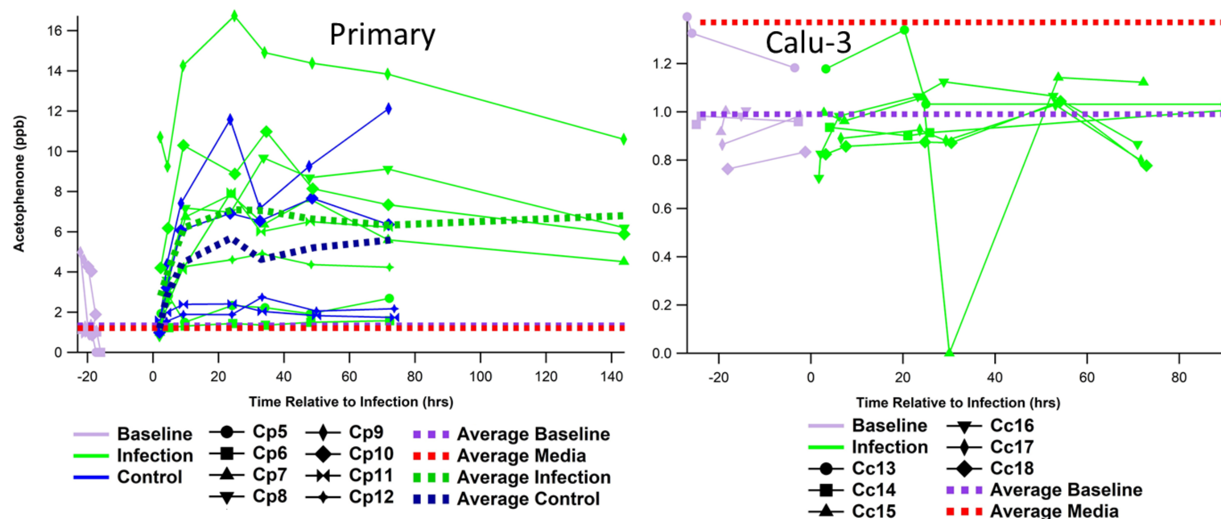


Figure 18. Concentrations of acetophenone for primary cell cultures Cp5-12 (left) and calu-3 cultures Cc13-18 (right) determined with GC-MS. Purple traces represent pre-infection baseline values. Blue traces correspond to 'Control' measurements. Green traces depict post-infection values. Dashed traces represent averaged values for "media", "pre-infection baseline", "control", and "infection".

Acetophenone – simplest aromatic ketone ($C_6H_5C(O)CH_3$, 120.151 amu), is known to be present in human breath, although its origin is not entirely understood. The GC-MS detection of acetophenone was

done in a tentative (qualitative) mode, with probability of detection at 97%. The typical GC retention time was 15 minutes which was distinct from the retention time of 4-ethyltoluene (17 min) that has a very similar molar mass of 120.195 amu and was detected in quantitative mode.

Figure 18 (left) shows a prominent rise of acetophenone concentration above average 'baseline' and 'media' levels for both 'control' and 'infection' experiments. The average increase in 'infection' is well above the 'control' increase, which can be seen in Figure 19, which shows the difference between 'infection' and 'control' measurements. All but one points in Figure 19 are positive, with a likely explanation that adenovirus infection increases the levels of acetophenone.

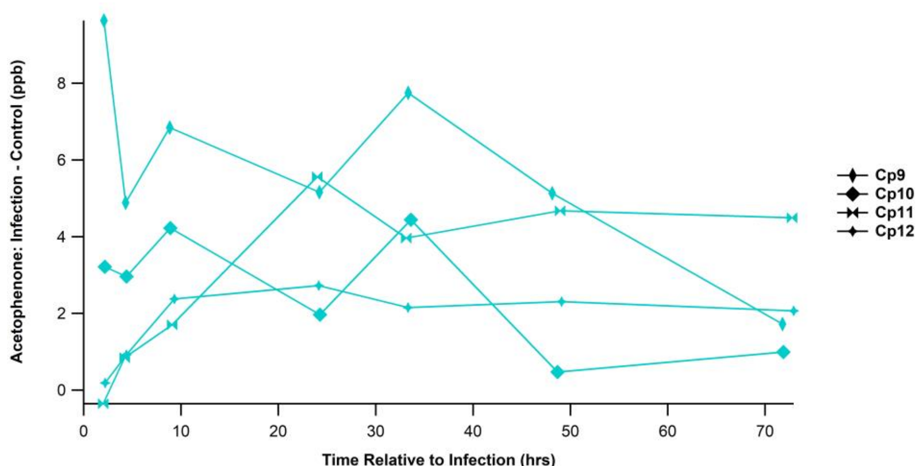


Figure 19. Difference between concentrations of acetophenone for 'Infection' and 'Control' experiments for primary cell cultures Cp5-12.

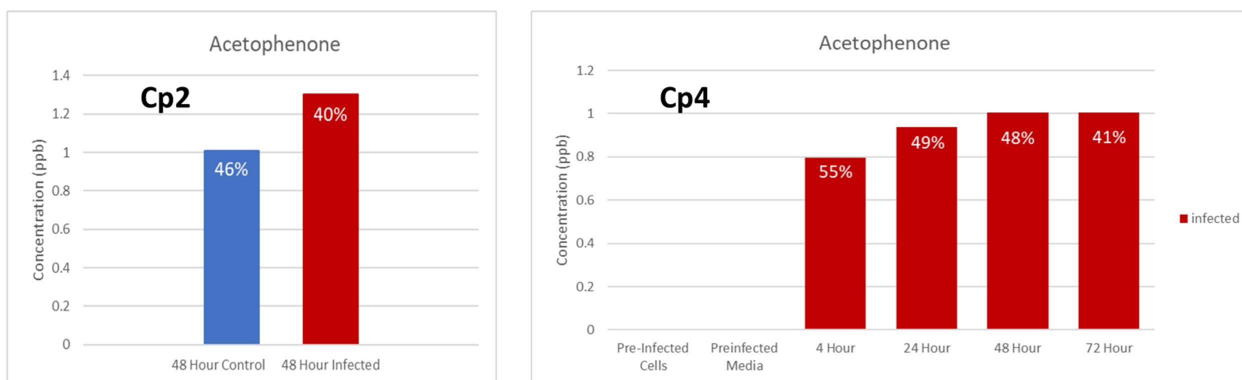


Figure 20. Detection of Acetophenone with GC-MS in cultures Cp2 and Cp4. For Cp2 measurements of control and infected metabolites at 48 hours are presented in the left pane, while Cp4 data represented on the right. Each bar is labeled with corresponding "Match Probability".

The significance of Acetophenone is further supported by its detection in cultures Cp2 and Cp4. Figure 20 shows a rise in concentration of acetophenone detected with GC-MS for culture Cp4. For culture Cp2 acetophenone was detected at slightly higher concentration in infected cells a 48h mark. Acetophenone

"$C_6H_5CCH_3$" is a good candidate for THz detection, with detection at a part per billion level of dilution feasible.

Methyl Ethyl Ketone, also known as 2-butanone, is also known to be present in human breath. Our experiments (Figure 21) did not reveal prominent patterns associated with adenovirus infection. Both 'control' and 'infected' levels were observed to be below 'baseline' and 'media'. The average value of 'infected' concentrations was comparable to average 'control' concentrations. Its high ranking in PCA classification is most likely caused by a couple outliers in 'pre-infection' measurements (purple trace in Figure 21 – left). Thus, we chose not to use methyl ethyl ketone in classification presented below.

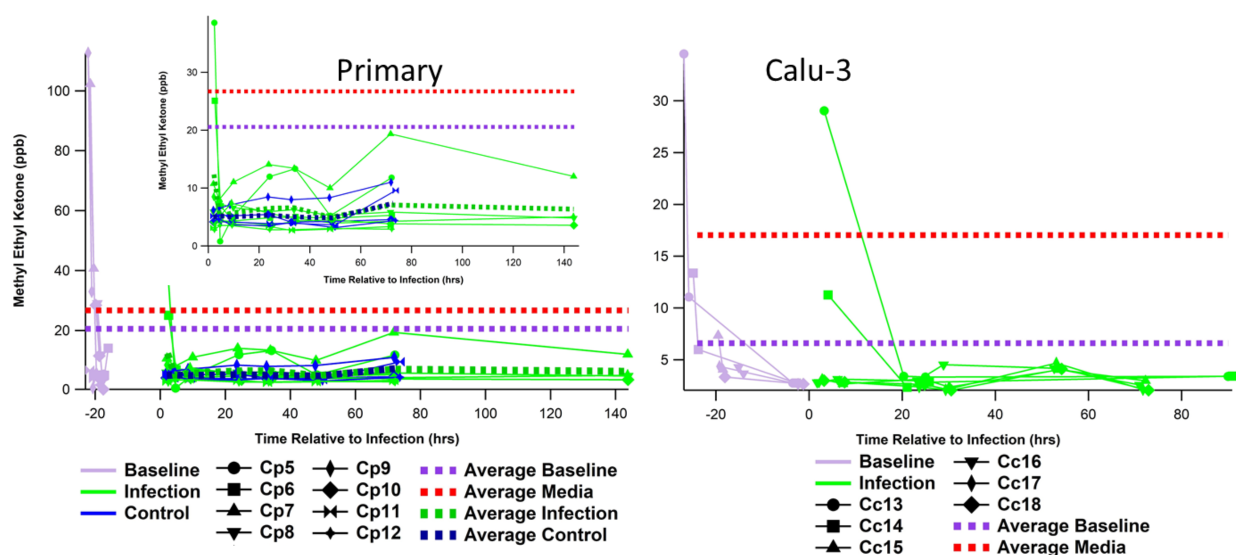


Figure 21. Concentrations of methyl ethyl ketone for primary cell cultures Cp5-12 (left) and calu-3 cultures Cc13-18 (right) determined with GC-MS. Purple traces represent pre-infection baseline values. Blue traces correspond to 'Control' measurements. Green traces depict post-infection values. Dashed traces represent averaged values for "media", "pre-infection baseline", "control", and "infection".

4-Ethyltoluene – an aromatic carbohydrate commercially produced by ethylation of toluene ($CH_3C_6H_4C_2H_5$, 120.195 amu), is known to be present in human breath, although its origin is not entirely understood. The GC-MS detection of 4-ethyltoluene was done in a quantitative mode with typical GC retention time of 17 minutes.

Figure 22 (left) shows a prominent rise of 4-ethyltoluene concentration for 'infection' experiment well above average 'baseline' and 'media' levels. The 'control' concentration rises above average 'media' concentration and then falls to average 'baseline' value over the course of 60 hours. The average increase in 'infection' is well above the 'control', which can be seen in Figure 23, which shows the difference between 'infection' and 'control' measurements. Notably almost no 4-ethyltoluene was detected in calu-3 cells (Figure 22 – right).

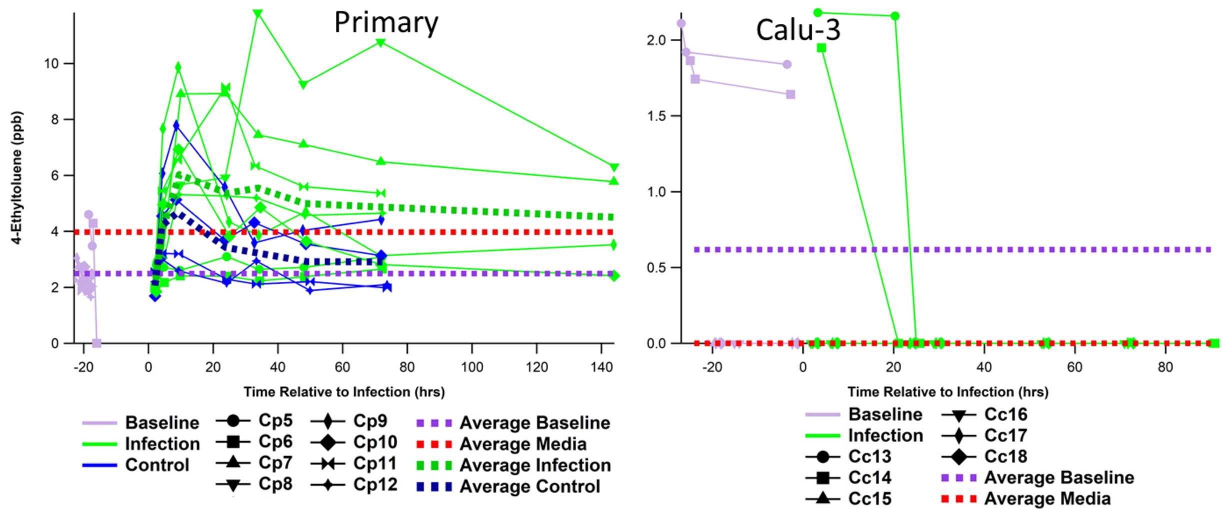


Figure 22. Concentrations of 4-ethyl toluene for primary cell cultures Cp5-12 (left) and calu-3 cultures Cc13-18 (right) determined with GC-MS. Purple traces represent pre-infection baseline values. Blue traces correspond to 'Control' measurements. Green traces depict post-infection values. Dashed traces represent averaged values for "media", "pre-infection baseline", "control", and "infection".

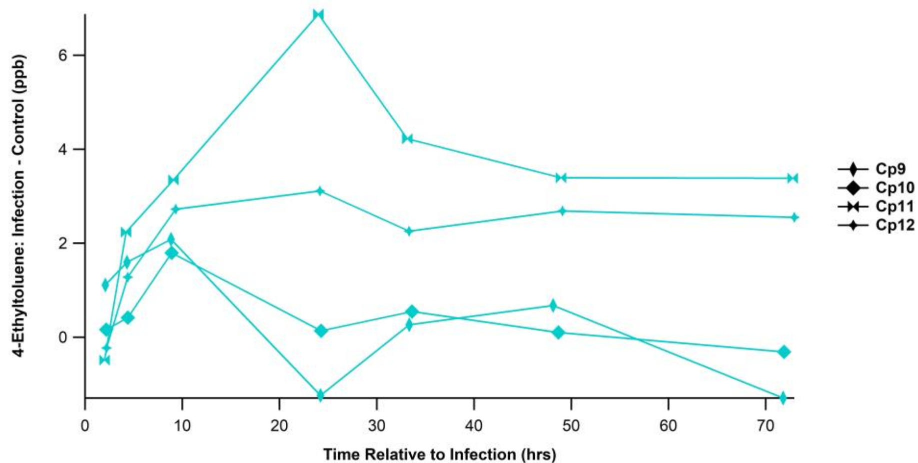


Figure 23. Difference between concentrations of 4-ethyl toluene for 'Infection' and 'Control' experiments for primary cell cultures Cp5-12.

Through analysis presented above we identified that styrene, 4-ethyltoluene, and acetophenone concentrations change systematically when primary epithelial cells are infected with adenovirus. Styrene concentrations drop below 'control', while acetophenone and 4-ethyltoluene concentrations rise well above baseline values. The pattern persists across cultures derived from 3 donor and multiple samples for each donor. Calu-3 cells exhibit very low levels of acetophenone and 4-ethyltoluene.

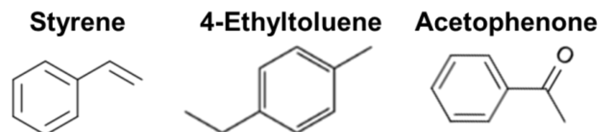


Figure 24. Chemical structures of styrene, 4-ethyltoluene, and acetophenone identified as possible biomarkers of adenovirus infection.

All three chemicals are aromatic compounds (Figure 24) with likely bio-chemical link associated with changes in metabolism induced by viral infection. Acetophenone is a ketone. Ketones are known to be associated with metabolism of fat in biological systems. This provides additional argument in favor of metabolic origins of our model.

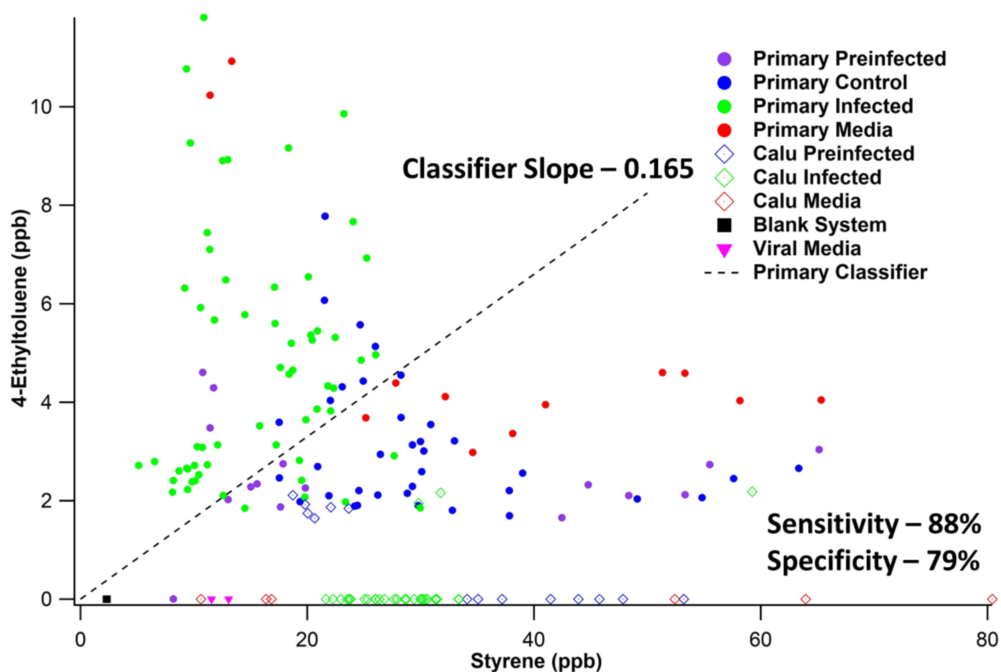


Figure 25. Classification of data for primary cultures Cp5-12 and calu-3 cultures Cc13-18 based on concentration of 4-ehtyltoluene and styrene. A linear classifier with a slope 0.165 divides the plane into 'infected' region (above the dashed line) and healthy 'control/baseline' region (below the dashed line). The sensitivity (true positive rate – probability of classifying infected correctly) is 89%. The specificity (true negative rate – probability of classifying healthy correctly) is 78%. Calu-3 cells are easily distinguishable from primary due to low levels of 4-ethyltoluene.

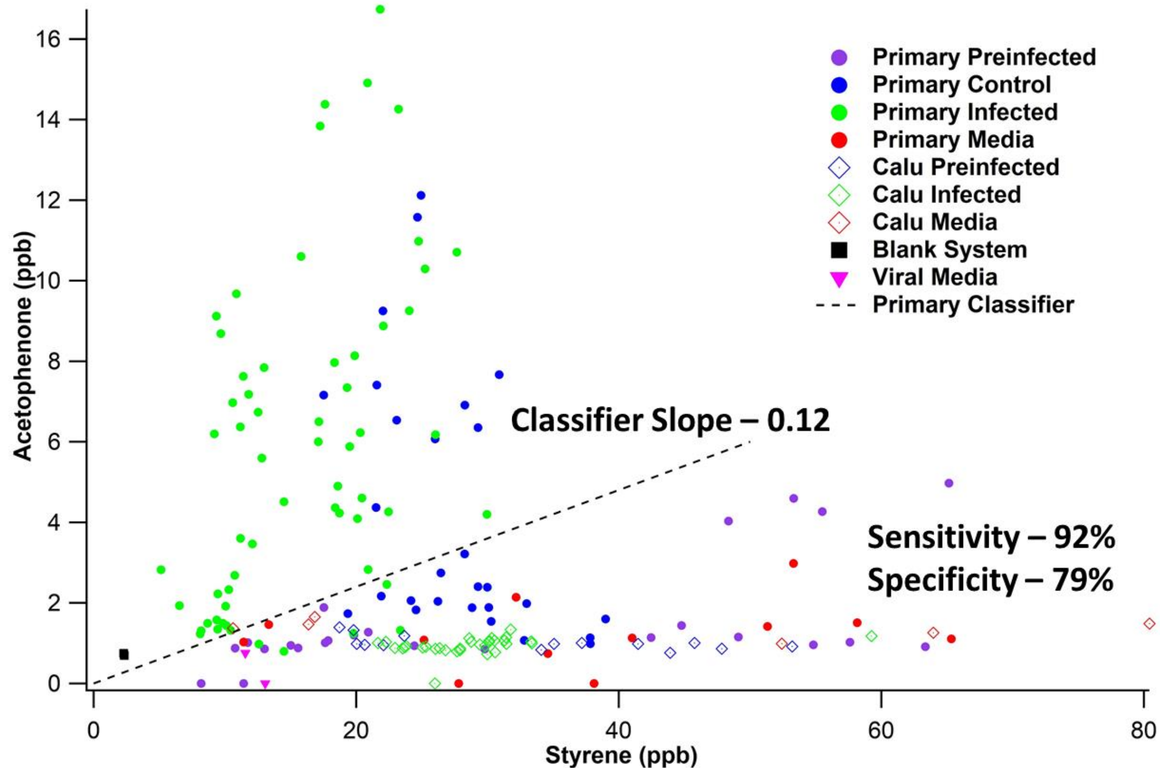


Figure 26. Classification of data for primary cultures Cp5-12 and calu-3 cultures Cc13-18 based on concentration of acetophenone and styrene. A linear classifier with a slope 0.12 divides the plane into 'infected' region (above the dashed line) and healthy 'control/baseline' region (below the dashed line). The sensitivity (true positive rate – probability of classifying infected correctly) is 92%. The specificity (true negative rate – probability of classifying healthy correctly) is 79%. Calu-3 cells are easily distinguishable from primary due to low levels of acetophenone.

Figures 25 and 26 show classification of all the data taken for primary cultures Cp5-12 and calu-3 cultures Cc13-18 based on concentration of styrene plotted against 4-ethyl toluene and acetophenone respectively. A linear classifier (dashed line in Figures 25, 26) divides the plane into 'infected' and 'control/baseline' regions.

4-ethyltoluene vs Styrene linear classifier has a slope of 0.165 (Figure 25), which corresponds to the threshold ratio of 4-ethyltoluene to styrene concentrations. That ratio and the threshold are shown in Figure 27-left. This classification for 112 primary culture samples yields sensitivity (true positive rate – probability of classifying infected correctly) of 88% and specificity (true negative rate – probability of classifying healthy correctly) of 79%

Acetophenone vs Styrene linear classifier has a slope of 0.12 (Figure 26), which corresponds to the threshold ratio of acetophenone to styrene concentrations. That threshold is shown in Figure 27- right. This classification for 112 primary culture samples yields sensitivity (true positive rate – probability of classifying infected correctly) of 92% and specificity (true negative rate – probability of classifying healthy correctly) of 79%.

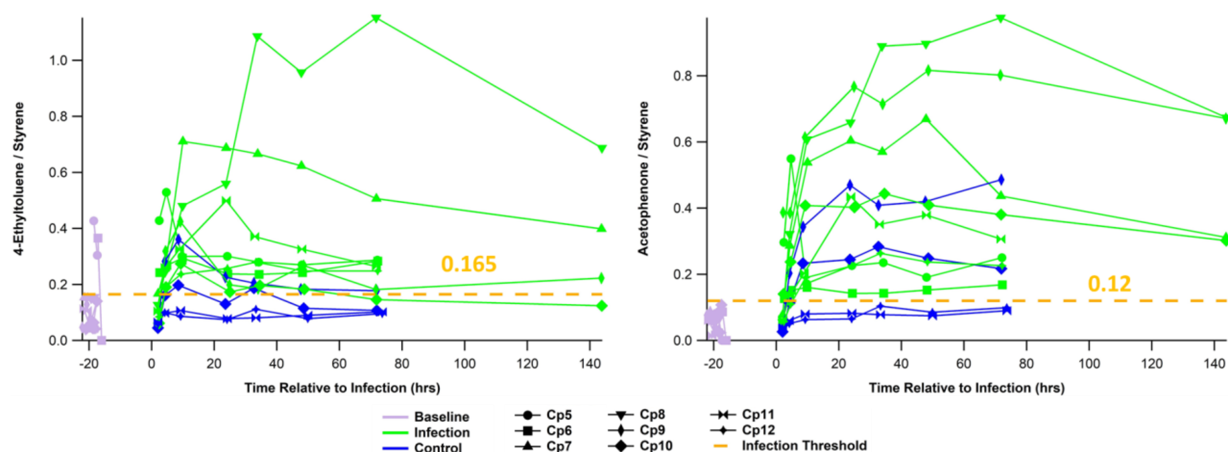


Figure 27. Ratio of concentrations of 4-ethyl toluene and styrene (left) and acetophenone and styrene (right) for cultures Cp5-12. The slope threshold classifiers from Figures 25 and 26 are shown in orange.

It is important to note that this classification separates 'infected' and 'healthy' primary cells, 'primary' and 'calu-3' cells, as well as signatures for blanc system (just enclosure shown in Figure 3) and viral media (a dish of media placed into enclosure). The results are persistent across 3 donors and multiple samples from each donor.

Conclusions

The main finding of this research project was the discovery of several gaseous chemicals (styrene, acetophenone, 4-ethyltoluene) whose concentrations, measured with GC-MS sensing, allowed us to classify the 'infected' vs 'healthy' cell cultures (112 samples in total) with sensitivity of ~90% and specificity of ~80%. These encouraging results call for further validation and investigation of variability of gaseous biomarkers for different types of infections.

We hypothesize that different viruses will exhibit different gaseous biomarkers, which may allow us to detect these infections pre-symptomatically. The ratios of acetophenone and 4-ethyltoluene to styrene peak 24-80 hours after the infection providing ample opportunity for health workers and military personnel to take measures necessary to protect the personnel and to curb the spread of the infection.

We demonstrated the use of THz sensing towards gaseous analysis of VOCs produced by cell cultures. Unlike GC-MS, THz sensing is amenable for miniaturization. More research and development is needed to extend the capability of THz sensors to detect heavier gaseous compounds using handheld formfactor THz devices.

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

"Nothing to Report."

How were the results disseminated to communities of interest?

"Nothing to Report."

What do you plan to do during the next reporting period to accomplish the goals?

"Nothing to Report."

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

Our results, outlined in this report, indicate the importance of 4-ethyltolune, acetophenone, and styrene as biomarkers of adenovirus viral infections. The finding will contribute to scientific understanding of viral biochemistry and provide a valuable modality for pre-symptomatic diagnostics of these illnesses. THz sensing technology used and developed in this project is an important tool for analytical gas sensing which can be miniaturized to fulfill the needs of military medical personnel in diagnosing viral diseases.

What was the impact on technology transfer?

"Nothing to Report."

What was the impact on society beyond science and technology?

"Nothing to Report."

5. **CHANGES/PROBLEMS:**

"Nothing to Report"

Changes in approach and reasons for change

"Nothing to Report"

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

We encountered a significant personnel reduction in the last year of performance. We intent to apply for expansion of the award to continue this research 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

"Nothing to Report"

Significant changes in use or care of vertebrate animals.

"Nothing to Report"

Significant changes in use of biohazards and/or select agents

"Nothing to Report"

6. PRODUCTS:

"Nothing to Report."

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

▪ *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

Name:	*Ivan Medvedev*
Project Role:	*PI*
Nearest person month worked:	*2*
Contribution to Project:	*Dr. Medvedev's laboratory performs THz analysis of the mixture, lead experimental design for the project. Dr. Medvedev performs overall coordination of the project.*
Funding Support:	

Name:	*Kate Excoffon*
Project Role:	*Co-PI*
Nearest person month worked:	*2*

Contribution to Project:	*Dr. Excoffon' s laboratory is responsible for grows of cell cultures, viral exposure and sample collection. Dr. Excoffon assists Dr. Medvedev in project coordination.*
Funding Support:	

Name:	*Jennifer Martin*
Project Role:	*Co-PI*
Nearest person month worked:	*0*
Contribution to Project:	*Dr. Martin performs GC-MS analyses of gaseous cellular samples.*
Funding Support:	

Name:	*Priyanka Sharma*
Project Role:	*Research Scientist*
Nearest person month worked:	*5*
Contribution to Project:	*Dr. Sharma is responsible for growth of cell cultures.*
Funding Support:	

Name:	*Daniel Tyree*
Project Role:	*Graduate Student*
Nearest person month worked:	*12*
Contribution to Project:	Mr. Tyree is responsible for design of experiments. He is responsible for THz sensing of cellular samples. In coordination with the PI he leads data analysis.
Funding Support:	

Name:	*James Readler*
Project Role:	*Graduate Student*
Nearest person month worked:	*3*
Contribution to Project:	*Mr. Readler is responsible for growth and cell cultures. He assists the PI and Mr. Tyree in design of experimental protocols.*
Funding Support:	NIH/NIAID 1R01AI127816-01A1

Name:	*Hannah Benston*
Project Role:	*Undergraduate Student*
Nearest person month worked:	*1*
Contribution to Project:	*Ms. Benston is assisting Mr. Tyree in performing THz gas sensing and associated data analysis*
Funding Support:	*STTR Phase II, AFOSR FA8650-16-C-6693*

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

NIH/NIAID 1R01AI127816-01A1 (NIH) Excoffon (PI) 9/25/17-8/31/22
"Prevention of adenovirus pathogenesis through downregulation of the apical adenovirus receptor"; The molecular mechanism and anti-adenovirus efficacy of novel molecules that downregulate the expression of the apical adenovirus receptor and adenovirus infection will be investigated in epithelia in vitro and in the cotton rat model for adenovirus pathogenesis in vivo.
Role: PI
No overlap

What other organizations were involved as partners?

"Nothing to Report."